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


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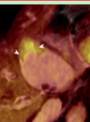
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WJCO 5th Anniversary Special Issues (2): Breast cancer

Current aspects of therapeutic reduction mammoplasty for immediate early breast cancer management: An update

Alexandre Mendonça Munhoz, Eduardo Montag, Rolf Gemperli

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it permits wider excision of the tumor, with a superior mean volume of the specimen and potentially reduces the incidence of margin involvement. Regardless of the fact that there is no consensus concerning the best TRM technique, the criteria is determined by the surgeon's experience, the extent/location of glandular tissue resection and the size of the defect in relation to the size of the remaining breast. The main advantages of the technique utilized should include reproducibility, low interference with the oncological treatment and long-term results. The success of the procedure depends on patient selection, coordinated planning and careful intra-operative management.

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Key words: Breast reconstruction; Conservative breast surgery; Partial mastectomy; Oncoplastic; Reduction mammoplasty; Outcome; Complications

Abstract

Breast-conservation surgery (BCS) is established as a safe surgical treatment for most patients with early breast cancer. Recently, advances in oncoplastic techniques are capable of preserving the breast form and quality of life. Although most BCS defects can be managed with primary closure, the aesthetic outcome may be unpredictable. Among technical options, therapeutic reduction mammoplasty (TRM) remains a useful procedure since the BCS defect can be repaired and the preoperative appearance can be improved, resulting in more proportional breasts. As a consequence of rich breast tissue vascularization, the greater part of reduction techniques have based their planning on preserving the pedicle of the nipple-areola complex after tumor removal. Reliable circulation and improvement of a conical shape to the breast are commonly described in TRM reconstructions. With an immediate approach, the surgical process is smooth since both procedures can be carried out in one operative setting. Additionally,

Core tip: Recently, advances in oncoplastic techniques are capable of preserving the breast form and quality of life. Among technical options, therapeutic reduction mammoplasty remains a useful procedure since the breast-conservation surgery defect can be repaired and the preoperative appearance can be improved. Additionally, it permits wider excision of the tumor, with a superior mean volume of the specimen and potentially reduces the incidence of margin involvement. The main advantages of the technique utilized should include reproducibility, low interference with the oncological treatment and long-term results. The success of the procedure depends on patient selection, coordinated planning and careful intra-operative management.

Munhoz AM, Montag E, Gemperli R. Current aspects of therapeutic reduction mammoplasty for immediate early breast cancer management: An update. *World J Clin Oncol* 2013; 5(1): 1-18

INTRODUCTION

Breast-conservation surgery (BCS) is an important component of early breast cancer treatment, with a survival outcome comparable to that of radical procedures^[1]. In fact, the long-term survival of BCS with radiation is not statistically different when compared with mastectomy in patients with Stage I or II breast cancer^[2].

BCS with concomitant reconstruction has been developed over the past decades. In essence defined as oncoplastic surgery, the procedure refers to a number of surgical techniques by which breast tumors are resected while the remaining glandular tissue is transposed to achieve a satisfactory aesthetic outcome^[2-4]. A variety of such techniques has been described, including volume replacement by local glandular flaps and breast reshaping by therapeutic reduction mammoplasty (TRM) or regional/distant flaps^[5-12].

In spite of the acceptance that most BCS defects can be managed with primary closure, some lesions are complex to resect without the risk of aesthetic deformity. In fact, favorable aesthetic outcome can be difficult to achieve and an example of this is in patients with large breast tumors in relation to breast size^[2,6,11]. In addition, radiation can also have a negative effect on the glandular and skin tissue. In our experience, the main clinical aspects are related to skin pigmentation changes, telangiectasia and skin fibrosis^[11,13-17].

Recently, clinical experience with oncoplastic surgery has demonstrated the freedom to perform wider tumor excisions, potentially reducing margin involvement. Additionally, another benefit is the possibility of allowing the excision of larger tumors without compromising cosmetic outcome^[6-18]. Thus, by means of customized techniques the surgeon ensures that oncological principles are not jeopardized while meeting the needs of the patient from an aesthetic point of view^[3].

In general, the oncoplastic procedures are related to volume displacement or replacement techniques and sometimes include contralateral breast surgery. Regardless of the fact that there is no consensus concerning the best approach, the criteria are determined by the surgeon's experience and the size of the defect in relation to the size of the remaining breast^[11]. The main advantages of the technique utilized should include low interference with the oncological treatment, reproducibility and long-term results. Probably, all these objectives are not achieved by any single procedure and each technique has advantages and limitations^[11].

LITERATURE SEARCH/DATA EXTRACTION

Two independent reviewers evaluated titles and abstracts

without language restrictions to assess eligibility in terms of outcome measures and study design. A literature search was carried out up to August 2013 to identify studies of breast cancer patients treated with oncoplastic surgery procedures and to determine if any use of mammoplasty techniques was recorded. In an attempt to minimize the omission of potentially relevant clinical studies, we also reviewed the reference lists of included studies and relevant reviews for additional eligible articles. Potential studies were identified by searches of Medline and PubMed databases using the terms "oncoplastic breast surgery", "conservative breast surgery reconstruction", "partial mastectomy reconstruction", "therapeutic mammoplasty" and "reduction mammoplasty". Studies identified were screened for those that focused on techniques, surgical and oncological outcomes after therapeutic mammoplasty reconstruction and references of each study were further investigated to include all relevant published data.

A total of 1382 potential articles were identified during the primary evaluation. After appraisal of the inclusion criteria, 311 articles were identified for potential inclusion and reviewed in detail. A total of 226 articles were excluded, leaving 77 articles to form the basis of this review.

THERAPEUTIC REDUCTION MAMMAPLASTY

Among the main technical options, TRM remains a useful procedure. Usually, the application of TRM involves resection of the tumor and remodeling the breast using an aesthetic breast reduction technique. As a consequence of rich breast tissue vascularization, the greater part of TRM has based their planning on preserving the pedicle of the nipple-areola complex (NAC) after tumor removal. Usually, the procedure is adequate for patients with moderate/larger breasts requiring excision of significant volumes of tissue and contralateral symmetrization. With TRM, the BCS defect can be repaired and the preoperative appearance can be improved, resulting in more proportional breasts^[15-17]. In addition, the technique reduces the difficulty of providing radiation therapy to the remaining breast tissues with acceptably low complication rates^[19-21]. In terms of local control and oncological outcome, the added removal of a substantial volume of breast tissue could add a significant amount of safety in terms of surgical margins^[22,23].

INDICATIONS OF TRM: TIMING, TECHNIQUES

Timing of reconstruction

With immediate reconstruction, the surgical process is smooth since BCS and TRM can be carried out in one operative setting. Additionally, because there is no scar and fibrosis tissue, breast reshaping is easier and the aes-

thetic is improved^[6,8,9,11,12,24-27]. In fact, Kronowitz *et al*^[9] observed that immediate repair is preferable to being delayed because of a decreased incidence of complications. In our previous experience utilizing TRM for BCS reconstruction, we observed that our post-radiation complication rate (delayed BCS reconstruction) was higher than that expected for TRM without radiotherapy (immediate BCS reconstruction)^[24]. This finding is similar to previous studies that suggest that delayed BCS reconstruction has a significantly higher complication rate compared with immediate procedures^[8,9].

In terms of oncological benefits and adjuvant treatment, immediate reconstruction can be advantageous. Some clinical series have observed that patients with large volume breasts present with more radiation related complications than patients with normal volume breasts^[19-21]. Additionally, some studies observed that there is an increased fat content in large breasts and the fatty tissue results in more fibrosis after radiotherapy than glandular tissue^[21]. Thus, TRM can increase the eligibility of large-breasted patients for BCS since it can reduce the difficulty of providing radiation therapy^[15-17,19,23]. Gray *et al*^[21], in a series of 257 patients, found that there was more retraction and asymmetry in the large-breasted *vs* the small-breasted group. Usually, these deformities are complicated to manage and habitually necessitate secondary reconstruction with autologous tissue.

Another aspect is the possibility of accomplishing a negative resection margin. In fact, the immediate reconstruction with TRM allows for wider local tumor excision, potentially reducing the incidence of margin involvement^[15-17,22,23]. Kaur *et al*^[22] compared patients submitted to oncoplastic procedures and to BCS. The immediate reconstruction permitted larger resections, with a superior mean volume of the specimen and negative margins.

In spite of the benefits, the immediate TRM reconstruction presents some negative aspects. The surgical time can be lengthened and require specialist training to learn and properly apply these procedures^[2,3,15]. Another point is related to the postoperative breast volume and shape^[26]. In fact, in some cases the final contour of the breast cannot be predicted at the time of the BCS and although the aesthetic result can be adequate, the outcome of the radiated breast is sometimes less favorable than the nonradiated breast^[5-8,23,26]. Despite there being no consensus, in delayed reconstruction, the plastic surgeon could wait until the postoperative changes in the deformed breast stabilize.

Another important point is related to the postoperative recovery. In theory, some complications of the immediate TRM reconstructions can unfavorably defer the adjuvant therapy. With delayed oncoplastic reconstruction, operative time is shortened and the surgical process is less extensive than an immediate one. Additionally, depending on the technique of reconstruction utilized, surgical techniques can be complicated and lengthy, and

potentially associated with relatively high postoperative complication rates. In fact, some surgeons are concerned that immediate application of TRM may delay adjuvant systemic and locoregional treatment and compromise prognosis. However, our previous experience with TRM^[11,14-17] and that of others^[8,18,23] has shown that an immediate approach does not compromise the start of radio and chemotherapy in the overall treatment of breast cancer. Besides the evidence in the literature, it has been our impression that nothing suggests that immediate application of TRM is not safe enough in terms of starting adjuvant therapy. In fact, Kahn *et al*^[28] in a series of 169 patients submitted to BCS provide evidence that immediate reconstruction does not lead to a delay in the commencement of adjuvant chemotherapy when compared to three adequate control groups from the same institution and time periods.

Breast defect definition

BCS reconstructive planning should include the breast volume, tumor location, the extent of glandular tissue resected, and chiefly address individual reconstructive requirements^[11]. Evaluation of BCS reconstruction must subsequently consider these important points and only then should the proper TRM procedure or a combination of procedures be chosen^[2,11]. It has been our experience that each BCS defect has its own special reconstructive necessities varying expectations for aesthetic outcome. On the basis of our 15 years of experience, it is possible to identify trends in types of breast defects and to develop an algorithm for immediate BCS reconstruction on the basis of the initial breast volume, the extent/location of glandular tissue resection and the remaining available breast tissue^[11]. To make possible development of a BCS reconstructive algorithm, immediate partial breast defects are classified into one of three types (Figure 1).

Type I : Defects include tissue resection in a smaller breast without ptosis. Type I A defects involve minimal defects that do not cause volume alteration/distortion in the breast shape and the tissue resected is less than 10%-15% of the total breast volume. Type I B defects involve moderate defects that do originate from moderate volume alteration/distortion in the breast shape or symmetry and the tissue resected is between 15% and 40% of the total volume. Usually, the skin above the tumor is resected with the tumor. Type I C defects involve large defects that do cause significant volume alteration/distortion in the breast shape and symmetry and the tissue resected is more than 40% of the total breast volume.

Type II : This group includes tissue resection in medium sized breasts with/without ptosis. Type II A involves small defects that do not cause enough volume alteration/distortion in the breast shape. Type II B defects involve moderate defects that cause minor/moderate volume alteration in the breast shape. Type II C defects

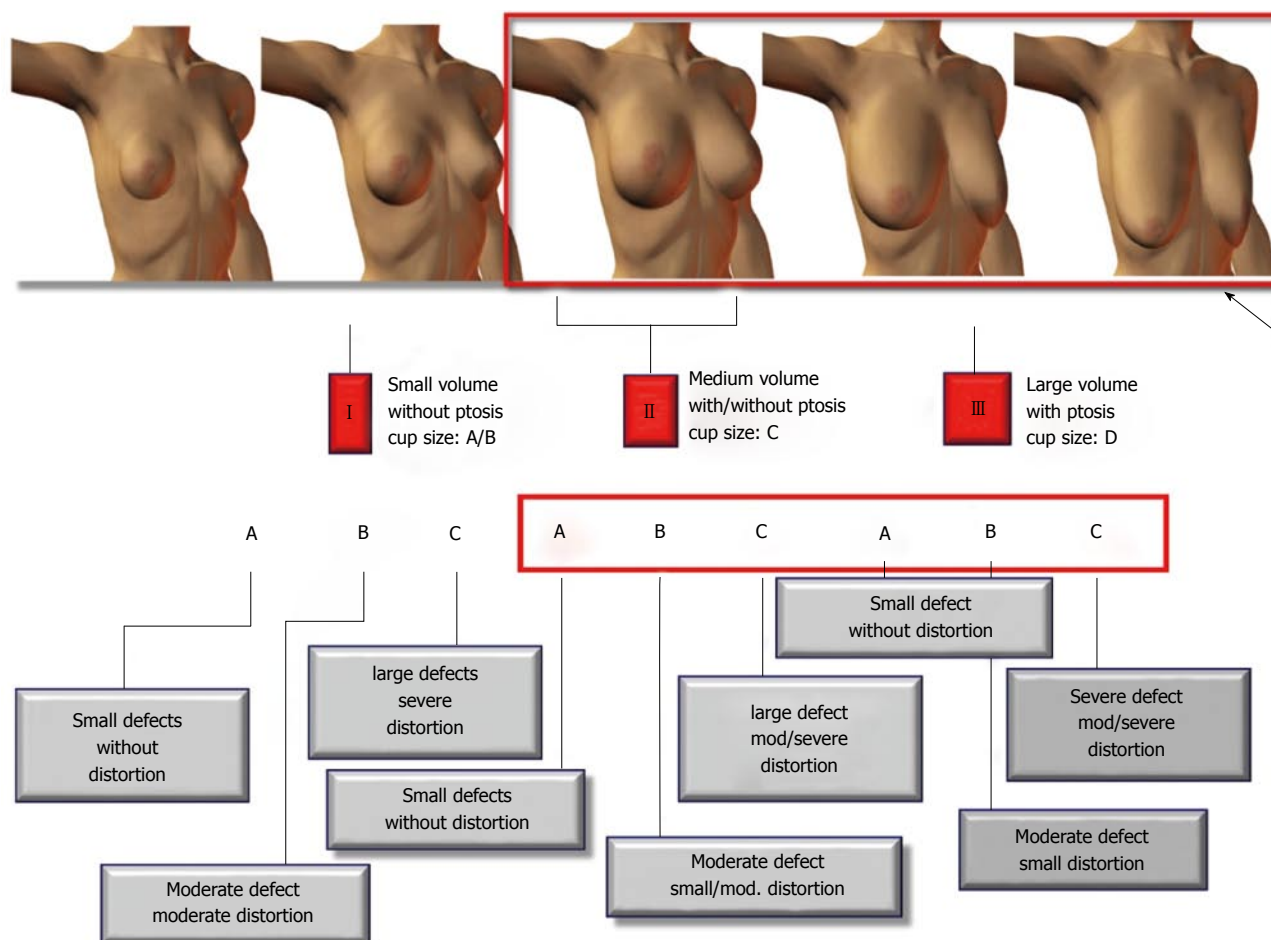


Figure 1 Algorithm for immediate conservative breast surgery reconstruction based on the type of breast and extent of defect.

involve large defects that cause moderate/large volume variations in the breast shape and symmetry.

Type III: This group includes tissue resection in large sized breasts with ptosis. Type IIIA involves small defects that do not cause enough aesthetic deformity. Type IIIB defects involve moderate defects that originate from minor/moderate volume alterations in the breast shape or symmetry. Type IIIC defects involve large defects that cause significant volume alteration in the breast.

Surgical techniques and the role of TRM

BCS defects can be scored and classified according to the proposed classification^[11]. In our experience, the majority of reconstruction techniques are performed with one of six surgical options: breast tissue advancement flaps (BAF), lateral thoracodorsal flap (LTDF), TRM (bilateral mastopexy and bilateral reduction mammoplasty), latissimus dorsi myocutaneous flap (LDMF) and abdominal flaps.

Habitually, TRM may be indicated if the patient has a moderate/large breast volume or if there is breast ptosis. Patient selection should be primarily limited to those who desire breast reduction and those who have at least moderately sized breasts with a defect that is suspected to be at least small and moderate in size (Figure 1). Surgi-

cal planning should include breast characteristics, extent of breast tissue resected, and chiefly address individual reconstructive requirements. Additionally, the decision is usually determined by the surgeon's preferences and the size of the defect in relation to the size of the remaining breast. In fact, it is important to identify trends in types of breast defects on the basis of the initial breast volume, the extent/location of glandular tissue resection and the remaining available breast tissue.

Type I A, II A and III A: Defects are usually repaired with BAF in which the defect created is usually spherical or rectangular. The breast tissue is advanced along the chest wall or beneath the breast skin flap to fill the tumor defect. Usually, in these patients no contralateral breast procedure is performed.

Type I B: In patients with lateral defects, the LTDF is performed. Previously described elsewhere^[14], this local flap is planned as a wedge-shaped triangle located on the lateral aspect of the thorax. Although additional scars are created, they will be placed in the lateral region. The defect margins are sutured to the margins of the flap and the donor site is closed primarily. In patients with central and medial tumors, the LDMF can be utilized^[11,13]. The flap is designed into a horizontal position and the width

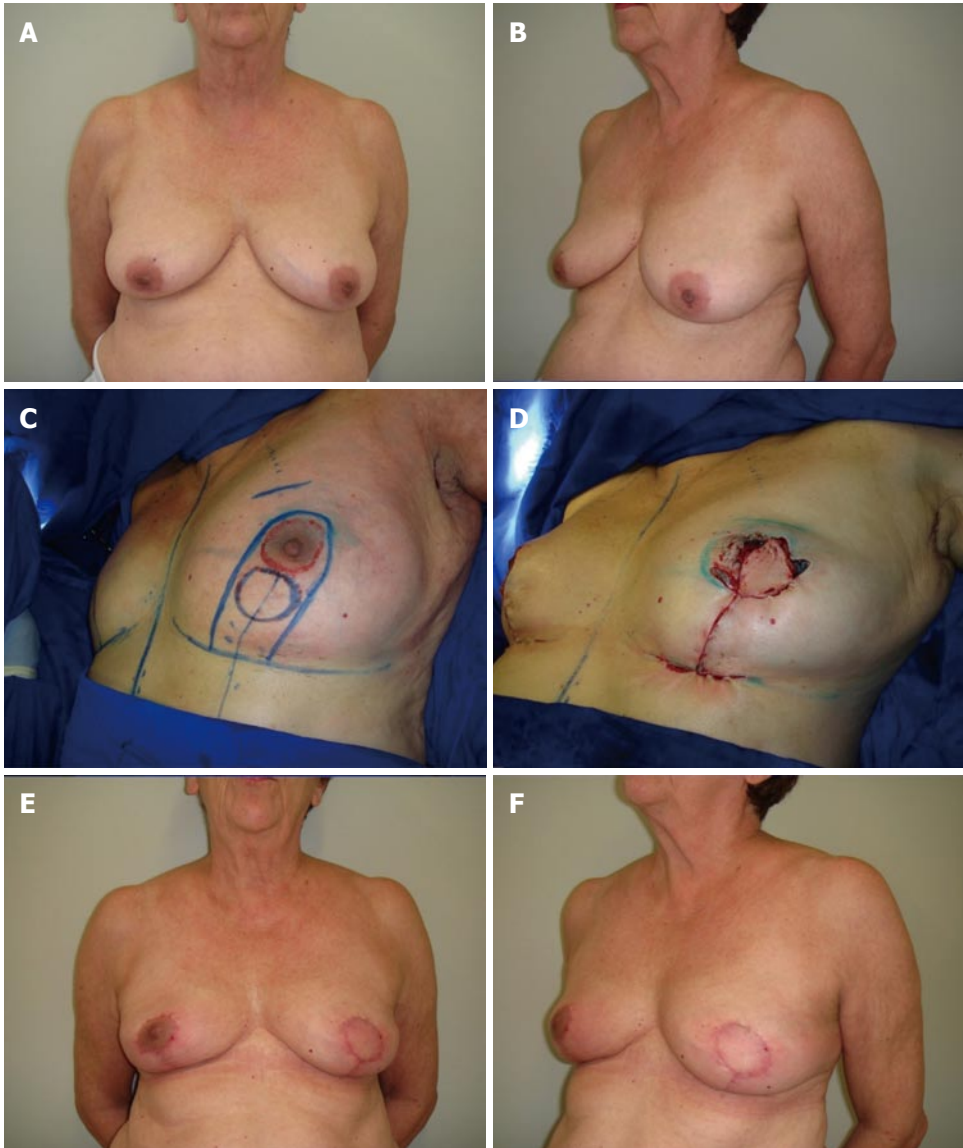


Figure 2 A 62-year-old patient with invasive ductal carcinoma (1.7 cm) of the left breast. The patient underwent a left central quadrantectomy and sentinel lymph node biopsy, immediately followed by a therapeutic reduction mammoplasty Inferior Pedicle Technique reconstruction (A-B, above left and right); A total of 78 g was removed from the left breast (C-D, center left and right); One year postoperative appearance after the radiotherapy (E-F, below left and right).

of the paddle is measured according to the skin previously resected.

Type I C: Defects are converted to a skin-sparing mastectomy (SSM) and reconstructed with an appropriate technique. In patients with enough abdominal tissue, an abdominal flap (pedicled/free TRAM or DIEP) can be an option according to the surgeon's preference. In patients without an adequate abdomen, a LDMF associated with an implant can be performed.

Type II B: Defects are frequently reconstructed with TRM techniques when there is sufficient breast tissue to perform the reconstruction (Figure 2).

Type II C: Defects are analyzed individually according to the size of the breast defect in relation to the remain-

ing breast tissue available. For this purpose, the patient is positioned upright to assess the amount of the remaining glandular tissue. Thus, type II C can be subclassified into favorable and unfavorable defects. If there is enough tissue to perform an adequate breast mound shaping, the defect is classified as favorable. For the lateral defects, the extended LTDF is most commonly employed. In patients with central and medial defects, the extended LDMF can be utilized^[13]. Conversely, if not enough breast tissue remains, the breast defect is classified as unfavorable and a SSM and total reconstruction is indicated.

Type III B: Defects are frequently reconstructed with TRM techniques when the patient presents with large volume breasts and there is a sufficient amount of breast tissue. The most favorable tumor location is in the lower breast pole where a conventional superior pedicle or

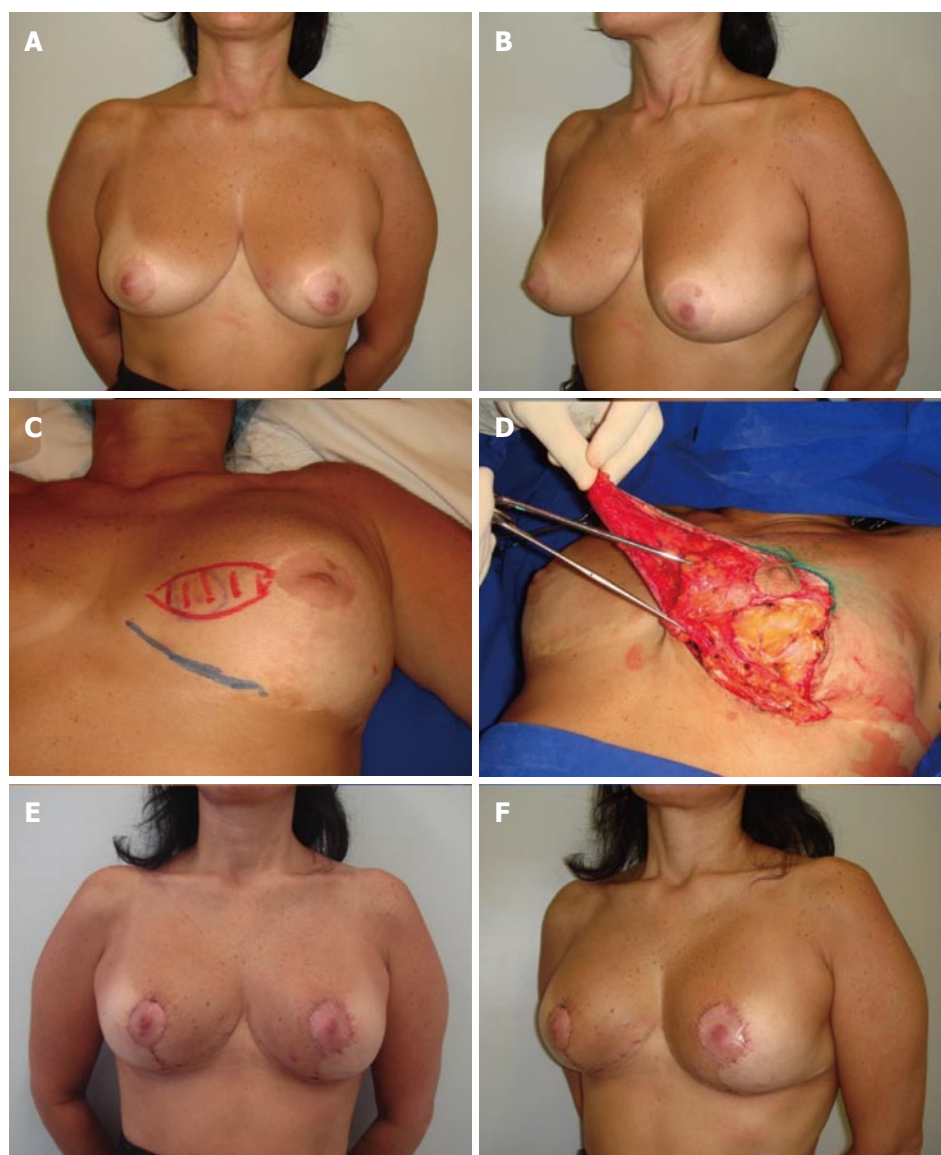


Figure 3 A 48-year-old patient with invasive ductal carcinoma (3.2 cm) of the inferior-medial quadrant of the left breast. The patient underwent a left inferior-medial quadrantectomy and sentinel lymph node biopsy, immediately followed by a therapeutic reduction mammoplasty Superior-Lateral Pedicle Technique reconstruction (A-B, above left and right); A total of 125 g was removed from the left breast (C-D, center left and right); Six months postoperative appearance after the radiotherapy with a very good outcome (E-F, below left and right).

superior-medial technique can be utilized^[15,16]. In patients with central tumors, an inferior pedicle is used to carry parenchyma and skin into the central defect^[17] (Figure 3).

Type III C: Breast defects are analyzed individually. When the defect is favorable, the deficiency is most frequently reconstructed with TRM (Figure 4). A marked reshaping of the breast with available tissue and a similar contralateral breast reduction is then performed. In patients in whom the relationship is not favorable, a skin-sparing mastectomy and total breast reconstruction can be indicated.

TYPES OF TRM TECHNIQUES

In spite of the reduction mammoplasty being a well

documented and commonly performed procedure for aesthetic objectives, less information is available regarding the outcome following immediate reconstruction for oncological objectives. To date, there are few large clinical reports that specifically address the use of the technique for immediate reconstruction and its outcome^[8,15-19] (Table 1). In addition, there is no consensus regarding the best TRM technique for immediate BCS reconstruction. Possibly, an ideal procedure does not exist and each case should be planned individually. The main advantages of the TRM technique utilized should include reproducibility, safety and long-term results. As any surgical technique, all these goals are probably not met by any single procedure and this is supported by the large number of TRM techniques available^[13].

Concerning the techniques, TRM procedures present

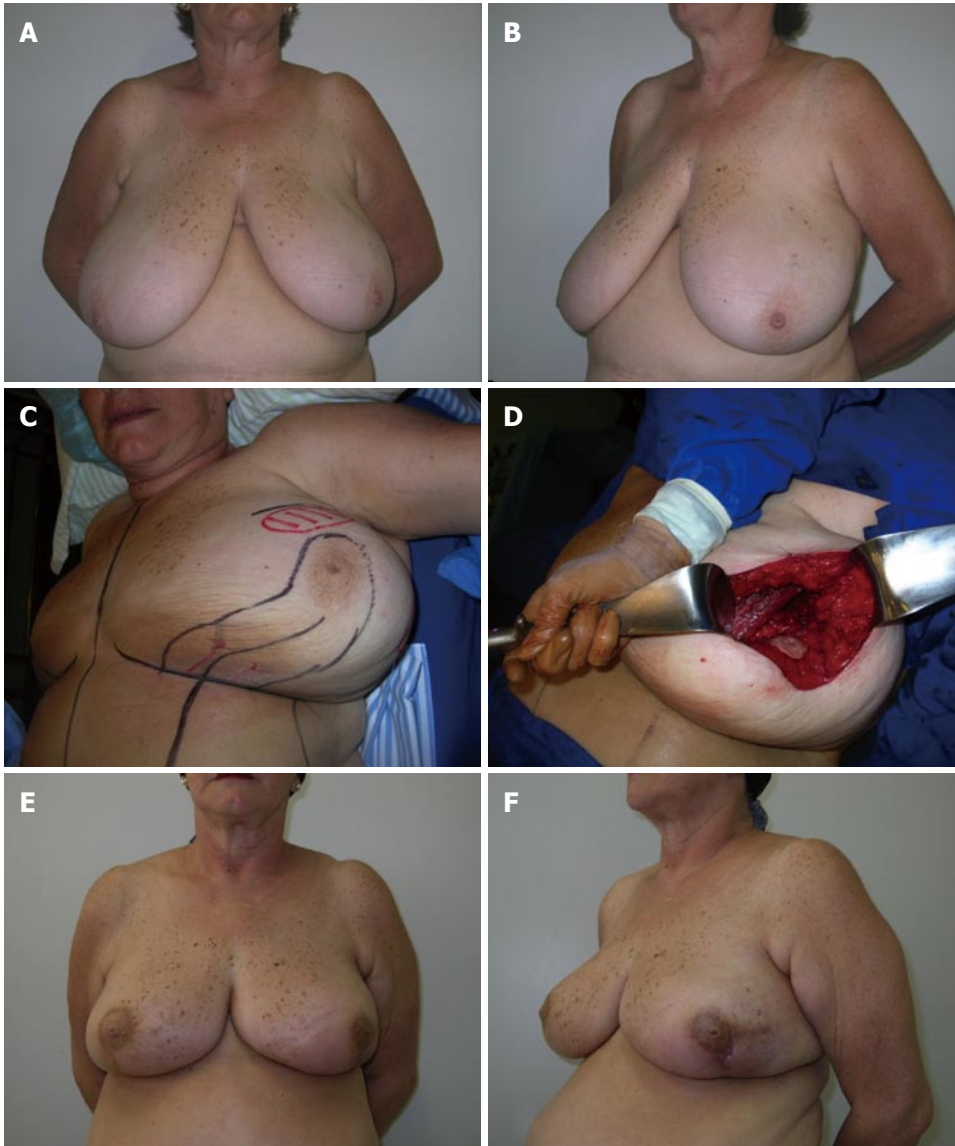


Figure 4 A 58-year-old patient with invasive ductal carcinoma (5.7 cm) of the superior quadrant of the left breast. The patient underwent a superior left quadrantectomy and axillary dissection, immediately followed by a therapeutic reduction mammoplasty Inferior Pedicle Technique reconstruction (A-B, above left and right); a total of 825 g was removed from the left breast (C-D, center left and right); One year postoperative appearance after the radiotherapy with a very good outcome (E-F, below left and right).

Table 1 Oncological and outcome using reduction mammoplasty techniques

Ref.	Year	Technique	n	Tumor size (cm)	Follow-up (mo)	Local recurrence (%)	Patient satisfaction (%)
Papp <i>et al</i> ^[12]	1998	Superior pedicle	10	NR	52	5	95
Nos <i>et al</i> ^[71]	1998	Superior pedicle	50	3.25	48	7	85
Spear <i>et al</i> ^[18]	2003	Superior pedicle	56	NR	46	6.9	91
Clough <i>et al</i> ^[41]	2003	Superior pedicle	101	3.2	24	0	88
Chang <i>et al</i> ^[19]	2004	Superior pedicle	37	0.6-5.2	NR	0	NR
Goffman <i>et al</i> ^[74]	2005	Superior pedicle	57	NR	18	13	82
Munhoz <i>et al</i> ^[15]	2006	Superior pedicle	74	2-4.0	22	0	93
Munhoz <i>et al</i> ^[16]	2006	Superior-medial pedicle	39	2-4.0	20	0	90
Munhoz <i>et al</i> ^[17]	2007	Inferior pedicle	26	2-4.0	21	0	89
Fitoussi <i>et al</i> ^[47]	2010	Superior pedicle	540	2.9	49	6.8	90

with a variety of glandular pedicle types with an inverted T scar design. Most of the techniques are predictable and permit management over the extent of resection

and the breast-shaping process. Because of rich breast tissue vascularization, the majority of techniques have based their planning on preserving the pedicle of the

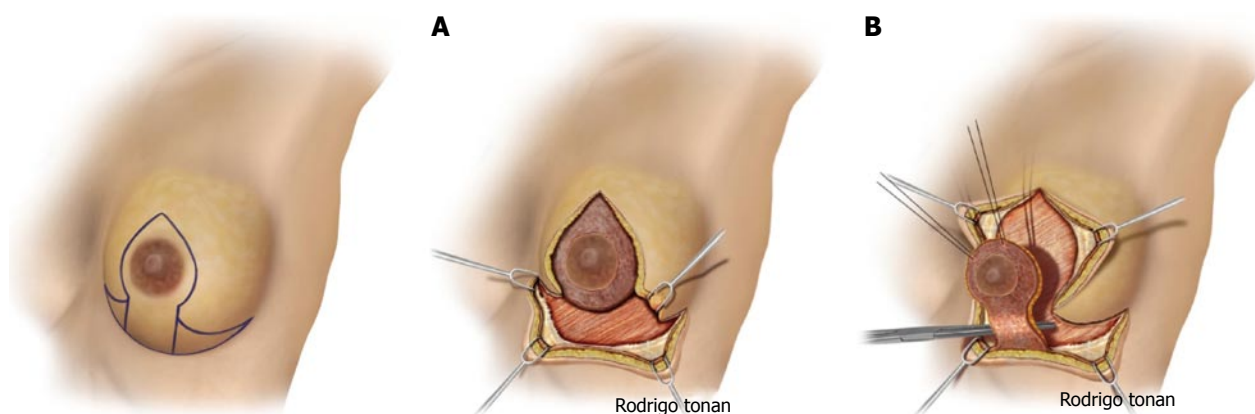


Figure 5 Schematic illustration of the therapeutic reduction mammoplasty surgical markings and Superior (A) and Inferior pedicle (B) techniques.

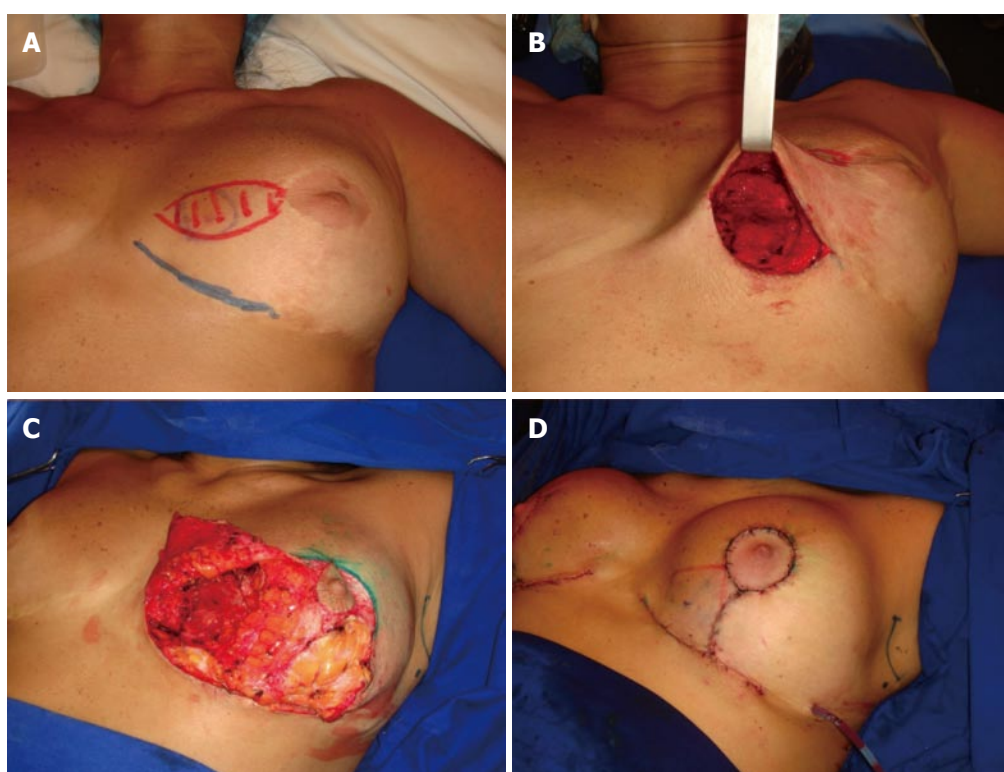


Figure 6 The superior-lateral pedicle technique for inferior-medial quadrantectomy reconstruction. For tumors located in the lower pole of the breast, the tumor resection can be incorporated into the sector of breast tissue removed as part of a superior pedicle mammoplasty (A-B); For inner region tumors, the reduction pattern can be rotated and a superior-lateral (C); The opposite breast surgery is usually performed to match the appropriate symmetry (D).

NAC after tumor removal. Each technique presents particular advantages for their indications, tumor location, additional skin and glandular resections and resultant scar^[15]. Typically, one of two procedures can be utilized: the superior pedicle and the inferior pedicle technique (Figure 5). A range of other approaches have been described, which are adaptations of the superior and inferior procedures.

Superior pedicle techniques

For tumors located in the lower pole of the breast, the tumor resection can be incorporated into the sector of breast tissue removed as part of a superior pedicle mam-

maplasty^[15,16]. For inner and outer region tumors, the reduction pattern can be rotated and a superior-lateral (Figure 6) or a superior-medial pedicle mammoplasty (SMDP) can be performed^[16] (Figure 7). The opposite breast surgery is usually performed to match the appropriate symmetry, particularly in breasts with severe ptosis. In our previous study analyzing different TRM for BCS reconstruction, the superior pedicle represented almost 90% of cases^[15].

In our clinical practice and with a well-trained surgical team, the procedure can be conducted on both sides at the same time, consequently reducing the operative time. When performing symmetrization, the surgeon can use

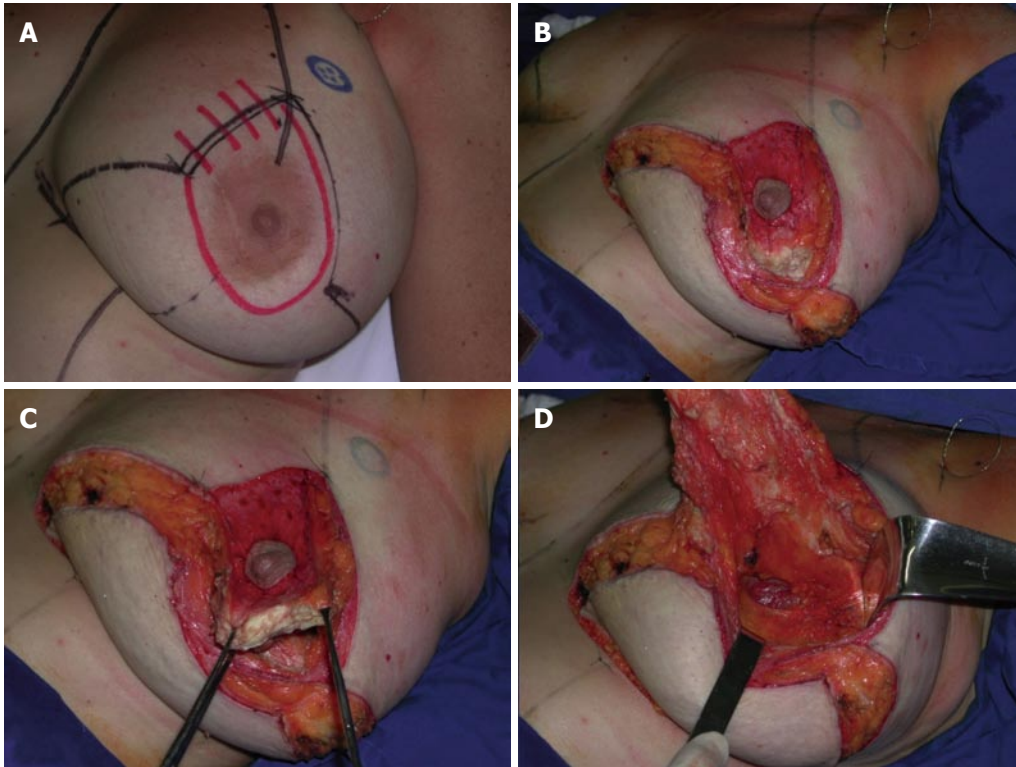


Figure 7 The superior-medial pedicle technique for superior-lateral quadrantectomy reconstruction. For superior and outer region tumors, the reduction pattern can be rotated and a superior-medial (A-D).

this opportunity to resect any suspicious breast lesion that may have been revealed by preoperative exams^[15,24].

Described originally by Orlando and Guthrie in the 1970s, the SMDP technique presents advantages in terms of pedicle safety and aesthetic outcome^[29]. The technique is designed to preserve the pedicle blood flow as well as to provide a better breast shape^[29-31]. The superior-medial pedicle receives its blood supply directly from the internal mammary vessels which are the main pedicle in the majority of patients^[31,32]. This anatomical characteristic permits a better NAC vascularization and can minimize vascular pedicle complications when planned and performed effectively.

In our previous experience, the SMDP was indicated in patients with medium or large volume breasts with ptosis^[16]. According to our algorithm, all tumors were located in the lower breast pole and the patients presented with small or moderate defects where there was enough breast tissue to perform the reconstruction^[11]. Similar to our previous study with superior pedicle techniques^[11,15], the planning of the SMDP procedure was based on preserving the pedicle of the NAC before tumor removal. In this situation, the tumor resection was incorporated into the sector of breast tissue removed as part of a conventional SMDP technique and it usually results in an inverted T scar pattern. Normally, the NAC is de-epithelialized and a minimum pedicle base of 4 cm is maintained, depending on the extension of tumor resection. If a large pedicle is maintained, it may be that more vascular supply can be incorporated. It is important to

maintain the distal aspect of the pedicle with a minimum 2 cm margin around the NAC in order to preserve the local vascular plexus. Concerning the final breast shape and projection, some authors in aesthetic reductions advocate the addition of extra glandular tissue in the pedicle to form a platform for the NAC so that it does not become depressed postoperatively. One important point is that the pedicle should rotate without difficulty into its new position^[31]. At this point, the most crucial aspect is to avoid some degree of kinking or compression^[32]. If too much glandular tissue is maintained in the pedicle, it will also make it more difficult to rotate and insert it in the new NAC position. Moreover, Hall-Findlay has suggested that there is a risk to the glandular tissue in excess drop down which might result in a pseudoptotic breast^[32].

In spite of the main advantages, the SMDP technique has some negative aspects. Since the NAC flap is not an axial flap, tissue vascularization to the most distant parts is difficult to foresee during the NAC pedicle planning and breast molding. This situation can predispose to partial necrosis of the NAC flap and even a total NAC loss. With the objective of improving the NAC vascularization and to avoid pedicle torsion, it is important to define the right position of the NAC pedicle according to the degree of breast ptosis and hypertrophy. Similar to the proposal by Cárdenas-Camarena *et al.*^[33], in the superior lateral technique we advocate locating the pedicle lower down when the degree of ptosis and migration of the NAC is greater to avoid marked rotation (Figure 8).

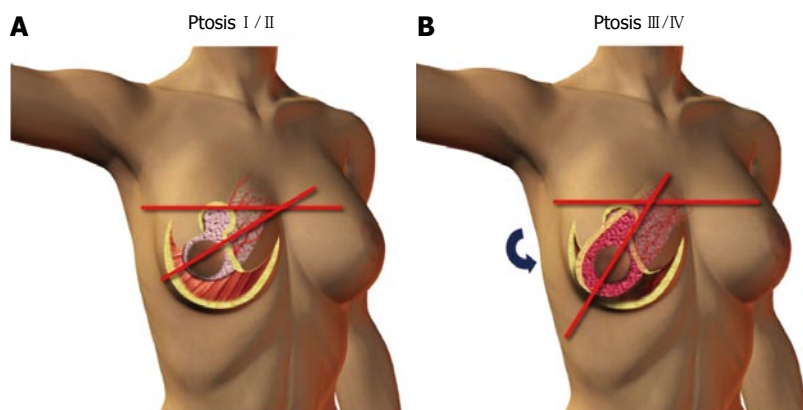


Figure 8 Schematic illustration of the superior-medial pedicle technique according to the degree of breast ptosis and the rotation of the pedicle. With the objective of improving the nipple-areola complex (NAC) vascularization and to avoid pedicle torsion, it is important to define the right position of the NAC pedicle according to the degree of breast ptosis and hypertrophy. It is important to locate the pedicle lower down when the degree of ptosis and migration of the NAC is greater to avoid marked rotation.

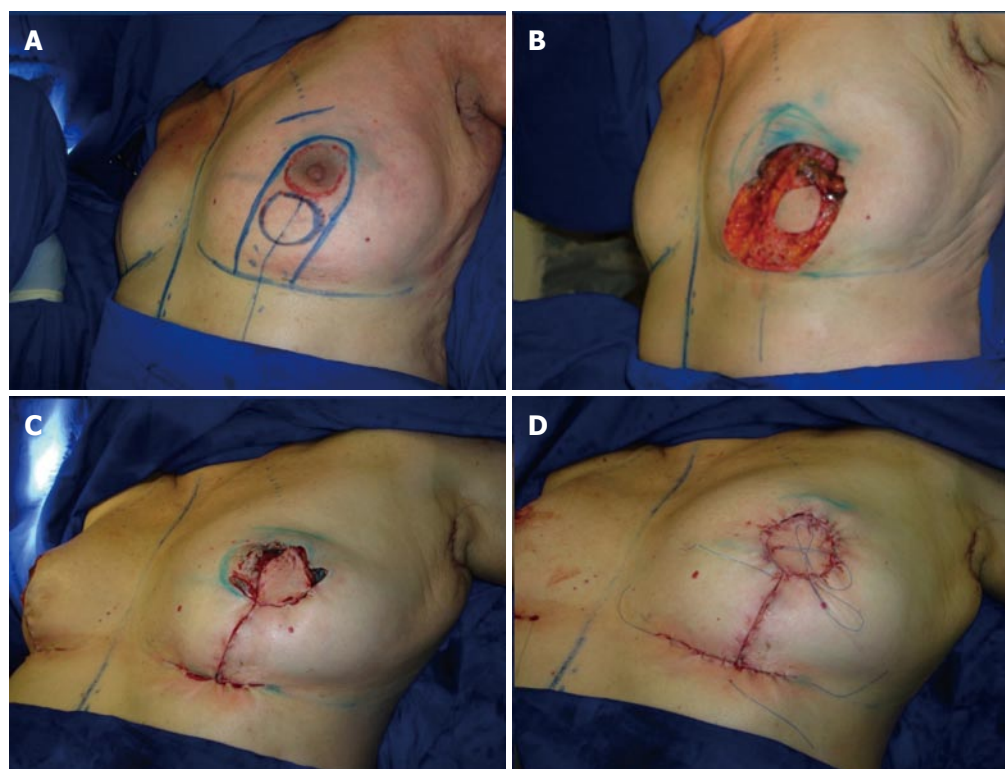


Figure 9 Inferior pedicle technique for central quadrantectomy reconstruction. For central region tumors (A), the lower breast and skin tissue may be moved into the defect as a skin-glandular flap and an inferior pedicle mammaplasty can be utilized (B-D).

Inferior pedicle techniques

For upper region tumors, the lower breast tissue may be moved into the defect as a glandular flap and an inferior pedicle mammaplasty (IPM) can be utilized^[17]. Introduced originally by Ribeiro^[34] in the 1970s and subsequently modified by Courtiss and Goldwyn^[35], the technique described the transposition of the NAC on an inferiorly based dermoglandular flap.

Concerning the IPM technique, there are some advantages in terms of pedicle safety and results. The inferior pedicle receives its blood supply directly from the fourth, fifth and sixth intercostal perforating vessels of the internal mammary arteries. In fact, Courtiss and Goldwyn^[35] demonstrated by cadaver dissections that the principal sources of blood flow are the perforating and intercostal branches of the internal mammary artery and the ex-

ternal mammary branches of the lateral thoracic artery. Thus, the IPM provides a breast that is easily shaped, without neurovascular changes to NAC^[36-39].

In our experience, the IPM was performed to repair skin and breast tissue and was indicated in patients with medium or large volume breasts with ptosis^[17]. All tumors were located in the upper and central breast pole and the patients presented with small or moderate defects where there was enough breast tissue to perform the reconstruction. In the central tumors, the NAC is resected and the lower pole tissue is preserved to perform the reconstruction (Figure 9). Despite no cases of contralateral tumor being observed in our series utilizing the IPM, the contralateral mammaplasty provides an opportunity for histological examination of tissue from the opposite breast. In fact, Petit *et al*^[40] reported a 4.5% incidence of

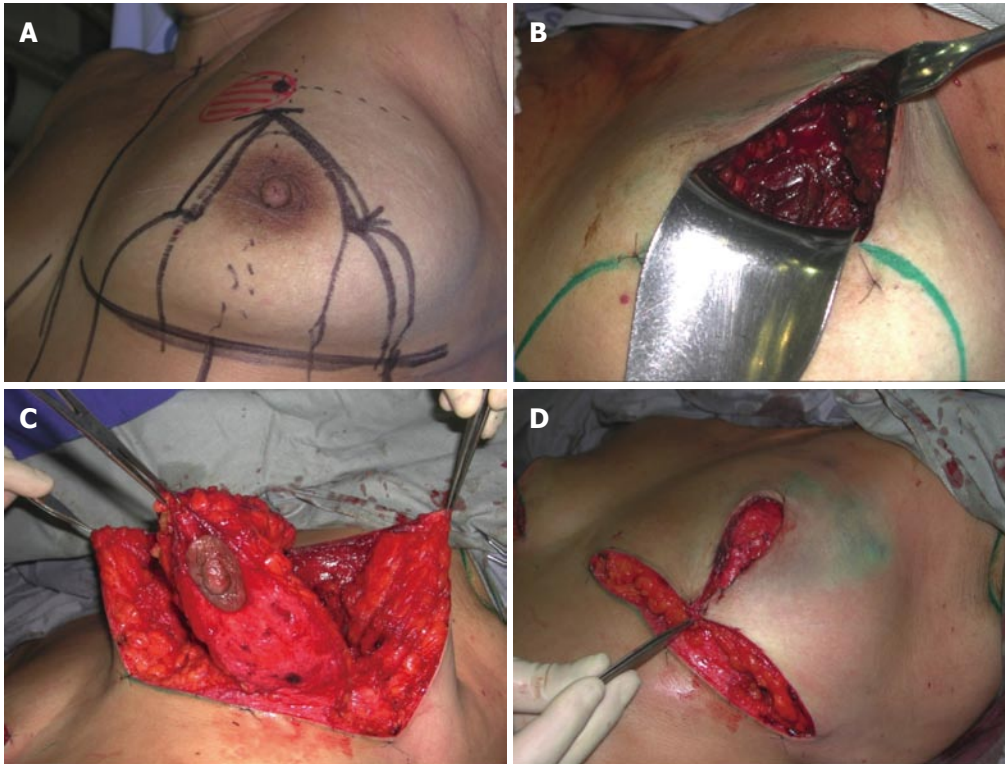


Figure 10 Inferior pedicle technique for upper quadrantectomy reconstruction. For upper region tumors (A), the lower breast and skin tissue associated with the nipple-areola complex may be moved into the defect as a skin-glandular flap and a conventional inferior pedicle mammoplasty can be utilized (B-D).

occult carcinomas in the contralateral breast in a series of 440 patients. Similarly, in our previous larger study, 4.3% of the patients submitted to contralateral mammoplasty presented with the diagnosis of occult breast cancer^[24].

IPM for BCS reconstruction can be a helpful technique for early breast cancer patients; nevertheless, important technical details must be considered. Preoperative evaluation can permit optimal positioning of the skin resection and to preserve the perforator vessels. Normally, the inferior pedicle is de-epithelialized and a minimum pedicle base of 6 cm is maintained. Concerning the breast contour close to the partial mastectomy area, it is practical to bend the inferior pedicle over itself to provide extra glandular tissue so that it does not become depressed postoperatively. For this purpose, the inferior pedicle should lie comfortably within the defect and it is important to avoid additional undermining to preserve the local vascular plexus (Figure 10). Even although we observed a low incidence of vascular complications, care must be taken in treating high risk patients, such as smokers and patients with a longer sternal notch to NAC distance. It has been our impression and that of some authors that the inferior pedicle length and the pedicle placement without tension are the main determinants of complications.

In spite of the advantages, the IPM presents some limitations. Although some authors have observed that the technique does not represent a problem with surveillance, with breast remodeling techniques it may be difficult to establish the location of a positive tumor

margin that is diagnosed postoperatively. In cases of re-exploration, it is rational to execute it in association with the plastic surgery team to identify the original tumor bed and to avoid injury to the inferior pedicle.

In our experience, we observed that the majority of the complications utilizing IPM for BCS reconstruction were immediate, minor and did not interfere with the adjuvant treatment^[17]. Similarly, as we have observed in superior pedicle techniques, the most frequent complications in the late period were related to skin disorders and fat necrosis which were diagnosed by mammography during the follow-up. According to Losken *et al.*^[23], postoperative surveillance is not impaired by immediate mammoplasty. In fact, calcifications and fat necrosis from postoperative scarring can mimic cancer recurrence; nevertheless, these changes can be distinguished on mammography, fine-needle aspiration or core biopsy.

CLINICAL RESULTS OF TRM IN BCS RECONSTRUCTION

Various oncoplastic procedures have been demonstrated to improve aesthetic outcome following BCS. Techniques range from simple glandular flaps to TRM and distant flaps^[11]. At the present time, optimal treatment should be correct, adequate and preventive by performing immediate reconstruction before radiotherapy^[9,24,25]. However, to date there is limited evidence in the plastic and breast surgery literature on the safety and aesthetic clinical results of the TRM techniques^[8,9,10,24,25,27,28]. In fact, most

of these clinical series are retrospective studies, generally based on a limited number of patients and sometimes only a single surgeon's experience. In addition, there are a small number of data on its impact on local recurrences, distant metastasis and overall survival^[9,41,42].

Some retrospective clinical studies with long-term follow-up have shown that TRM is not associated with a higher rate of recurrence or lower survival rate than conventional techniques. Clough *et al.*^[41] with a median follow-up of 46 mo reported 101 patients who underwent BCS and oncoplastic reconstruction. Local recurrence developed in 11 cases (5 years local recurrence rate was 9.4%). Thirteen patients developed metastases and eight died of their disease (5 years metastasis-free survival of 82.8% and an overall survival rate of 95.7%). Similarly, Kronowitz *et al.*^[9] in a review of 69 patients observed local recurrence in 2% of immediate oncoplastic reconstructions and in 16% of delayed ($P = 0.06$). The difference observed between the two groups can be explained by the advanced tumor stage for the patients who had a delayed reconstruction. Recently, Rietjens *et al.*^[42] reported the long-term oncological results of the oncoplastic reconstruction in a series of 148 patients. With a median follow-up of 74 mo, 3% developed an ipsilateral breast cancer recurrence and 13% developed distant metastasis. According to the authors, the rate of local recurrence after 5 years was low in their series when compared with the 14.3% of cumulative incidence in the NSABP trial, the 9.4% after 5 years in the Institut Curie study and the 0.5% after 5 years in the Milan I trial. Consequently, the oncoplastic approach associated with BCS can be considered as safe as mastectomy in tumors less than 2 cm and possibly safer than the BCS.

Another important point is related to tumor size and volume resection. In fact, the use of TRM presents a significantly higher volume of tissue excised compared to standard wide local excision. Kaur *et al.*^[22] in 2005 compared quadrantectomy with TRM procedures and observed a higher mean volume of tissue excised during TRM. However, they demonstrated no significant difference in margins between the two groups. Similarly, Giacalone *et al.*^[43] demonstrated mean specimen volumes of 190 cm³ for oncoplastic techniques and 99 cm³ for standard quadrantectomy.

There is limited evidence of the oncoplastic procedures concerning the aesthetic outcome. In addition, the methods of aesthetic evaluation vary significantly^[9,10]. Some authors reported that the volume of tissue resection is directly associated with the aesthetic outcome^[7,44-52]. Gendy *et al.*^[44] compared the aesthetic outcomes of 106 patients. Although the panel scored the aesthetic outcome as high, the aesthetic failure rate was 18% on breast retraction assessments. The authors demonstrated an advantage for the BCS reconstruction with regards to the incidence of complications (8% *vs* 14%), additional surgery (12% *vs* 79%) and restricted activities (54% *vs* 73%). Olivotto *et al.*^[45] and Mills *et al.*^[46] have docu-

mented that excision of a volume greater than 70 cm³ in medium-size breasts often leads to unsatisfactory aesthetic results. Clough *et al.*^[41], in a panel of three, assessed cosmetic results at 2 and 5 years. At 2 years, 88% and at 5 years, 82% of patients had a fair to excellent outcome. A significantly worse aesthetic outcome was observed in patients that received pre-operative radiotherapy compared to the remainder which were given radiotherapy post-operatively.

Fitoussi *et al.*^[47] retrospectively used a similar aesthetic evaluation method as proposed by Clough *et al.*^[41], with a panel made up of a surgeon, a nurse and a layman, using a five-point scale from excellent to poor. The cosmetic outcome in this retrospective study was satisfactory in 98% of patients at 12 mo and in 90% of patients at 5 years following BCS reconstruction.

Although oncological safety remains the primary objective of BCS, surgical management is increasingly focusing on improved aesthetic outcomes. Immediate application of TRM has evolved to meet this need and the extensive body of literature published in recent years demonstrates the widespread use of these techniques. Recognizing that there is a small risk for local recurrence, we believe that immediate application of TRM could be a safe option for early breast cancer patients who desire BCS.

LIMITATIONS OF TRM

Adjuvant treatment

There is evidence that oncoplastic breast conservation surgery and immediate application of TRM does not lead to a delay in the commencement of adjuvant chemotherapy^[15,18,23,25,28,42,50,53-56]. Although this is an integral part of overall oncological safety, few controlled studies have been published. In fact, most of the studies focus on recurrence rates and long-term survival; however, the evidence described is mostly based on single center retrospective analyses with relatively low patient numbers and no control groups (Table 2).

Time between BCS and commencement of adjuvant chemotherapy is only one aspect of oncological safety after early breast cancer management. In fact, patients who undergo complex surgery may be more susceptible to immunosuppression caused by chemotherapy. This may increase surgery related complication rates, which can lead to an internal delay between chemotherapy cycles, requiring frequent administration of granulocyte colony-stimulating factor or repeat hospital admissions^[28].

In a recently published meta-analysis, the average complication rate in the TRM group was 16% and 14% in the flap reconstruction group^[49]. However, it does not seem that complications in the oncoplastic groups, although potentially higher, have any negative impact on patient care from an oncological point of view. In fact, adequate technique and patient selection is crucial in order to minimize morbidity when this oncoplastic tech-

Table 2 Oncological and outcome evidence for delivery of adjuvant chemotherapy after immediate breast-conservation surgery reconstruction

Ref.	Year	n	Tumor size (cm)	Adjuvant chemotherapy n (%)	Delay in chemotherapy	Delayed adjuvant chemotherapy n (%)
Nos <i>et al</i> ^[71]	1998	50	Tis-T4	5 (10)	+	3 (6)
Losken <i>et al</i> ^[23]	2002	20	Tis-N/D	N/D	-	0
Clough <i>et al</i> ^[41]	2003	101	T1-T4	0	+	4 (4)
Spear <i>et al</i> ^[18]	2003	22	N/D	22 (100)	-	0
McCulley <i>et al</i> ^[53]	2005	50	Tis-N/D	23 (46)	-	0
Munhoz <i>et al</i> ^[15]	2006	74	T1-T2	22 (29.7)	-	0
Thornton <i>et al</i> ^[54]	2006	6	T1-T2	0	-	0
Kronowitz <i>et al</i> ^[50]	2007	41	Tis-T2	18 (44)	-	0
Losken <i>et al</i> ^[25]	2007	63	Tis-N/D	N/D	-	0
Rietjens <i>et al</i> ^[42]	2007	148	T1-T3	89 (60)	-	0
Meretoja <i>et al</i> ^[72]	2010	90	Tis-T3	60 (67)	+	2 (2)
Fitoussi <i>et al</i> ^[47]	2010	540	T1-T3	N/D	+	10 (1.9)
Song <i>et al</i> ^[55]	2010	28	Tis	N/A	-	0
Romics <i>et al</i> ^[56]	2012	31	T1-T3	31 (100)	-	0
Kahn <i>et al</i> ^[28]	2013	169	T1-T3	N/D	-	0

N/D: Not disclosed; N/A: Not applicable; (+): Positive; (-): Negative.

nique is selected. Despite the poor scientific evidence, nothing suggests that TRM is unsafe in terms of starting adjuvant therapy on time. In fact, Kahn *et al*^[28] provide evidence that immediate BCS reconstruction does not lead to a delay in the commencement of adjuvant chemotherapy when compared to three adequate control groups from the same institution and time periods.

Concerning adjuvant radiotherapy, one might surmise that techniques that involve rearrangement of breast tissue may jeopardize the boost radiation dose delivery since the target area is defined as the site of the original tumor^[15,57-59]. For this reason, coordinated planning with the radiotherapy center is important since TRM modifies the normal architecture of the breast^[15,16]. To locate the original tumor area, we advocate orienting the original tumor area by skin markings and also placing surgical clips at the tumor margins. It has been our impression, similarly observed by other authors^[57,58], that identification of the original tumor bed based only on physical exam, without precise imaging information, can result in missing the primary tumor bed. In our previous studies^[15-17], clips have not interfered with mammography and have actually helped recognize areas at risk for recurrence. Additionally, clips have not been mentioned as interfering with physical examination or aesthetic outcome^[57]. On the other hand, Poortmans *et al*^[60] mentioned that the location of clips can be misleading in cases where volume displacement techniques are utilized, especially where the boost area is not the entire surgical cavity. According to other authors, this usually results in larger boost volumes than actually necessary, potentially leading to local fibrosis and poorer aesthetic outcome^[60,61].

Another important issue is related to delayed reconstruction following BCS and radiotherapy. Frequently, the appearance of the radiated breast is less pleasing than the nonradiated one and the total dose, boost therapy and number of radiation fields may be involved^[19,21,23,24].

Spear *et al*^[18] mentioned that, besides the differences observed in the normal healing process, the radiated breast has a longer induration and swelling than the opposite breast^[18]. In our previous experiences utilizing TRM, the majority of patients presented with good or very good results in terms of breast shape and symmetry^[15-17]. In spite of this, almost 10% of patients presented with complications of breast skin necrosis and wound dehiscence, 92.3% of patients were either very satisfied or satisfied with their result and none regretted the surgery. Losken *et al*^[23] advocated that when radiation is expected, the possibility of fibrosis and glandular atrophy should be taken into account in an attempt to preserve breast symmetry. The authors suggested less aggressive reductions on the ipsilateral breast to accommodate for any additional size distortion. Additionally, some authors advocated that oncoplastic reconstruction with radiation is best achieved using autologous, nonirradiated flaps^[6,9,11,24].

Postoperative surveillance

According to Losken *et al*^[49,62] postoperative surveillance is not significantly affected by the rearrangement of breast tissue. These results corroborate with the findings of Roberts *et al*^[63] concerning the incidence of abnormal mammograms after reduction mammoplasty. In fact, these authors observed that, despite the substantial mobilization of tissue, postoperative mammography did not lead to more diagnostic interventions than nonoperative controls^[63,64].

Concerning late complications, the most common event is related to fat necrosis and this aspect is well defined in conventional mammograms. In our previous experience comparing immediate and delayed BCS reconstruction with reduction mammoplasty techniques, this complication was significantly higher in the delayed group^[24]. It has been our impression that radiation therapy played a significant role and contributed to de-

Table 3 Immediate breast-conservation surgery reconstruction and therapeutic reduction mammoplasty techniques: surgical margins and outcome

Ref.	n	Follow-up (mo)	Tumor size (cm)	Positive margins (%)	Local recurrence (%)
Clough <i>et al</i> ^[41] 2003	101	46	3.2 (0.1-7)	10.9	6.9
Clough <i>et al</i> ^[7] 1999	20	54	NS	0	5
Nos <i>et al</i> ^[71] 1998	50	48	3.5 (1.5-6)	10	7
Papp <i>et al</i> ^[12] 1998	10	52	NS	0	5
Masetti <i>et al</i> ^[75] 2000	56	23	NS	NS	0
Spear <i>et al</i> ^[18] 2003	11	24	NS	0	0
Chang <i>et al</i> ^[19] 2004	37	NS	0.6-5.2	2.7	0
Losken <i>et al</i> ^[23] 2002	14	23	1.5 (0.6-3)	28.6	0
Munhoz <i>et al</i> ^[15] 2006	74	22	1.9 (0.6-3.9)	9.5	0
Fitoussi <i>et al</i> ^[47] 2010	540	49	2.9	5	6.8

NS: Not specified.

velopment of fat necrosis. One might surmise that in delayed reconstructions, a slower reestablishment of a local blood supply to rearranged breast tissues from the underlying irradiated chest wall can be observed. In addition, previous breast tissue scarring and local effects of radiotherapy can also disrupt the local blood supply and the ability to create a safe parenchymal pedicle^[9,24]. Thus, in these patients, careful surveillance is prudent since the risk of local recurrence is always possible. According to Losken *et al*^[25,49,62], postoperative surveillance is not impaired by simultaneous TRM. In some cases, calcifications and fat necrosis can simulate tumor recurrence; however, these aspects can be distinguished on mammogram or core biopsy^[15-17,25].

Final surgical margins assessment and immediate reconstruction

When margins are found to be positive following BCS reconstruction, there is no consensus as to whether or not additional re-excision is possible as there may be difficulty in identifying the area of the original quadrantectomy. Some breast surgeons therefore indicate skin-sparing mastectomy if margins are compromised following definitive pathology evaluation.

In fact, TRM involves rearrangement of glandular tissue and could make re-excision difficult in cases where close or positive margins are observed^[64,65]. This fact could make it difficult to locate the residual tumor and to perform margin re-excision. In our previous studies^[14-17,65], intraoperative margin evaluation was assessed by pathological monitoring, which is based on macroscopic, radiological and histological examination of frozen sections. In our experience, positive margins discovered on permanent pathology in a previously negative margin patient were observed in 5.5%^[65]. Overall, the oncological outcome of our data corroborate the study of Fitoussi *et al*^[47], which is the largest study describing the oncological outcomes of 540 patients over a period of two decades. This study described clear margins in 438 patients (81%), focal involvement in 77 patients (14%)

and tumor-involved margins in 25 patients (5%). According to the authors, eleven patients underwent re-excision (2%), 40 patients received an additional radiotherapy boost (7%) and 51 patients required a mastectomy (9% overall) (Table 3).

Previous studies have investigated risk factors to identify patients with a high probability of having positive margins^[25,64-75]. Age (younger)^[25,65,67,68], tumor type (in situ carcinoma)^[25,64-75] and larger tumor size^[65,69] have all been associated with positive margins. Our results were comparable to those of the previous studies, with young patients and larger tumor size as more likely to have positive margins^[64]. Concerning the reoperative rates, Weinberg *et al*^[69] observed that 6.2% had later re-excisions and Cendán *et al*^[70] reported that 19.6% of subjects required additional operations to clear surgical margins. Despite these aspects, the positive margins can be effectively managed with either re-excision with/without reconstruction or with skin-sparing mastectomy and total reconstruction, depending on the extension of tissue resection, preference and pathology. The decision to re-operate depends on the extent of tumor involvement, whether the dissection had already been extended to the chest wall or skin, or whether the patient had opted to proceed with a total reconstruction. It has been our impression that reoperation was not a negative aspect and the disadvantage of a more extensive surgery is negligible. In cases of re-exploration, it is rational to carry it out in association with the plastic surgery team to identify the original tumor bed and to avoid injury to the NAC pedicle^[15-17,64].

Thus, intraoperative assessment of surgical margins requires multidisciplinary cooperation among oncological and plastic surgeons and pathologists. According to Losken *et al*^[25], all patients should be informed preoperatively of the potential need for a delayed-immediate approach. Additionally, these high-risk patients can be better managed by staged procedures and confirmation of negative margins prior to CBS reconstruction.

Opposite breast surgery

Another important issue is related to the OB surgery. Previous studies have differed with regard to the timing of opposite breast symmetrization. In fact, Gray *et al*^[21] described an association between breast irradiation in large volume breasts or obese patients and greater retraction at 5 years and an inferior overall cosmetic result. Another concept advocated was to perform symmetrization 6 mo after BCS following neoadjuvant treatment, as they suspected an unpredictable effect of radiotherapy on the breast and an alteration of body weight during chemotherapy.

In our previous experiences, almost all patients submitted to TRM had bilateral procedures^[15-17]. In fact, Kronowitz *et al*^[50] observed a significant relationship between the reconstructive technique and the need for an OB reduction. This aspect can be viewed as a negative point; however, it also has the advantages of allowing for sampling of glandular tissue^[15-17,19,24,27,50]. In our previ-

ous study^[24], we reported our experience with surgical management and outcome in BCS reconstruction with TRM techniques with regards as to whether immediate or delayed reconstruction is better in terms of complication rates. In this series, in 2.8% of patients, an unexpected cancer in the OB was observed in immediate TRM reconstruction. Although the diagnosis of occult cancer is not a reason to perform an OB reduction, this procedure can be advantageous for high-risk patients and especially for patients with previous breast cancer^[24].

Delayed BCS reconstruction and outcome

Another important issue is related to the complication rates and the timing of reconstruction. In our previous series, delayed reconstruction complication rates have been shown to be higher than immediate reconstruction (31% *vs* 22% respectively)^[24]. However, this aspect was not significant ($P = 0.275$). Thus, our results indicate that timing of reconstruction is not a significant predictor of complications following BCS reconstruction with TRM. This finding is contradictory to published reports that suggest that delayed BCS reconstruction has a significantly higher complication rate compared with immediate procedures. In fact, Kronowitz *et al*^[9] observed that delayed reconstruction was associated with a complication rate almost twice that of immediate. In our study, the relatively small number of patients and especially the small number of obese patients in the delayed group (21.7% *vs* 10.5%) may have influenced this comparison. Thus, a large number of patients and a prospective and controlled sample are necessary for definitive conclusions.

CONCLUSION

BCS defects represent an anatomic variety that ranges from small defects that may repair with primary closure to large defects that involve skin, NAC and a significant amount of glandular tissue. Each defect has its own special reconstructive necessities and varying expectations for aesthetic outcome. Recently, increasing attention has been focused on the oncoplastic approach and although surgical techniques have advanced, BCS reconstruction remains a challenging impasse. It has been our impression that a number of procedures have been described involving primary closure, breast reshaping, local and distant flaps. In addition, some different classifications have been proposed to describe the extent of resection, which has consequently created a wide-range of surgical options with different indications.

TRM in combination with BCS is not a new concept but is becoming increasingly accepted by oncological breast surgeons. In selected patients, this approach has allowed us to perform wide resections and obtain good oncological control with favorable aesthetic outcome. The majority of the complications were immediate, minor and comparable to other aesthetic reduction techniques. The main indication is in patients with enough breast tissue to perform the reconstruction. Although the combined

approach requires more preoperative planning and intra-operative care, the concept can reduce deformities, favor the oncological treatment and optimize the aesthetic outcome in most early stage cancer patients.

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Temozolomide for treatment of brain metastases: A review of 21 clinical trials

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Key words: Temozolomide; Solid tumours; Brain metastases; Clinical trials; Clinical outcomes

Core tip: Temozolomide has been used in glioblastoma multiforme as well as melanoma. Recently, studies showed that it might be also effective in patients with brain metastases from various malignancies. In this study, we carried out a review of 21 published clinical trials to determine whether temozolomide would benefit patients with brain metastases from solid tumours. As a result, a modest therapeutic effect was observed when temozolomide was used as a single agent, however, the combination of temozolomide with whole-brain radiotherapy and/or other anticancer drugs exhibited encouraging activity. This study for the first time provided a systematic evaluation of temozolomide in the treatment of brain metastases.

Abstract

Brain metastases from solid tumours are associated with poor prognosis despite aggressive treatment. Temozolomide can be used for the treatment of glioblastoma multiforme as well as melanoma. It has also been shown to have activity in patients with brain metastases from various malignancies, since it can cross the blood-brain barrier. To better understand the efficacy of temozolomide in the treatment of brain metastases, we carried out a review of 21 published clinical trials to determine whether temozolomide would benefit patients with brain metastases from solid tumours. Information regarding complete response, partial response, stable disease, objective response and objective response rate were collected to assess clinical outcomes. A modest therapeutic effect was observed when temozolomide was used as a single agent, however, the combination of temozolomide with whole-brain radiotherapy and/or other anticancer drugs exhibited encouraging activity. Thus, future high quality studies are warranted to confirm our findings.

Zhu W, Zhou L, Qian JQ, Qiu TZ, Shu YQ, Liu P. Temozolomide for treatment of brain metastases: A review of 21 clinical trials. *World J Clin Oncol* 2013; 5(1): 19-27 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i1/19.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i1.19>

INTRODUCTION

Brain metastases are a common complication in patients with cancer, occurring in approximately 25% of patients with disseminated diseases. The most common primary tumours, which metastasize to the brain, are lung cancer (25%-30%)^[1,2], breast cancer (10%-15%)^[3,4] and melanoma (12%-20%)^[5]. Over two thirds of patients with brain metastases suffer debilitating neurologic symptoms, including headaches, focal weakness, cognitive dysfunction, and seizures. Prognosis in these patients is particularly

grim, and without treatment, median survival period is 1-2 mo, depending on the extent of cranial disease and the degree to which it can be controlled.

No standard systemic therapy exists for patients with brain metastases. Researchers continue looking for optimal treatments. Whole-brain radiotherapy was the standard of care in patients with multiple or inoperable brain metastases. Nevertheless, radiosurgery was becoming an increasingly attractive option for patients with up to four lesions^[6,7], especially young patients with good performance status and satisfactory control of extra-cranial disease. Chemotherapy options were limited and generally used as salvage therapy in patients who failed to respond to whole-brain radiotherapy or radiosurgery^[8,9]. However, the assumption that the blood-brain barrier prevented the passage of chemotherapy agents into the brain discouraged the use of systemic chemotherapy in treating brain metastases, even though the blood-brain barrier might already be disrupted by the presence of brain metastases and/or the treatment with whole-brain radiotherapy^[10,11].

Temozolomide is an orally administered alkylating agent. It belongs to the imidazotetrazines and reaches the central nervous system in therapeutic concentrations. It can be rapidly absorbed following oral administration and undergoes spontaneous conversion at physiological pH to the active metabolite (3-methyltriazen-1-yl) imidazole-4-carboxamide in the tissues^[12]. Clinical activity of temozolomide is closely linked to the activity of O6-alkylguanine-DNA alkyltransferase, a DNA repair protein which removes O6-alkylguanine adducts in DNA^[13]. More importantly, temozolomide's ability to cross the blood-brain barrier has been demonstrated^[14]. Temozolomide also has a good toxicity profile. The dose-limiting toxicity is non-cumulative myelosuppression that rarely requires treatment delay or dose reduction and may improve patients' life quality. Currently, temozolomide is a standard therapy in patients with refractory anaplastic astrocytomas. However, the optimal treatment of brain metastases is still under investigation. Theoretically, an efficacious and well-tolerated chemotherapeutic, such as temozolomide, might also show great potential to treat brain metastases. Therefore, we carried out a review of published clinical trials to better understand the efficacy of temozolomide in the treatment of brain metastases from solid tumours.

IDENTIFICATION OF ELIGIBLE STUDIES

We searched the PubMed database (until 5 April 2012) for the relevant articles by using the term "Temozolomide" with no limitations. In addition, another search strategy was employed by using the key term "Temozolomide", limited to "Humans", "Clinical Trial" and "Cancer". All relevant publications were reviewed and repetitive articles were eliminated. The articles in reference lists were also hand-searched for potentially relevant publications.

INCLUSION CRITERIA

All human-associated studies, regardless of tumour type, were included once they met the following criteria: (1)

solid tumour with brain metastases (except primary brain tumour); (2) monotherapy or combination therapy with temozolomide; (3) histological confirmation; (4) relatively stable administration dosage of temozolomide; and (5) sufficient data of clinical outcomes.

DATA EXTRACTION

For each study, the following information was collected: first author, year of publication, country of the first author, the number of total and evaluable patients, median age, gender, cancer type, prior treatment, name of drugs, dose regimen, median cycle of treatment, clinical outcomes including the number of patients who achieved complete response, partial response, stable disease, objective response and progressive disease. For studies including different types of tumour, data were separately extracted by tumour type if information was enough.

LITERATURE SEARCH

A total of 2448 articles were identified from the PubMed database, of which 1987 were excluded due to repeated and unrelated contents. Among the rest, phase I studies were excluded as the administration dosage of temozolomide was not relatively stable. In addition, only trials on solid tumours with brain metastases were included in this review. Therefore, 21 papers were chosen from the remaining 461 articles for this study. Among these 21 articles, seven used temozolomide as a single agent while the others combined temozolomide with other anti-tumour drugs and/or radiotherapy.

Because of the heterogeneity of patients, regimens, clinical settings and a variety of outcome measurement in these trials, conducting a meta-analysis was inappropriate. Results were therefore analyzed qualitatively.

STUDY CHARACTERISTICS

Study characteristics information was summarized in Tables 1 and 2. Among all first authors, seven were from United States, five from Greece, four from Italy, two from Germany and one from each of United Kingdom, France, and Poland. The number of patients in these trials ranged from 11 to 157, with median age from 48 to 66. There were 12 cancer types described in these 21 trials, including non-small cell lung cancer, breast cancer, melanoma, small cell lung cancer, colorectal cancer, ovarian cancer, endometrial cancer, oral cavity cancer, renal cancer, bladder cancer, gastric cancer and carcinoma of the head and neck. Besides, there were five studies including unknown primary tumours. Most patients had received prior therapies and had metastatic or recurrent diseases at baseline.

TREATMENT ADMINISTRATION

In Tables 3 and 4, treatment administration information for each therapy type was recorded. In trials of mono-

Table 1 Study characteristics of the studies that used temozolomide as a single agent

Trial	Publication information (author/yr/country)	Patient characteristic			Cancer	Prior treatment
		Total number	Median age	Gender (male/ female)		
1	Abrey <i>et al</i> ^[16] /2001/United States	41 (34 ¹)	60	11/30	NSCLC (<i>n</i> = 22) Breast (<i>n</i> = 10) Melanoma (<i>n</i> = 3) SCLC (<i>n</i> = 2) Rectal (<i>n</i> = 2) Ovarian (<i>n</i> = 1) Endometrial (<i>n</i> = 1)	WBRT (<i>n</i> = 41) Stereotactic RT (<i>n</i> = 9) Chemotherapy (<i>n</i> = 35) Surgery (<i>n</i> = 11)
2	Siena <i>et al</i> ^[20] /2009/Italy	157	51.1 ⁴ /53.9 ³ /59.1 ²	72/85	Melanoma (<i>n</i> = 53) Breast cancer (<i>n</i> = 51) NSCLC (<i>n</i> = 53)	WBRT (<i>n</i> = 41) Chemotherapy (<i>n</i> = 98) Radiotherapy (<i>n</i> = 34) Chemotherapy (<i>n</i> = 21)
3	Schadendorf <i>et al</i> ^[21] /2006/Germany	45 (40 ⁵ /37 ⁶)	54.5	29/16	Melanoma	Chemotherapy (<i>n</i> = 21)
4	Giorgio <i>et al</i> ^[19] /2005/Italy	30	65	23/7	NSCLC	WBRT (<i>n</i> = 30) Stereotactic radio surgery (<i>n</i> = 1) Chemotherapy (<i>n</i> = 30)
5	Christodoulou <i>et al</i> ^[17] /2000/Greece	28 (24 ¹)	56	19/9	NSCLC (<i>n</i> = 12) SCLC (<i>n</i> = 5) Breast (<i>n</i> = 4) Other (<i>n</i> = 7)	WBRT (<i>n</i> = 23) Radiation (other sites) (<i>n</i> = 5) Surgery (<i>n</i> = 4) Chemotherapy (<i>n</i> = 22) Biologic therapy (<i>n</i> = 1) Chemotherapy (<i>n</i> = 34)
6	Agarwala <i>et al</i> ^[15] /2004/United States	151 (122 ¹)	53 ⁷ /46.5 ⁸	95/56	Melanoma	Immunotherapy (<i>n</i> = 23 ⁷ /21 ⁸) WBRT (<i>n</i> = 4) Chemotherapy (<i>n</i> = 1) Surgery (NA) Radiotherapy (NA)
7	Dziedzic <i>et al</i> ^[18] /2003/Poland	12 (11 ¹)	57	6/6	NSCLC	

¹The number of patients eligible for the assessment of clinical outcomes; ²Non-small cell lung cancer (NSCLC); ³Breast cancer; ⁴Melanoma; ⁵The number of patients eligible for the assessment of brain lesion response; ⁶The number of patients eligible for the assessment of extracerebral response; ⁷No prior chemotherapy; ⁸Prior chemotherapy. NA: Not available; SCLC: Small cell lung cancer; WBRT: Whole-brain radiotherapy.

therapy, temozolomide was administered at a dose of 150-200 mg/m² per day on days 1-5 of a 28-d cycle^[15-19] or 125-150 mg/m² per day on days 1-7, 15-21 of a 28- or 35-d cycle^[20,21]. The median cycle of each treatment is summarized in Tables 1 and 3.

In trials that combined temozolomide with radiotherapy, patients were treated as follows: (1) 30 Gy of whole-brain radiotherapy with concomitant temozolomide (75 mg/m² per day) for 10 d, and subsequent temozolomide at a dose of 75 mg/m² per day for 21 d every 4 wk^[22]; (2) A total dose of 30 Gy with ten daily fractions of 3.0 Gy was given 5 d per week over 2 wk, and temozolomide was administered at a dose of 95 mg/m² per day for the entire radiation treatment duration including days without radiation treatment^[23]; (3) temozolomide was given at 60 mg/m² per day (days 1-16) concomitantly with whole-brain radiotherapy (36 Gy/12 fractions given in 16 d), and subsequent temozolomide at a dose of 200 mg/m² per day for 5 consecutive days every 28 d^[24]; (4) temozolomide was given at 200 mg/m² per day on days 1-5 every 28 d. This therapy regimen was combined with stereotactic radiotherapy (20 Gy) or whole-brain radiotherapy (30 Gy)^[25]; and (5) temozolomide was administered at a dose of 75 mg/m² per day concurrent with 40 Gy fractionated conventional external-beam radiotherapy (2 Gy, 5 d/wk) for 4 wk, and subsequent temozolomide

at a dose of 200 mg/m² per day for 5 consecutive days every 28 d^[26].

Among those studies that combined temozolomide with other drugs and radiotherapy or with other drugs alone, three added cisplatin^[17,27,28], two combined thalidomide^[29,30], and the rest used vinorelbine^[31], lomustine^[32], doxorubicin^[33], arsenic trioxide^[34] or docetaxel^[27] as a part of chemotherapy protocol or radiation. The median cycle of each treatment is summarized in Tables 2 and 4.

CLINICAL OUTCOMES

Response criteria were used as defined by World Health Organization criteria, response evaluation criteria in solid tumors criteria, Macdonald criteria, Eastern Cooperative Oncology Group criteria or Standard response criteria. Objective response was based on the total number of patients who achieved complete response or partial response. Objective response rate was defined as the proportion of patients who got complete response or partial response. There were seven studies mentioning extracerebral or global responses as an endpoint^[17,21,25,27,28,30,32]. The extracerebral and global objective response rates ranged from 0.027 to 0.291, and 0.088 to 0.428, respectively. Seventeen trials evaluated cerebral response. Interestingly, efficacy of monotherapy and combination therapy was

Table 2 Study characteristics of the studies that combined temozolomide with radiotherapy and/or other agents

Trial	Publication information (author/yr/country)	Patient characteristic			Cancer	Prior treatment
		Total number	Median age	Gender (male/ female)		
8	Addeo <i>et al</i> ^[22] /2008/Italy	27	55	13/14	NSCLC (<i>n</i> = 15) Breast (<i>n</i> = 12)	Chemotherapy (<i>n</i> = 20) Surgery (<i>n</i> = 20) Radiotherapy (<i>n</i> = 12)
9	Mikkelsen <i>et al</i> ^[23] /2010/ United States	17	65.4	10/7	Lung (<i>n</i> = 13) Colon (<i>n</i> = 1) Melanoma (<i>n</i> = 1) Mixed (prostate, bladder, lung) (<i>n</i> = 1) Unknown (probably lung) (<i>n</i> = 1)	Chemotherapy (<i>n</i> = 7) Surgery (<i>n</i> = 1) Stereotactic radiosurgery (<i>n</i> = 2)
10	Kouvaris <i>et al</i> ^[24] /2007/ Greece	33	66	22/11	SCLC (<i>n</i> = 4) NSCLC (<i>n</i> = 10) Breast (<i>n</i> = 7) Rectal (<i>n</i> = 5) Melanoma (<i>n</i> = 5) Oral cavity (<i>n</i> = 1) Unknown (<i>n</i> = 1)	NA
11	Hofmann <i>et al</i> ^[25] /2006/ Germany	35 (34 ¹)	53	19/16	Melanoma	Chemotherapy (<i>n</i> = 7) Immunotherapy (<i>n</i> = 4) Chemoimmunotherapy (<i>n</i> = 3) Surgery/radiosurgery (<i>n</i> = 4)
12	Antonadou <i>et al</i> ^[26] /2002/ Greece	25 (24 ¹)	49	25/14	Melanoma	NA
13	Atkins <i>et al</i> ^[29] /2008/ United States	39	61	29/10	Melanoma	Immunotherapy (<i>n</i> = 12) Radiotherapy (<i>n</i> = 7)
14	Cortot <i>et al</i> ^[28] /2006/ France	50 (47 ² /33 ³ /47 ⁴)	57	40/10	NSCLC	Surgery (<i>n</i> = 40) Radiotherapy (<i>n</i> = 3)
15	Iwamoto <i>et al</i> ^[31] /2007/ United States	38 (36 ¹)	57	15/23	NSCLC (<i>n</i> = 17) SCLC (<i>n</i> = 3) Breast (<i>n</i> = 11) Colon (<i>n</i> = 2) Renal (<i>n</i> = 2) Endometrial (<i>n</i> = 1) Bladder (<i>n</i> = 1) Head and neck (<i>n</i> = 1)	Chemotherapy (<i>n</i> = 37) WBRT (<i>n</i> = 30) Surgery (<i>n</i> = 20) Stereotactic radiosurgery (<i>n</i> = 18)
16	Larkin <i>et al</i> ^[32] /2006/ United Kingdom	26 (14 ¹)	50	14/12	Melanoma	Immunotherapy (<i>n</i> = 7) Radiosurgery (<i>n</i> = 1) Surgery (<i>n</i> = 1)
17	Caraglia <i>et al</i> ^[33] /2005/ Italy	19	63	7/12	Breast (<i>n</i> = 8) NSCLC (<i>n</i> = 6) Colo-rectal (<i>n</i> = 3) Melanoma (<i>n</i> = 1) Ovarian (<i>n</i> = 1)	Systemic treatment (<i>n</i> = 12) Radiotherapy(out of brain) (<i>n</i> = 3) WBRT (<i>n</i> = 13)
18	Bael <i>et al</i> ^[34] /2007/United States	11 (5 ¹)	50	8/3	Melanoma	Immunotherapy (<i>n</i> = 3)
19	Christodoulou <i>et al</i> ^[35] /2005/Greece	32 (21 ¹)	53	11/21	Breast (<i>n</i> = 15) NSCLC (<i>n</i> = 11) SCLC (<i>n</i> = 1) Gastric (<i>n</i> = 1) Melanoma (<i>n</i> = 3) Unknown (<i>n</i> = 1)	Chemotherapy (<i>n</i> = 27) Radiotherapy (<i>n</i> = 17) Surgery (<i>n</i> = 1)
20	Hwu <i>et al</i> ^[30] /2005/United States	26 (14 ² /15 ³)	60	14/12	Melanoma	WBRT (<i>n</i> = 8) Stereotactic radiosurgery (<i>n</i> = 4)
21	Bafaloukos <i>et al</i> ^[27] /2004/ Greece	25 (24 ¹)	48	15/10	Melanoma	Surgery (<i>n</i> = 4) NA

¹The number of patients eligible for the assessment of clinical outcomes; ²The number of patients eligible for the assessment of brain lesion response; ³The number of patients eligible for the assessment of extracerebral response; ⁴The number of patients eligible for the assessment of global response. TMZ: Temozolomide; NA: Not available; NSCLC: Non-small cell lung cancer; WBRT: Whole-brain radiotherapy.

quite different among these studies.

Out of the seven trials using temozolomide as a single agent and cerebral response as an endpoint, objective responses were observed in six studies with objective

response rates ranging from 0.042 to 0.1. In the trial conducted by Giorgio *et al*^[19], a total of 30 patients who had brain metastases from non-small cell lung cancer were included, and 2 patients got complete response, 1 partial

Table 3 Treatment administration and clinical outcomes of the studies that used temozolomide as a single agent

Trial	Cancer	Treatment administration			Clinical outcomes						
		Drug	Dose regimen	Median cycles	CR	PR	OR	ORR	SD	PD	Other (median ¹)
1	NSCLC (<i>n</i> = 22) Breast (<i>n</i> = 10) Melanoma (<i>n</i> = 3) SCLC (<i>n</i> = 2) Rectal (<i>n</i> = 2) Ovarian (<i>n</i> = 1) Endometrial (<i>n</i> = 1)	TMZ	150-200 mg/m ² per day, days 1-5/28-d cycle	NA	0	2 ²	2	0.059	15 (8 ² /4 ³ /3 ⁴)	17 (9 ² /3 ³ /5 ⁴)	TTP: 1.97 mo OS: 6.62 mo
2	Melanoma (<i>n</i> = 53) Breast cancer (<i>n</i> = 51) NSCLC (<i>n</i> = 53)	TMZ	150 mg/m ² per day, days 1-7, 15-21/28- or 35-d cycle	NA	1 ²	9 (5 ⁵ /2 ³ /2 ²)	10	0.064	31 (12 ⁵ /8 ³ /11 ⁵)	116 (36 ⁵ /41 ³ /39 ²)	PFS: 56 d ⁵ /58 d ³ /66 d ² OS: 100 d ⁵ /172 d ² OS: 4.1 mo (3.6 mo ⁵ /4.3 mo ⁷)
3	Melanoma	TMZ	125-150 mg/m ² per day, days 1-7, 15-21/28-d cycle	48 d	0/0 ⁶	2/1 ⁶	2/1 ⁶	0.044/ 0.027 ⁶	5/5 ⁶	33/31 ⁶	
4	NSCLC	TMZ	150-200 mg/m ² per day, days 1-5/28-d cycle	6	2	1	3	0.1	3	24	TTP: 3.6 mo OS: 6 mo
5	NSCLC (<i>n</i> = 12) SCLC (<i>n</i> = 5) Breast (<i>n</i> = 4) Other (<i>n</i> = 7)	TMZ	150 mg/m ² per day, days 1-5/28-d cycle	NA	0	1	1	0.042	4	19	TTP: 3 mo OS: 4.5 mo
6	Melanoma	TMZ	150-200 mg/m ² per day, days 1-5/28-d cycle	NA	1	8	9	0.074	40	73	PFS: 1.2 mo ⁷ /1.0 mo ⁸ OS: 3.5 mo ⁷ /2.2 mo ⁸
7	NSCLC	TMZ	200 mg/m ² per day, days 1-5/28-d cycle	1	0	0	0	0	3	8	NA

¹Median: Data here are median values; ²NSCLC; ³Breast cancer; ⁴Other cancer; ⁵Melanoma; ⁶Extracerebral response; ⁷No prior chemotherapy; ⁸Prior chemotherapy. TMZ: Temozolomide; NA: Not available; NSCLC: Non-small cell lung cancer; SCLC: Small cell lung cancer; SD: Stable disease; PR: Partial response; CR: Complete response; OR: Objective response; ORR: Objective response rate; PD: Progressive disease; PFS: Progression-free survival; TTP: Time to progression; OS: Overall survival.

response, and 3 stable disease. However, in another study of advanced non-small cell lung cancer with brain metastases^[18], no objective response was achieved.

In trials that used combination therapy, objective response was observed in all studies with objective response rates greater than 0.1 except two studies. One trial evaluated vinorelbine and intensive temozolomide in patients with recurrent or progressive brain metastases with an objective response rate of 0.055^[31]. Another one was temozolomide, thalidomide, and whole brain radiation therapy for patients with brain metastasis from metastatic melanoma with an objective response rate of 0.077^[29]. The highest objective response rate in trials that combined temozolomide with radiotherapy was 0.959, achieved in the study of temozolomide and concurrent radiotherapy in patients with brain metastases from advanced lung cancer and breast cancer^[26]. Whereas, in the trial of concurrent temozolomide and whole brain radiation therapy for multiple brain metastases which was conducted by Mikkelsen *et al.*^[23], objective response rate was 0.176 with 3 patients achieving partial response. In two studies that combined temozolomide with other drugs and radiotherapy, objective response rates were 0.077^[29] and 0.128^[28]. Among those studies that used combination chemotherapy, the highest objective response rate was observed in the trial of temozolomide plus pegylated liposomal doxorubicin in the treatment of brain metastases from solid tumours^[35,36]. Stable disease was achieved in all studies. Other evaluation data,

such as median overall survival, progression-free survival and time to progression, were also collected, if available and summarized in Tables 1-4.

DISCUSSION

Brain metastases from solid tumours are associated with poor prognosis despite aggressive treatment. Also, the majority of patients have suffered debilitating neurological symptoms. Standard systemic therapy for patients with brain metastases is still under investigation. However, many clinical investigations had been discouraged by the concern that although chemotherapy drugs would have efficacy against the primary tumour (*e.g.*, lung cancer), they would not cross the blood-brain barrier. Therefore, chemotherapy would not be active against the metastatic brain disease. Even though data suggested that the blood-brain barrier was disrupted when brain metastases were present and chemotherapy could be effective against brain metastases from chemosensitive solid tumours^[37], unfortunately, the severe adverse events would be very difficult for pre-treated patients who had already received radiation and multiple regimens of myelosuppressive chemotherapy to tolerate. Temozolomide, a derivative of imidazotetrazine, is the prodrug of 3-methyl-(triazene-1-yl) imidazole-4-carboxamide. The therapeutic benefit of temozolomide depends on its ability to alkylate/methylate DNA, which most often occurs at the N-7 or O-6 positions of guanine residues. This methylation

Table 4 Treatment administration and clinical outcomes of the studies that combined temozolomide with radiotherapy and/or other agents

Trial	Cancer	Treatment administration			Clinical outcomes						
		Drug	Dose regimen	Median cycles	CR	PR	OR	ORR	SD	PD	Other (Median ¹)
8	NSCLC (<i>n</i> = 15) Breast (<i>n</i> = 12)	TMZ, WBRT	WBRT 30 Gy, TMZ 75 mg/m ² per day, days 1-10; subsequent TMZ 75 mg/m ² per day, days 1-21/28-d cycle	4.2	2 (1 ² /1 ³)	11 (5 ² /6 ³)	13	0.481	6 (3 ² /3 ³)	8 (6 ² /2 ³)	PFS: 6 mo OS: 8.8 mo
9	Lung (<i>n</i> = 13) Colon (<i>n</i> = 1) Melanoma (<i>n</i> = 1) Mixed (prostate, bladder, lung) (<i>n</i> = 1) Unknown (probably lung) (<i>n</i> = 1)	TMZ, WBRT	WBRT 30 Gy, TMZ 95 mg/m ² per day, days 1-14	NA	0	3	3	0.176	10	4	PFS: 2.4 mo OS: 4.1 mo
10	SCLC (<i>n</i> = 4) NSCLC (<i>n</i> = 10) Breast (<i>n</i> = 7) Rectal (<i>n</i> = 5) Melanoma (<i>n</i> = 5) Oral cavity (<i>n</i> = 1) Unknown (<i>n</i> = 1)	TMZ, WBRT	WBRT 36Gy, TMZ 60 mg/m ² per day, days 1-16; subsequent TMZ 200 mg/m ² per day, days 1-5/28-d cycle	NA	8 (3 ² /2 ⁴ / 1 ³ /2 ⁵)	11 (5 ² /2 ⁶ /1 ³ / 1 ⁵ /1 ⁷ /1 ⁸)	19	0.545	2 (1 ⁵ /1 ⁶)	12 (3 ² /1 ⁴ / 2 ⁶ /5 ³ / 1 ⁵)	PFS: 11 mo OS: 12 mo
11	Melanoma	TMZ, WBRT	WBRT 20 or 30 Gy, TMZ 200 mg/m ² per day, days 1-5/28-d cycle	NA	1 ⁹	2 ⁹	3 ⁹	0.088 ⁹	9 ⁹	17 ⁹	OS: 8 mo mixed response ¹⁰ : 5
12	NSCLC (<i>n</i> = 16) SCLC (<i>n</i> = 5) Breast (<i>n</i> = 2) Unknown (<i>n</i> = 2)	TMZ, WBRT	WBRT 40 Gy 5 d/wk, TMZ 75 mg/m ² per day, days 1-28; subsequent TMZ 200 mg/m ² per day, days 1-5/28-d cycle	NA	9	14	23	0.959	1	0	OS: 8.6 mo
13	Melanoma	TMZ, WBRT, Thalidomide	WBRT 30 Gy, days 1-5/8-12; TMZ 75 mg/m ² per day, Weeks 1-6; thalidomide 100 mg/d, Weeks 1-4, 100-400 mg/d Weeks 5, 7, 9	NA	1	2	3	0.077	7	29	TTP: 7 wk OS: 4 mo
14	NSCLC	TMZ, WBRT, Cisplatin	WBRT, TMZ 200 mg/m ² per day, days 1-5/28-d cycle, cisplatin 75 mg/m ² , day 1/28-d cycle	NA	1 ² /0 ³ /0 ⁴	5 ² /6 ³ /8 ⁴	6 ² /6 ³ /8 ⁴	0.128 ² / 0.181 ³ / 0.17 ⁴	21 ² /16 ³ / 10 ⁴	20 ² /11 ³ / 2 ⁹	TTP: 2.3 mo OS: 5 mo
15	NSCLC (<i>n</i> = 17) SCLC (<i>n</i> = 3) Breast (<i>n</i> = 11) Colon (<i>n</i> = 2) Renal (<i>n</i> = 2) Endometrial (<i>n</i> = 1) Bladder (<i>n</i> = 1) Head and neck (<i>n</i> = 1)	TMZ, Vinorelbine	TMZ 150 mg/m ² per day, days 1-7, 15-21/28-d cycle; vinorelbine 25 or 30 mg/m ² per day, days 1, 8/28-d cycle	2	1 (NSCLC)	1 (breast)	2	0.055	5	29	PFS: 1.9 mo OS: 5 mo
16	Melanoma	TMZ, Lomustine	TMZ 150 mg/m ² per day, days 1-5/28-d cycle; lomustine 60 mg/m ² per day, day 5/56-d cycle	NA	0 ⁹	0 ⁹	0 ⁹	0 ⁹	1 ⁹	13 ⁹	OS: 2 mo
17	Breast (<i>n</i> = 8) NSCLC (<i>n</i> = 6) Colo-rectal (<i>n</i> = 3) Melanoma (<i>n</i> = 1) Ovarian (<i>n</i> = 1)	TMZ, Doxorubicin	TMZ 200 mg/m ² per day, days 1-5/28-d cycle; pegylated liposomal doxorubicin 35 mg/m ² per day, day 1/28-d cycle	NA	3	4	7	0.368	8	4	PFS: 5.5 mo OS: 10.0 mo
18	Melanoma	TMZ, arsenic trioxide (ATO)	ATO 0.25 mg/kg per day, days 1-5 in week 0 + 0.35 mg/kg twice weekly/8-wk cycle; TMZ 200 mg/m ² per day, days 1-5 in weeks 1, 5/8-wk cycle	NA	0	0	0	0	0	5	NA
19	Breast (<i>n</i> = 15) NSCLC (<i>n</i> = 11) SCLC (<i>n</i> = 1) Gastric (<i>n</i> = 1) Melanoma (<i>n</i> = 3) Unknown (<i>n</i> = 1)	TMZ, Cisplatin	TMZ 150-200 mg/m ² per day, days 1-5/28-d cycle; cisplatin 75 mg/m ² per day, day 1/28-d cycle	3	1 ⁹ (NSCLC)	1/8 ⁹	9 ⁹	0.428	5 ⁹	6 ⁹	TTP: 2.9 mo OS: 5.5 mo

20	Melanoma	TMZ, Thalidomide	TMZ 75 mg/m ² per day, days 1-42/8-wk cycle; thalidomide 200-400 or 100-250 mg/d, days 1-42/8-wk cycle	1	2/0 ¹¹	1/0 ¹¹	3/0 ¹¹	0.214 /0 ¹¹	7/5 ¹¹	4/10 ¹¹	OS: 6 mo
21	Melanoma	A: TMZ, Docetaxel B: TMZ C: TMZ, Cisplatin	A: TMZ 150 mg/m ² per day, days 1-5/28-d cycle, docetaxel 80 mg/m ² per day, day 1/28-d cycle; B: TMZ200 mg/m ² per day, days 1-5/28-d cycle; C: TMZ 200 mg/m ² per day, days 1-5/28-d cycle, CDDP 75 mg/m ² per day, day 1/28-d cycle	NA	0/2 ¹¹	6/5 ¹¹	6/7 ¹¹	0.25/ 0.291 ¹¹	5	13	TTP: 2 mo OS: 4.7 mo

¹Median: Data here are median values; Drugs: Chemotherapy and/or radiotherapy; ²NSCLC; ³Breast cancer; ⁴SCLC; ⁵Melanoma; ⁶Rectal cancer; ⁷Unknown; ⁸Oral cavity cancer; ⁹Global response; ¹⁰Mixed response: PR or SD in the brain and PD at other locations; ¹¹Extracerebral response. TMZ: Temozolomide; NA: Not available; NSCLC: Non-small cell lung cancer; SD: Stable disease; PR: Partial response; CR: Complete response; OR: Objective response; ORR: Objective response rate; PD: Progressive disease; PFS: Progression-free survival; TTP: Time to progression; OS: Overall survival.

agents as treatment for brain metastases. Studies combining temozolomide with whole-brain radiotherapy reported more favourable response rates ranging from 0.176 to 0.959 with median overall survival ranging from 4.1 to 12 mo. In these trials, temozolomide might be shown to possess a radiosensitizing effect^[37,38]. In a large review of 1292 patients to define the prognostic factors in patients with brain metastases, Lagerwaard *et al.*^[39] concluded that the three strongest prognostic factors were performance status, response to steroids, and evidence of systemic disease^[40]. In the trial conducted by Addeo *et al.*^[22], a promising objective response rate of 0.48 (13 of 27 patients) was observed. A possible explanation would be that in this study, 11 of 27 patients were included in first RPA class according to RTOG classification. An objective response rate of 0.82 and two (8%) cases of stable disease were obtained in this group. In contrast, among the 6 patients (22%) included in the third RPA class, no objective response was observed. Besides, the proportion of metastases of lung origin was significantly higher in several studies which got favourable outcomes. Selection bias could have occurred in these trials.

Studies that combined temozolomide with other drugs had also been reported to yield high response rates in patients with brain metastases. The difference between monotherapy and combination therapy could be attributed to the efficacy of other agents. For example, pegylated liposomal doxorubicin had the ability to accumulate in both brain tissue and tumour tissue within the brain^[41,42]. Cisplatin, an active cytotoxic drug in solid tumours, might enhance the anti-tumour activity of temozolomide by reducing the activity of the DNA repair enzyme. However, most of patients involved in the studies were heavily pre-treated and failed prior therapy. Perhaps, the chemotherapeutic sensitization of temozolomide could be attributed to the improvement of the therapeutic effect in combination therapy. In addition, the dosage of other chemotherapeutic agents might be reduced to alleviate toxic reaction and improve patients' quality of life. The trial of temozolomide plus pegylated liposomal doxorubicin indicated that this chemotherapy regimen was well tolerated in elderly patients. This implied that temozolomide/pegylated liposomal doxorubicin could

be an effective therapeutic strategy for patients who were not suitable for conventional treatments because of the presence of brain metastases or old age.

In conclusion, since the studies in which temozolomide was used as a single agent usually achieved minimal outcomes, monotherapy might not be an optimal therapeutic strategy. However, the combination of temozolomide with whole-brain radiotherapy or other agents showed the potential to improve clinical outcomes of patients with brain metastases. It is worth re-examining the effects of temozolomide combined with other drugs or whole-brain radiotherapy on survival of patients with brain metastases from solid tumours in a randomized phase III study.

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Clitoris metastasis from a retroperitoneal leiomyosarcoma: A case report

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is the first case of clitoral and brain metastases originating from a retroperitoneal leiomyosarcoma.

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Key words: Leiomyosarcoma; Clitoris; Brain; Lung; Metastasis

Core tip: This is the first case of a clitoral metastasis originating from a retroperitoneal leiomyosarcoma. Furthermore, this report highlights the importance of atypical metastases in leiomyosarcomas.

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Abstract

Leiomyosarcoma is a rare form of cancer commonly found in the retroperitoneum, uterus, stomach, small intestine and vascular tissue. Surgery with a wide margin of resection is the most effective treatment. Nevertheless, metastasis is common and generally occurs within the first 3 years. The liver and lungs are the most common sites of metastasis in leiomyosarcoma. Other sites of metastasis include bone, spleen, soft tissues and brain. Metastatic tumours of the clitoris are extremely rare. As cited in the literature, the most common cancers that metastasize to the clitoris are breast, bladder, renal and gastric. Here, we report a case of a clitoral mass in a 64-year-old woman who received an operation for retroperitoneal leiomyosarcoma 4 years prior. Mass resection was performed. The pathological diagnosis was a leiomyosarcoma metastasis. The patient also presented with brain and lung metastases at the time of the clitoral metastasis. This

INTRODUCTION

Leiomyosarcoma is the third most frequently diagnosed soft-tissue sarcoma in adults, after malignant fibrous histiocytoma and liposarcoma^[1]. Leiomyosarcomas can arise at any site in the human body where smooth muscles are present; however, the tumours primarily arise in the retroperitoneum, subcutaneous tissue of the extremities, uterus, gastrointestinal tract and large vessels^[1,2]. Leiomyosarcomas have a propensity for haematogenous spread and rarely metastasize to the lymph nodes. The liver and lungs are the most common sites of metastasis for leiomyosarcomas^[2] and most patients develop distant metastases within long years following primary tumour resection^[2-4]. Here, we report a case of a metastasis to the clitoris an unexpected metastasis site, in a 64-year-old woman who received an operation for retroperitoneal



Figure 1 Macroscopic appearance of clitoral leiomyosarcoma.

leiomyosarcoma 4 years prior.

CASE REPORT

A 64-year-old woman was referred with a 1-mo history of a painful mass in the clitoris. A 2 cm polypoid mass with regular margins in the clitoris was revealed upon vaginal examination (Figure 1). The computed tomography of the chest indicated multiple bilateral pulmonary metastases (Figure 2), and abdominal magnetic resonance imaging showed a mass lesion on the left psoas muscle (Figure 3A). In addition, brain magnetic resonance imaging showed a metastatic mass lesion located in the left temporo-parietal region (Figure 3B). The patient's medical history revealed that she had undergone a total hysterectomy and bilateral salpingo-oophorectomy at the age of 60 years. During the surgery, a retroperitoneal mass lesion on the psoas muscle under the left kidney had been incidentally determined. The mass lesion was completely removed. A histological examination revealed chronic cervicitis, endocervical polyps, endometrial polyps, corpus albicans in the right and left ovary, paratubal cysts, bilateral chronic salpingitis in total abdominal hysterectomy and bilateral salpingo-oophorectomy specimens, and retroperitoneal leiomyosarcoma in the mass lesion on the psoas muscle under the left kidney. Surgical margins were clearly detected. Distant metastasis was not determined. The patient had refused to undergo chemotherapy and radiotherapy.

Based on this information, a diagnosis of metastatic retroperitoneal leiomyosarcoma was proposed, and the mass in the clitoris was removed with a wide local excision. The diameter of the clitoral metastatic mass was 2 cm × 1.8 cm, and 6-8 mitoses were microscopically detected per 10 high-power fields. The tumour was well differentiated and immunoreactive for α -smooth muscle actin and desmin (Figure 4A). The histological diagnosis was low-grade leiomyosarcoma of the clitoris. A retroperitoneal leiomyosarcoma specimen was re-examined to distinguish whether the clitoral mass was a metastasis or the primary tumour. The retroperitoneal tumour was composed of spindle-shaped cells with abundant eosinophilic cytoplasm and elongated nuclei (Figure 4B).

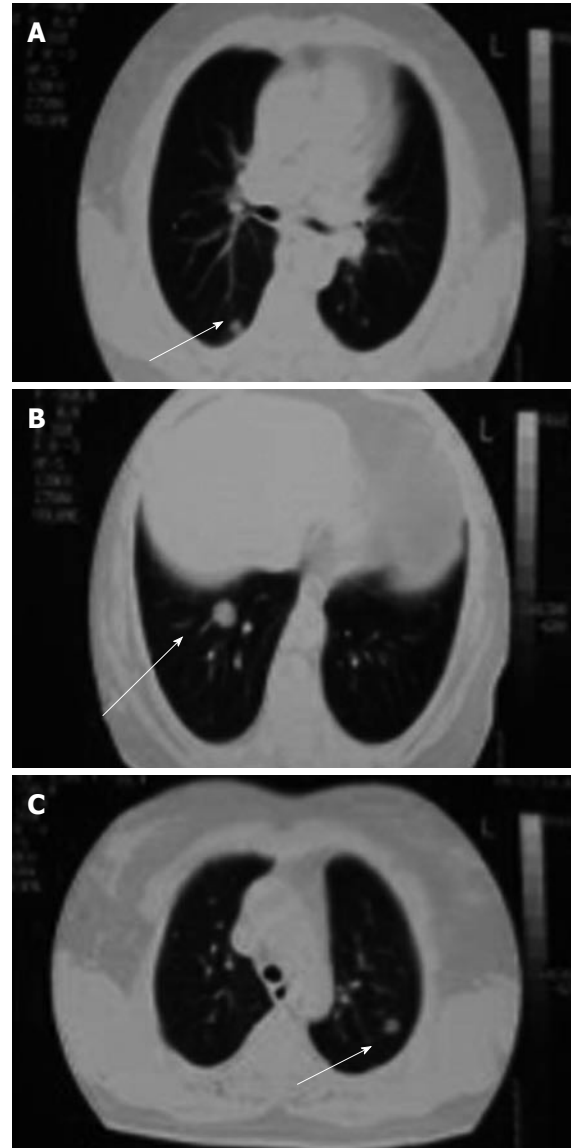


Figure 2 Bilateral pulmonary metastases in the chest computed tomography image.

Most nuclei were centrally located, blunt-ended and “cigar-shaped”. In some areas of the tumour, the nuclear hyperchromatism and pleomorphism were notable. The tumour was composed of compact cellular areas with focal myxoid change. Coagulative necrosis was evident. Mitotic figures were frequent (7-8/10 high-power fields), and atypical mitoses were common. The atypical spindle cells appeared yellow after histochemical staining using the Van Gieson elastic stain. Upon immunohistochemical analysis, the tumour cells were diffusely and strongly positive for smooth muscle actin (Figure 4C), caldesmon and vimentin. In contrast, the tumour cells were negative for desmin (Figure 4D), S-100 protein, epithelial membrane antigen, CD117 and CD 34 (Figure 4E). Approximately 15% of the tumour cells were Ki-67 positive. The surgical margins were clear. Thus, the histopathological and immunohistochemical findings were consistent with leiomyosarcoma of the retroperitoneum. The morpho-

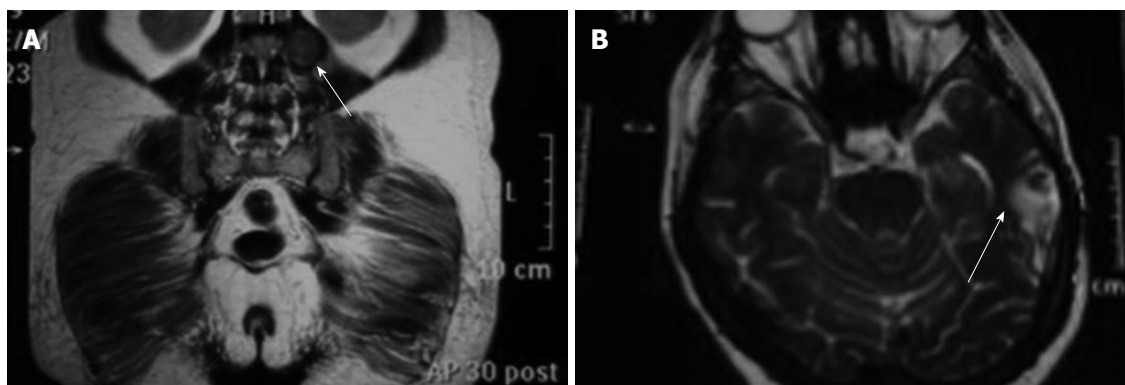


Figure 3 Magnetic resonance imaging. A: A contrasted mass on the left psoas muscle identified from abdomen magnetic resonance imaging (arrow); B: A metastatic brain lesion located in the left temporo-parietal region identified from brain magnetic resonance imaging (arrow).

logical and immunohistochemical results of the clitoral mass were similar to that of the retroperitoneal mass except for desmin positivity. Based on these findings, chemotherapy and radiotherapy were advised, but the patient refused.

DISCUSSION

Leiomyosarcomas can originate throughout the body, but the most common site is the retroperitoneum (20%-67% of cases)^[1,2]. Retroperitoneal leiomyosarcomas are more frequent in females and typically affect middle-aged to older adults^[3]. Diagnosis is delayed because most retroperitoneal leiomyosarcomas are asymptomatic. The primary tumour masses are typically large, and distant metastases are present at the time of diagnosis in approximately 40% of cases^[4]. In our patient, retroperitoneal sarcoma was detected incidentally during a gynaecological operation. Although the mass was 8 cm, the patient had no complaints, and distant metastasis was not evident at the time of the diagnosis.

Surgery was the main treatment. Given that the survival of patients with retroperitoneal sarcomas depends on complete resection of the tumour, extensive surgery is recommended^[5,6]. Nevertheless, the prognosis of retroperitoneal sarcoma is poor due to a high incidence of local recurrence and distant metastases^[6]. However, the following characteristics are associated with a favourable prognosis: tumours less than 5 cm in diameter, low histological grade tumours, and bladder tumours^[7]. Tumours that occur in the retroperitoneum have the worst prognosis. Mitotic activity is the primary prognostic factor; specifically, tumours with 5 mitoses per 10 high-power fields are considered malignant^[8]. Accordingly, the retroperitoneal leiomyosarcoma in our patient has poor prognostic factors (8 cm size, low grade, retroperitoneal site). Although the mass in the retroperitoneal region was fully removed and the surgical margins were histologically negative, local recurrence and lung, brain and clitoris metastases were detected approximately 4 years after the patient's first surgery. The recurrent mass detected in our patient can be explained by microme-

tastasis or tumour seeding. Furthermore, it has been reported that local invasion may be an important factor for tumour recurrence and distant metastasis^[7].

The distant spread of these tumours occurs via the bloodstream principally to the lung, liver, and peritoneal cavity. Metastasis may also be found in the bone, spleen, and soft tissues^[2-7]. A clitoris metastasis from retroperitoneal leiomyosarcoma have not been reported previously. The brain is an uncommon metastasis site in prior case reports; metastases from previously reported leiomyosarcomas were generally located in the uterus^[9,10]. The diameter of the clitoral metastasis was 2 cm × 1.8 cm and 6-8 mitoses per 10 high-power fields were observed. The tumour was well differentiated and immunoreactive for α -smooth muscle actin and desmin. Histopathological examination of the resected lesion revealed features consistent with leiomyosarcoma; therefore, based on the patient's past medical history and these histological similarities, the patient was diagnosed as having a clitoris metastasis to the from the retroperitoneal leiomyosarcoma. We discussed a potential mechanism of metastasis to the clitoris and brain in the patient. Soft-tissue sarcomas rarely show lymphatic spread; therefore, we hypothesize that clitoris and brain metastases occurred by the haematogenous route.

Other case reports of tumours that metastasize to the clitoris include breast, bladder, renal and gastric cancers^[11-14]. Metastatic leiomyosarcomas are treated with surgery and/or adjuvant chemotherapy, but no standard treatment has been established given the small number of patients^[6,15]. Increased survival has been reported in patients undergoing a metastasectomy^[15]. Because our case refused the chemotherapy or radiotherapy, we only performed the clitoral mass excision. Despite surgery, the patient died six months after metastasis identification.

Herein, we report the case of a 64-year-old woman who was diagnosed with a clitoral metastasis form a retroperitoneal leiomyosarcoma. In addition lung and brain metastases were detected in the work-up examination. In a known case of leiomyosarcoma, a newly developed clitoral mass should be considered as a metastasis and

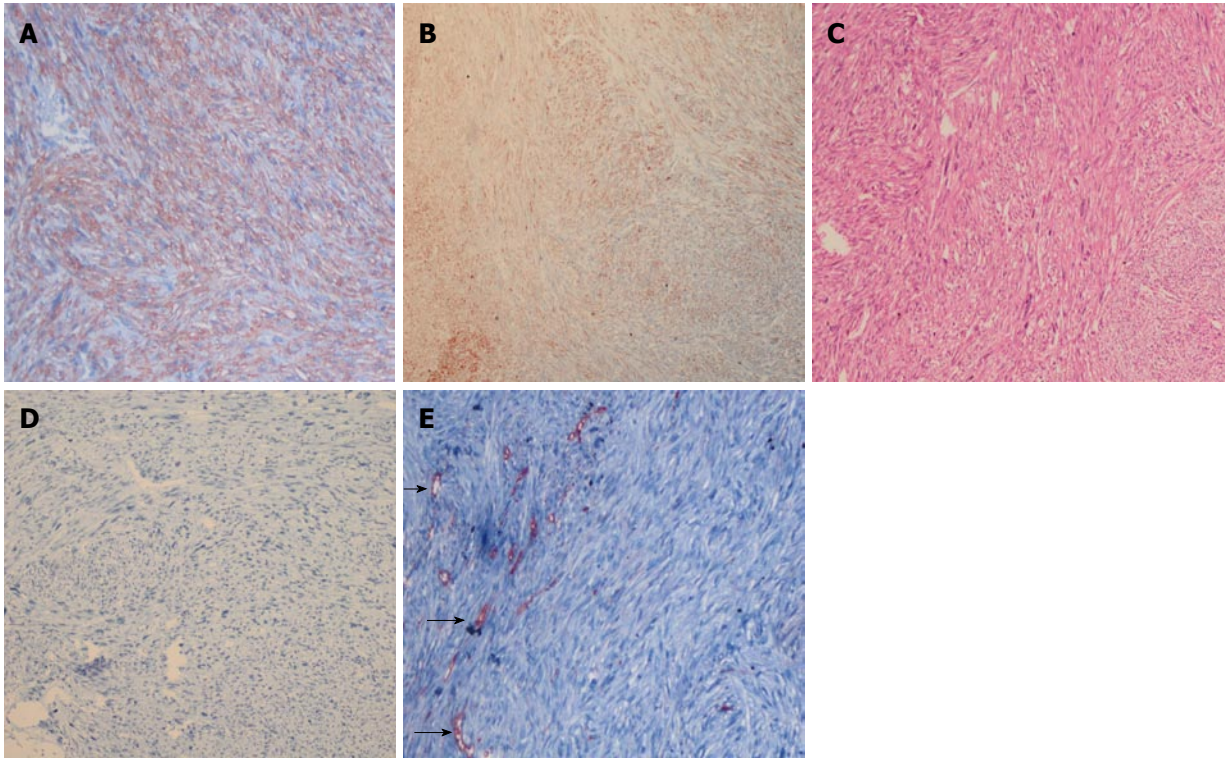


Figure 4 Diagnosis of metastatic retroperitoneal leiomyosarcoma. A: Strong positive SMA immunohistochemical staining in clitoral tumor ($\times 100$); B: Immunohistochemically, spindle-shape tumour cells revealed staining for desmin in the clitoral tumor ($\times 100$); C: Spindle shaped tumour cells with eosinophilic cytoplasm and nuclear polymorphism in retroperitoneal leiomyosarcoma ($\times 100$ magnification, Haematoxylin-Eosin staining); D: The retroperitoneal tumour cells were negative for desmin in retroperitoneal leiomyosarcoma ($\times 100$); E: Immunohistochemistry reveals CD34 negative tumour cells and CD34 positive vascular endothelial cells (arrows) in retroperitoneal leiomyosarcoma ($\times 200$).

histologically confirmed. Leiomyosarcomas usually recur within several years from the initial diagnosis. Therefore, patients should be observed and clinicians should be aware of areas of atypical metastasis.

COMMENTS

Case characteristics

A 64-year-old woman has a painful mass in the clitoris.

Clinical diagnosis

A 2 cm polypoid mass with regular margins in the clitoris was revealed upon vaginal examination.

Differential diagnosis

The differential diagnosis of the clitoral mass should be performed from infective organisms, cysts, invasion from cancers in the vicinity and metastasis from distant organs and should be confirmed with biopsy.

Laboratory diagnosis

The diagnosis of clitoral tumors includes radiological diagnosis, surgical procedures (biopsies) and histo-pathological examination.

Imaging diagnosis

The computed tomography of the chest indicated multiple bilateral pulmonary metastases, abdominal magnetic resonance imaging showed a mass lesion on the left psoas muscle, and brain magnetic resonance imaging showed a metastatic mass lesion located in the left temporo-parietal region.

Pathological diagnosis

The retroperitoneal and clitoral tumour were composed of spindle-shaped cells with abundant eosinophilic cytoplasm and elongated nuclei and most nuclei were centrally located, blunt-ended and "cigar-shaped" and immunohistochemically, tumor cells were diffusely and strongly positive for smooth muscle actin, caldesmon and vimentin, S-100 protein, epithelial membran antigen, CD117 and CD 34.

Treatment

Because our case refused the chemotherapy and radiotherapy, the authors only performed clitoral tumour excision.

Experiences and lessons

Leiomyosarcoma usually recurs within several years from the initial diagnosis. Therefore, patients should be subject to such an observation period at least and clinicians should be aware of areas of atypical metastasis.

Peer review

The authors observed a clitoris metastasis originated from retroperitoneal leiomyosarcoma. This is a very interesting case and should be published so that the observation can be shared with colleagues in the field. There are some spelling mistakes in the article.

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- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

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- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee.

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Evolving role of skin sparing mastectomy

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simple mastectomy. New techniques such as the use of acellular dermal matrix a and cell-assisted fat transfer have enhanced the use of implants for volume replacement following SSM.

Kasem A, Mokbel K. Evolving role of skin sparing mastectomy. *World J Clin Oncol* 2014; 5(2): 33-35 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i2/33.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i2.33>

Abstract

Skin sparing mastectomy (SSM) can facilitate immediate breast reconstruction and is associated with an excellent aesthetic result. The procedure is safe in selected cases; including invasive tumours < 5 cm, multi-centric tumours, ductal carcinoma in situ and for risk-reduction surgery. Inflammatory breast cancers and tumours with extensive involvement of the skin represent contraindications to SSM due to an unacceptable risk of local recurrence. Prior breast irradiation or the need for post-mastectomy radiotherapy do not preclude SSM, however the aesthetic outcome may be compromised. Preservation of the nipple areola complex is safe for peripherally located node negative tumours. An intraoperative frozen section protocol for the retro-areolar tissue should be considered in these cases. The advent of acellular tissue matrix systems has enhanced the scope of implant-based immediate reconstruction following SSM. Cell-assisted fat transfer is emerging as a promising technique to optimise the aesthetic outcome.

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Key words: Skin sparing mastectomy; Breast reconstruction; Acellular dermal matrix; Implants; Flaps

Core tip: Skin sparing mastectomy (SSM) is oncologically safe in selected cases and is aesthetically superior to

INTRODUCTION

Despite the increasing popularity of breast conserving surgery, approximately one third of women diagnosed with breast cancer still require or opt to have total mastectomy as a loco regional treatment. There is a growing body of evidence that immediate reconstruction following total mastectomy is associated with aesthetic and psychological benefits^[1]. The preservation of the skin envelope of the breast facilitates immediate reconstruction with a superior aesthetic result compared with conventional mastectomy. In standard skin sparing mastectomy (SSM), the nipple areolar complex is sacrificed and reconstructed at a later date. However, the nipple areolar complex can be preserved in certain cases [nipple sparing mastectomy (NSM)] and this further enhances the aesthetic outcome and is associated with psychosexual benefits^[1,2].

ONCOLOGICAL SAFETY

The preservation of the skin envelope of the breast had previously raised concerns regarding the oncological safety of SSM. This concern has been based on the fact that the skin envelope of the breast contains residual glandular breast tissue and can harbour a residual disease^[3,4]. However, numerous retrospective and prospective studies have shown that SSM is oncologically safe with no compromise of loco-regional control or overall survival

(OS). This is particularly true for ductal carcinoma in situ (DCIS) and T1 and T2 invasive breast cancer. There are limited data regarding the oncological safety in patients with T3 tumours, however, the published data show no compromise of clinical outcome^[1,5].

A recent meta-analysis of all published studies (9 studies, 3739 SSMs) related to SSM demonstrated a similar disease-free survival to non-SSM. In fact, the meta-analysis showed that the OS was slightly superior in the SSM group, however, this observation should be interpreted with caution since the meta-analysis do not include the tumour grade in the pooled analysis^[6].

Furthermore, NSM has been growing in popularity, due to increasing data supporting its oncological safety. A recent meta-analysis of the NSM ($n = 6615$) was published in 2013 and demonstrated an acceptable incidence of local recurrence, distant relapse and nipple-related complications^[2]. Preservation of the nipple areolar complex is oncologically safe provided that the tumour-nipple distance exceeds 2.5 cm and the local recurrence is lowest for node-negative, unifocal tumours, which are estrogen receptor-positive and human epidermal growth factor receptor 2 negative. It is very important that an intra-operative frozen section protocol is in place when the nipple areolar complex preservation is considered and if intra-operative frozen section analysis of the sub-areolar tissue demonstrates malignancy, then the nipple areolar complex is sacrificed^[1]. Prophylactic mastectomy for risk reduction represents a good indication for NSM.

SSM has been found oncologically safe for extensive DCIS. However, it is important that adequate surgical margins are obtained and the DCIS does not extensively involve the surgical margins. If there is significant DCIS involvement of the margins (< 1 mm at more than one site), then surgical excision of the overlying skin flap should be considered and, if this is not feasible, then post-mastectomy radiation should be considered as an adjuvant treatment after multidisciplinary discussion especially for high grade DCIS. For focally positive or close margins, post-mastectomy radiation can be omitted since the incidence of local recurrence is lower than the risk of developing cancer in the contra-lateral breast^[7].

Due to the fact that it is not feasible to conduct randomised controlled trials, there is a continuous need to publish updated meta-analyses from time to time, in order to ensure that the oncological safety of SSM remains intact^[2]. Such meta-analyses should assess the risk of bias (selection bias, detection bias, attrition and reporting bias) and study heterogeneity. OS and disease-free survival (DFS) should be the primary end points of the meta-analysis while the secondary endpoints should include surgical complications and quality of life.

In relation to T3 tumours, it is feasible to downstage the tumour with neo-adjuvant treatments, such as neo-adjuvant systemic therapy or even radiation prior to carrying out SSM. Neither prior radiotherapy nor post-mastectomy radiation represent contraindications to SSM, however, one has to accept that the aesthetic outcome will be compromised by radiation treatment, due to a higher incidence of capsule formation, which will require

surgical intervention, if it becomes symptomatic^[2,8].

BREAST RECONSTRUCTION FOLLOWING SSM

Following SSM, volume replacement is most commonly carried out using a mammary tissue expander or a fixed volume implant. The advent of the acellular dermal matrix devices has enhanced the scope of using implants in the context of immediate reconstruction and increased the rate of single-stage SSM and immediate reconstruction^[9]. Other options for breast reconstruction include free autologous tissue transfer with the free deep inferior epigastric perforator flap being the commonest^[10]. In relation to conventional pedicled flaps, the latissimus dorsi myocutaneous has its role in the field of immediate reconstruction.

Finally, the advent of cell-assisted fat transfer has been recently introduced, in order to improve the aesthetic outcome in women undergoing SSM and immediate reconstruction. The cell-assisted fat transfer is useful in improving the aesthetics of the breast contours and providing soft tissue covering in areas where the implant is palpable and visible. Furthermore, there is evidence suggesting that the use of cell-assisted fat transfer is associated with improvement of the severity of the capsule that develops in some patients undergoing breast reconstruction^[11].

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Large cell neuroendocrine carcinoma of the ovary: A pathologic entity in search of clinical identity

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Core tip: Large cell neuroendocrine carcinomas of diverse organs are rare. A brief overview of characteristics, diagnosis and treatment of this tumor type when occurring in the ovary is provided in this editorial.

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Abstract

Large cell neuroendocrine carcinoma (LCNEC) of the ovary is a rare diagnosis and only a few dozen cases have been reported in the literature. It is characterized by large pleomorphic cells with large round or oval nuclei, presence of mitoses and staining for neuroendocrine (NE) markers such as chromogranin A, synaptophysin, neuron specific enolase. This editorial gives a brief overview of this histologic type of ovarian carcinomas. LCNEC of the ovary is a pathologic entity that may not be diagnosed purely on clinical grounds due to the similarity of its clinical features with those of the more common epithelial ovarian cancers. Nevertheless the diagnosis is worth-making from a practical point of view in order to consider treatments tailored towards the NE component if it is dominant or it becomes dominant during the natural evolution of the disease. Establishment of an international tumor registry with an accompanying tumor tissue bank of ovarian LCNEC could be a means of obtaining further knowledge on clinical characteristics and advance research on this rare entity. This will further inform on treatment strategies and could identify future molecular treatment targets.

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INTRODUCTION

Large cell neuroendocrine carcinoma (LCNEC) of the ovary is a rare diagnosis and only a few dozen cases have been reported in the literature. It is characterized by large pleomorphic cells with large round or oval nuclei, presence of mitoses and immunohistochemical staining for one or more neuroendocrine (NE) markers such as chromogranin A, synaptophysin, neuron specific enolase or CD56^[1]. LCNEC was first described in the lung and, although initially classified as a variant of large cell carcinoma (which is a non-small cell carcinoma), was noticed to behave more similarly to small cell carcinomas^[2]. Similar neoplasms have been described arising from uterine body and cervix as well as other organs such as stomach, gallbladder, kidney, urinary bladder, prostate and parotid glands^[3-8]. Rare metastatic cases with an unknown primary have been reported^[9]. In most cases a concomitant epithelial ovarian component is present while presentation with pure large cell NE histology is less common^[10,11].

Presentation of LCNEC of the ovary is similar to the usual presentation of epithelial ovarian cancer with an abdominal mass, pain or distention and the diagnosis of

Table 1 Summary of basic characteristics of ovarian large cell neuroendocrine carcinoma

Pathologically distinct entity from epithelial ovarian cancers
Clinically similar presentation with common epithelial ovarian cancers
Often co-exists with non-neuroendocrine components
Several lines of data argue for a common origin of neuroendocrine and epithelial components (common co-existence, monoclonality analysis, neuroendocrine features arising in epithelial prostate cancer following treatment)
Treatment; suggested to be addressing the epithelial component except if neuroendocrine component is clearly dominant

a tumor is confirmed after radiologic evaluation (Table 1). Many of the cases are stage III or IV but earlier cases are often reported. Metastatic sites include the abdominal cavity and liver, typical of epithelial ovarian cancer, while other sites such as lung, brain and bone have been reported less commonly^[10,12]. Thus a more diverse metastatic pattern in LCNEC of ovary compared to epithelial cancers is encountered. This is also exemplified in the skin metastasis seen in the accompanied case report. In some cases with available information metastatic deposits are solely of NE histology^[12].

Diagnostic pathology shows large cells usually with significant pleiomorphism, large nuclei with coarse and granular chromatin, prominent nucleoli, often significant mitotic activity and palisading with rosette formation. Immunohistochemistry confirms the diagnosis with positivity for one or more of the standard NE markers. Almost all cases evaluated have elevations of Ca-125 tumor marker. The great majority of cases have an adjacent epithelial ovarian cancer component, most often endometrioid and more rarely serous^[13]. Of note the epithelial component often, but not always, expresses NE markers despite its differing morphology^[1,13]. The pathologist needs to be alert to the diagnosis and include LCNEC in the differential diagnosis of undifferentiated carcinomas of both the ovary and the endometrium^[14].

Prognosis of LCNEC of the ovary is difficult to ascertain because of the rarity of the disease, the small number of reported cases and the lack of systematic population based studies or registry data. These shortcomings in addition to pathologic diagnosis inconsistencies prevent a solid data-based prognostication in comparison with epithelial ovarian cancer. The experience of many authors is that LCNEC of the ovary is more aggressive than epithelial ovarian cancer and may not respond as well to chemotherapy^[13]. Nevertheless others have observed LCNEC to display chemosensitivity similar to other epithelial ovarian cancers^[1,15].

Pathogenesis could be an informative element for prognosis but most importantly for therapy. The fact that the great majority of ovarian LCNEC are diagnosed as part of a biphasic tumor combined with epithelial elements implies that the two components have a common cellular origin and represent two divergent clones of the same neoplastic process. Indeed a monoclonality analysis using human androgen receptor analysis disclosed a com-

mon origin of the two components in a case of composite LCNEC and mucinous epithelial ovarian carcinoma^[16]. Similarly monoclonality was shown by the same authors in a case of composite LCNEC and cervical adenocarcinoma^[17]. Thus, based on both the fact of common concurrence with epithelial components and the monoclonality studies, it appears that ovarian LCNEC and the even more rare endometrial LCNEC^[18] represent a dedifferentiated clone of an epithelial carcinoma. This reminds the case of endometrial carcinosarcomas (malignant mixed müllerian or mesodermal tumors) in which the sarcomatous component is derived from an epithelial to mesenchymal transition program during which epithelial malignant cells obtain mesenchymal morphologic and functional properties that allow them to become mobile and metastasize^[19]. The case of LCNEC in the prostate may be informative for the pathogenesis of LCNEC in general, arguing also for a common origin of epithelial and NE components. LCNEC of this origin often arise after patients are on androgen repression therapy for an epithelial prostatic carcinoma and may represent a result of therapy pressure on the tumor cells^[7]. According to this theory prostatic LCNEC represent clones of the initial epithelial cancer that have become dominant following endocrine therapy because of their innate resistance to it. The minority of ovarian LCNEC cases in which no epithelial component is discernible (pure LCNEC) may represent either epithelial tumors in which the totality of cancer cells have undergone NE transition or true NE tumors derived from resident NE cells. A dual origin with concomitant transformation of epithelial cells and NE cells may be true in the exceedingly rare case of composite LCNEC and serous ovarian cancer^[20].

Regarding therapy, these pathogenic considerations imply that the optimal first line treatment for mixed epithelial and LCNEC ovarian tumors should be against the epithelial component. In case of pure LCNEC or NE element preponderance or selection of the NE clone post-treatment consideration should be given to a NE type combination (platinum-etoposide).

In conclusion, LCNEC of the ovary is a pathologic entity that may not be diagnosed purely on clinical grounds due to the similarity of its clinical features with those of the more common epithelial ovarian cancers. Nevertheless the diagnosis is worth-making from a practical point of view in order to consider treatments tailored towards the NE component if it is dominant or it becomes dominant during the natural evolution of the disease. Establishment of an international tumor registry with an accompanying tumor tissue bank of ovarian LCNEC could be a means of obtaining further knowledge on clinical characteristics and advance research on this rare entity. This will further inform on treatment strategies and could identify future molecular treatment targets.

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WJCO 5th Anniversary Special Issues (1): Lung cancer

Targeted immunotherapy for non-small cell lung cancer

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Abstract

Targeted therapies that deliver the expected anti-tumor effects while mitigating the adverse effects are taking the cancer world by storm. The need for such therapies in non-small cell lung cancer (NSCLC), where systemic cytotoxic chemotherapies still remain the backbone of management, is felt more than ever before. Runway success of immunotherapies such as Ipilimumab for melanoma has brought excitement among oncologists. Immune-based treatments are in various stages of evaluation for NSCLC as well. Immunotherapies using strategies of antigen based or cell based vaccines, and blocking immune checkpoints are of substantial interest. Meaningful clinical responses are yet to be reaped from these new treatment modalities.

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Key words: Immunotherapy; Non-small cell lung cancer; Programmed death-1; Programmed death ligands 1; Cytotoxic T-Lymphocyte Antigen-4

Core tip: Lung cancer is the leading cause of cancer-death worldwide. Majority of these patients have non-small cell lung cancer (NSCLC). Traditional chemotherapy is limited by its high toxicity. Emerging data have

demonstrated promising outcome of immunotherapy in NSCLC. This review delineated the rationale and potential targets of cancer immunotherapy, with a summary of immunotherapeutic agents for treatment of NSCLC. Protein/peptide-based and cell-based vaccines, as well as immune checkpoint targeted agents such as Ipilimumab and PD-1 pathway inhibitors were discussed. In addition, we reviewed ongoing immunotherapy-based studies including several major phase II/III clinical trials, results of which will be available soon for incorporation into clinical practice.

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INTRODUCTION

Lung cancer remains the leading cause of death in the United States with about 160000 estimated deaths in 2013^[1]. Majority of these patients have non-small cell lung cancer (NSCLC). Over the past few decades, platinum based chemotherapy is the standard of care for advanced stages of NSCLC. These systemic therapies have significant toxicities and confer unacceptable morbidity. A decade ago, it was realized that advancement in treating NSCLC could not be reached with the use of cytotoxic agents alone^[2]. While entering the era of personalized medicine, focus of cancer therapy has been recently shifted to identifying and targeting certain driver mutations. This has been successful in certain solid tumors including NSCLC, wherein identifying the genetic mutation in epidermal growth factor receptor (EGFR) and fusion gene rearrangement in anaplastic lymphoma kinase has become the standard of care. Historically lung cancer was not seen as an “immunologic malignancy”. However recent success of Ipilimumab in melanoma^[3] and Sipuleucel-T in prostate cancer^[4] opened up a new realm

of cancer immunotherapy. Emerging data demonstrate promising outcome of immunotherapy in lung cancer. Herein we review the basics of cancer immunotherapy and development of immunotherapeutic agents for management of NSCLC.

We conducted a review of articles in the past 10 years, that harbored the terms “immunotherapies”, “non small cell lung cancer” in the Pubmed and Medline database as well as trials that are ongoing in clinicaltrials.gov.

BASIC IMMUNOLOGY PRINCIPLES AND ITS APPLICATION IN NSCLC

Immunity acts as a double edged sword when it comes to cancer. The cross talk between tumor and immunity is complex. Cancer immunoediting plays an important role in this context. Three phases of cancer immunoediting have been described: elimination, equilibrium and escape^[5].

In the initial phase of elimination, innate and adaptive immune systems work in concert to eradicate tumor cells, after which the survived cells enter a state of dormancy called the equilibrium phase. Under immune pressure, tumor cells may undergo phenotypic or genomic modification, resulting in the survival and proliferation of tumor variants that are capable of escaping immune attack. Important modulations of this immune escape include down-regulation of HLA class I, loss of tumor antigens, lack of death receptor signaling, insufficient costimulation, and negative regulation pathways including regulatory T cells, inhibitory cytokines and molecules involved in immune checkpoints.

The potential targets for immunotherapy can be tumor specific antigen or each component of the cross talk between the immune system and tumor. It has been proposed that targeting tumor specific antigen may be ideal in treating early stage cancers when the tumor cells are highly immunogenic; whereas targeting non antigen specific immune pathways may be optimal in managing advanced stage cancers. Advancement of our knowledge in cancer immunology has resulted in better cancer vaccine designs with hope to improve clinical outcomes^[6].

Previously lung cancer was not seen as immunogenic. However recent studies have shown the association of immune response with overall outcome, indicating a role of immunotherapy in lung cancer management. Histochemical analysis of lung tumors in retrospect, showed that increased infiltration of the stroma by CD4⁺/CD8⁺ cells was an independent favorable prognostic factor^[7]. In addition, it has been observed that in advanced NSCLC, favorable prognosis has been associated with higher number of macrophages and CD8 T in tumor nests as compared with surrounding stroma^[8]. In contrast, increased infiltration by Foxp3⁺ lineage Treg cells is associated with poor outcome. Furthermore depletion of Treg achieves prolonged survival in mouse models^[9,10]. Current development of NSCLC immunotherapy is mainly focused on tumor vaccines and blockade of immune checkpoint pathways.

CANCER VACCINES

A therapeutic cancer vaccine intends to treat an existing cancer by strengthening the body's natural defense against cancer. Broadly, cancer vaccines can be divided into protein/peptide based vaccines and cellular vaccines.

PROTEIN/PEPTIDE BASED VACCINES IN NSCLC

L-BLP25 vaccine

MUC1 is a transmembrane glycoprotein that is upregulated in many solid tumors including NSCLC. It is purported that aberrant up regulation of MUC1 in tumor cells favors tumor angiogenesis *via* activating Erk and Akt pathways^[11]. L-BLP25 is a peptide vaccine that targets the exposed core peptide of MUC1^[12].

The vaccine was studied in an open label, phase II randomized trial in 171 patients with stage III B/IV NSCLC with response or stable disease after first line therapy^[13]. The trial evaluated effect of L-BLP25 liposome vaccine on survival and toxicity in the above patients. Quality of life and immune related responses due to the vaccine were the secondary end points. Patients were prestratified by stage and randomly assigned to either L-BLP25 plus best supportive care (BSC) or BSC alone. Patients in the L-BLP25 arm received a single intravenous dose of cyclophosphamide 300 mg/m² followed by eight weekly subcutaneous immunizations with L-BLP25; and then every 6 wk, as this had been previously shown to boost immune response in certain other cancers^[14]. Though the study failed to achieve the primary end point of overall survival (OS); subgroup analysis of patients with stage III B disease showed strong positive trend towards 2 years survival. Update on these patients published later showed continued improved survival in patients on the L-BLP-25 arm^[15]. These results were achieved with minimal toxicity.

Based on the above results, a phase III trial, Stimulated Targeted Antigenic response to NSCLC (START, NCT00409188) was undertaken. One thousand two hundred and thirty-six patients with stable unresectable stage III disease were randomized to receive either intravenous cyclophosphamide followed by weekly BLP-25 *vs* placebo. The trial did not meet its primary end point of OS, however the subgroup that was pretreated with prior chemoradiation (either concurrent or sequential) had significant improvement in OS^[16]. They reported the vaccine to be well tolerated with some flu-like symptoms, but no significant immune associated adverse effects.

Other clinical trials of L-BLP25 include the multinational, double blinded, placebo controlled trial in Asian population, with unresectable stage III NSCLC who have been stable or responded to primary chemoradiation, L-BLP25 trial In Asian NSCLC Patients: Stimulating Immune Response^[17].

A phase II study of L-BLP-25 is looking in combination with bevacizumab in patients who have undergone

chemoradiation for stage III NSCLC is ongoing as well^[18].

Melanoma-associated antigen-A3 vaccine

Melanoma-associated antigen (MAGE) is a family of tumor specific antigens that is expressed on variety of tumor cells and specifically the MAGE-A3 is detected in about 35%-50% of NSCLC^[19,20]. It is also expressed on cells of other tumors such as melanoma, renal, bladder and liver cancer^[21]. MAGE-A3 is also expressed on normal testicular and placental. However with unique immune tolerance mechanisms these organs were able to escape immune attack. Hence MAGE-A3 is a unique tumor antigen and the vaccine against it should be well tolerated in theory^[22,23]. Presence of MAGE-A3 is independently associated with poor prognosis in NSCLC^[24].

MAGE-A3 vaccine is composed of recombinant fusion protein, in combination with immune-enhancing adjuvant. A phase II trial studying the efficacy and safety of the vaccine was performed in 182 patients with MAGE-A3 positive, resected stage I B/ II NSCLC. This was an international, double blinded, placebo controlled trial, where patients were randomized to receive either MAGE-A3 vaccine or placebo. The results were encouraging as the long term analysis showed a positive trend in OS, disease progression time and disease-free survival in those receiving the MAGE-A3. The vaccine was very well tolerated leading to good compliance^[25]. These encouraging results lead to the ongoing randomized trial in lung cancer, MAGE-3 as Adjuvant Non-Small Cell Lung Cancer Immunotherapy. It is a phase III trial looking at MAGE-A3 vaccine *vs* placebo used in adjuvant setting for patients with MAGE-A3 positive stage I B, II or IIIA resected NSCLC. Disease free survival is the primary end point and OS, lung cancer specific survival and adverse events (AE) amongst others are secondary end point. The results of the study are eagerly expected in early 2014^[26].

Epidermal growth factor vaccine

EGFR is a transmembrane receptor tyrosine kinase belonging to the Erb family of receptors and is activated by binding of its specific ligand epidermal growth factor (EGF), hypothesized to be responsible for pathways that regulates cell survival, cell death *via* controlling the extracellular growth factors^[27]. EGFR expression is altered in many cancer, including NSCLC. In NSCLC harboring mutated EGFR, use of EGFR tyrosine kinase inhibitors as first line therapy has been shown to improve survival and safety profile in a number of clinical trials and are approved for this indication^[28-30].

Recombinant human EGF vaccine is an antigen-based vaccine which prevents binding of the endogenous EGF to the receptors by stimulating production of anti-EGF antibodies that clear it from circulation.

A phase I clinical trial conducted in 43 patients with advanced NSCLC was randomized to receive either single or double dose EGF vaccination. Patients receiving double dose of vaccination had higher antibody titers,

were found to have a positive survival trend. Antibody titers and serum EGF levels appear to correlate with patient survival^[31]. Significant positive outcome between higher antibody response and increased survival was confirmed in a phase II trial by García *et al*^[32]. A phase III trial for safety and efficacy of EGF vaccine in inoperable advanced stage NSCLC is currently ongoing^[33].

WHOLE TUMOR CELL VACCINE

Belagenpumatucel vaccine

Transforming growth factor $\beta 2$ (TGF $\beta 2$) converts CD4⁺CD25⁻ naïve T cells to CD4⁺CD25⁺Treg cells by inducing transcription factor Foxp3^[34] which in turn mediate immunosuppression in NSCLC by blocking dendritic cells, natural killer cells and lymphokine activated killer cells. Higher levels of TGF $\beta 2$ are associated with worse outcomes in NSCLC^[35]. Belagenpumatucel is a non-viral gene based allogeneic vaccine that carries TGF $\beta 2$ antigen modification of tumor cells. It consists of 4 cell lines of NSCLC (2 adenocarcinoma, 1 squamous carcinoma and 1 large cell carcinoma)^[36] which downregulates TGF $\beta 2$ upon administration.

The vaccine was investigated in early as well as advanced NSCLC where patients were randomly assigned to receive a dose of either 1.25, 2.5 or 5×10^7 cells/injection. Patients with any prior therapy were able to participate in the trial. Antibodies reactive against the cell lines were evaluated using enzyme-linked immunosorbent assay. The vaccine was well tolerated with minimal side effects. Survival was significantly improved in cohorts who received higher doses of the vaccine ($\geq 2.5 \times 10^7$ cells/injection); however difference across dose cohorts for advanced NSCLC was only marginal. Estimated probabilities of surviving 1 and 2 years were 68% and 52% respectively for higher dose groups combined and 39% and 20% respectively for the low dose group. Patients in the advanced stage disease had a 15% response rate^[36]. A smaller phase II trial by the same group in 21 patients confirmed the safety and efficacy of the vaccine. They also suggested the possibility of using CTC (circulating tumor cells) as surrogate of OS^[37].

Encouraged by the above results, a phase III trial of belagenpumatucel is underway as maintenance therapy for patients with T3N2-III A, IIIB and IV NSCLC who did not progress after front line chemotherapy (STOP trial)^[38]. Findings were reported in September 2013 at the European Cancer Congress from the STOP trial. Of 532 patients enrolled, 42 had stage II A and 490 had stage IIIB/IV disease. Patients were randomized 1:1 to receive either the vaccine or placebo until disease progression or withdrawal. Though STOP did not meet its predefined primary end point in the entire population, it was noted that patients who were randomized to receive the vaccine within 12 wk of the end of their first line chemotherapy had better OS (20.7 mo) as compared to those with placebo (13.4 mo). In addition, pretreatment radiation showed improved median OS with treatment

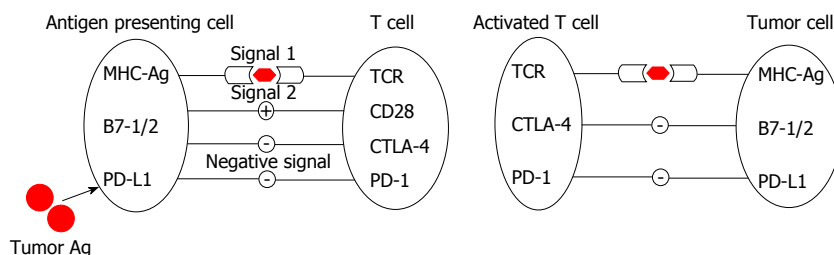


Figure 1 Interaction between T cells and antigen presenting cells or tumor cells. The quality and amplitude of T cells depends on the balance of the above co-stimulatory and inhibitory signals and ultimate orchestration of death of tumor cells. Agonists of costimulators or antagonist of inhibitors are the main topic of the study as described. MHC-Ag: Major histocompatibility complex antigen; TCR: T cell receptor; PD-1: Programmed death-1; PD-L1: Programmed death ligand 1; CTLA-4: Cytotoxic T lymphocyte antigen-4.

arm OS of 40.1 mo compared to 10.3 mo receiving placebo. The trial will be continued with focus on these specific subgroups^[39].

Immune checkpoints inhibitors

T cell receptors activate antigen specific cytotoxic T cells after recognition of the antigen peptide along with major histocompatibility complex. This activation usually requires costimulatory signal obtained *via* interaction of CD28 expressed on T cells with molecules expressed on antigen presenting cells (Figure 1). CD28 can also interact with inhibitory receptors on antigen presentation cells or tumor cells such as Cytotoxic T-Lymphocyte Antigen (CTLA)-4, PD-1, PD-L1 and B- and T-lymphocyte attenuator^[40], therefore inhibits T cell functions, a process called immune check point^[41,42]. Two promising strategies for immune check point modulation are currently being investigated in NSCLC.

CTLA-4 inhibitors

CTLA-4 inhibitors have been extensively studied and are thought to be responsible for initiating and maintaining peripheral tolerance as a part of physiological immune mechanism. Ipilimumab is a fully humanized mAb targeting the CTLA-4 inhibitory coreceptor, thus rescuing cytotoxic T cell activity and potentiating tumor death^[43]. It was approved for malignant melanoma^[3] and since then has been evaluated in various other malignancies.

A phase II study of Ipilimumab enrolled 204 patients with stage III B/IV NSCLC who had not received any prior chemotherapy^[44]. Patients were randomly assigned 1:1:1 to receive a concurrent Ipilimumab regimen (four doses of Ipilimumab plus paclitaxel and carboplatin followed by two doses of placebo plus paclitaxel and carboplatin), a phased Ipilimumab regimen (two doses of placebo plus paclitaxel and carboplatin followed by four doses of Ipilimumab plus paclitaxel and carboplatin), or a control regimen (up to six doses of placebo plus paclitaxel and carboplatin). In a previous study, lower dose of paclitaxel was found to be equally efficacious, with prospects of decreased toxicity when combined with Ipilimumab^[45]. Patients who tolerated the treatment well without evidence of progression then went on to receive either Ipilimumab or placebo for another 12 wk until disease progression or death. Clinical response patterns to Ipilimumab differs

from conventional cytotoxic therapies and hence a novel parameter, immune related response criteria (irRC) has been recently established to capture this phenomenon. Ipilimumab may cause regression of index lesions in the face of new lesions and initial progression followed by tumor stabilization or decrease in tumor burden. The irRC uses the total tumor burden obtained by adding measurable new lesions to index lesions in determining the tumor response. Changes in non-index or nonmeasurable lesions are discounted. Thresholds for immune-related complete response (CR, complete disappearance of all lesions), immune-related partial response (PR, decrease of total tumor burden from baseline by $\geq 50\%$), immune-related progressive disease (PD, increase of total tumor burden from nadir by $\geq 25\%$), and immune-related stable disease (all other settings including a slow steady decline in total tumor burden from baseline) were the same as for the CR, PR, PD, and stable disease^[44,46]. Here, immune related progression free survival (irPFS) which was defined as time from randomization to immune related progression or death was used as the primary end point.

Patients receiving phased Ipilimumab and carboplatin/paclitaxel showed improved irPFS as compared to carboplatin/paclitaxel alone, however no such benefit was seen in the group receiving concurrent Ipilimumab and carboplatin/paclitaxel. Also further subgroup analysis showed better outcome in patients with squamous histology. Hence the timing of Ipilimumab with cytotoxic chemotherapy seems to play an important role in the outcome.

Incidence of grade 3 or 4 AE was similar in all 3 arms. Though non hematologic AE related to carboplatin (alopecia, fatigue, nausea/vomiting, neuropathy) were similar across all arms, immune mediated adverse effects (rash, pruritis, diarrhea, colitis, transaminitis and pituitary dysfunction) showed a trend for increased incidence in Ipilimumab containing arms. These immune related side effects from Ipilimumab are thought to be secondary to CTLA4 inhibition. It is to be noted that though dose of Ipilimumab used in this trial was higher than that used in melanoma (10 mg/kg *vs* 3 mg/kg), the incidence of AE was comparable^[47].

A larger phase III trial of phased carboplatin/ paclitaxel and Ipilimumab in patients with stage IV NSCLC is underway^[48]. In addition, a safety and efficacy study of

Table 1 Ongoing clinical trials of programmed death-1 antibodies in non-small cell lung cancer

Therapy	Trial	Study population	Study design	Ref.
Nivolumab phase I safety study	NCT01454102 (checkmate 012)	Advanced NSCLC	Nivolumab as single agent <i>vs</i> combination with various chemotherapies	[55]
Nivolumab phase III study to determine OS	NCT01673867 (checkmate 057)	Previously treated (failed platinum based) advanced NSCLC	Nivolumab compared to docetaxel in previously treated patients	[56]
Nivolumab phase III study to look for tumor size and OS	NCT01642004 (checkmate 017)	Previously treated (failed platinum based) advanced NSCLC	Nivolumab compared to docetaxel in previously treated patients	[57]
Nivolumab phase III study assessing tumor size after treatment	NCT01721759 (checkmate 063)	Previously treated and failed 2 lines of chemotherapy	Assess response rate objectively (monitoring tumor size) in patients receiving Nivolumab	[58]
Nivolumab phase II study to determine Response	NCT01928576	Previously treated, advanced/recurrent NSCLC	Assess objective response with Nivolumab, preceded by epigenetic therapy (azacitidine IV or oral, entinostat) priming	[59]
MK-3475 (lambrolizumab) phase I dose limiting study followed by part B to assess safety and tolerability	NCT01928576	Any solid tumor, or advanced NSCLC	Patients with any solid tumor will receive lambrolizumab to assess dose; followed by part B where patients with advanced NSCLC will receive this therapy in combination with chemotherapy	[60]

NSCLC: Non-small cell lung cancer; OS: Overall survival.

Ipilimumab in stage IV NSCLC *vs* Pemetrexed in recurrent stage IV NSCLC who have not progressed after first line platinum based chemotherapy is also ongoing^[49]. A similar study of Ipilimumab and carboplatin/paclitaxel is also being conducted in Japan^[50].

Programmed death-1 pathway inhibitor

Programmed death-1 (PD-1) is another key receptor that can mediate immunosuppression by interacting with PD ligands 1 (PD-L1) and PD-L2. The anti-tumor activity of cytotoxic T cells can be enhanced by blocking this pathway^[51,52].

Anti PD-1 pathway agents gained momentum when a phase I dose escalation study of Nivolumab, a humanized IgG4 mAb, was performed in 39 patients with various cancers (melanoma, colorectal, prostate, NSCLC and renal cell). Among the 6 patients with NSCLC, all of whom had received multiple chemotherapies in the past, 1 patient achieved partial remission for over 14 mo, and the other 5 patients had stable disease post treatment^[53].

A larger phase I study then was conducted in patients with NSCLC, melanoma, castration resistant prostate cancer, colorectal cancer or renal cell cancer who largely had multiple lines of chemotherapy in the past. Nivolumab was administered Intravenous every 2 wk of 8 wk until CR, disease progression, unacceptable side effects or consent withdrawal. Of the 129 NSCLC patients, 17% had objective responses with best responses (24%) at the 3 mg/kg dose. The responses were rapid, durable and the unprecedented OS rate of 24% at 2 years was provocative and is termed “landmark OS”. Major AEs were rash/pruritis (16%), colitis (12%) and specifically pneumonitis (6%). Drug related pneumonitis was severe in 3 patients resulting in 2 early deaths. Better management and monitoring strategies have been introduced since then to prevent such AE related deaths in future. This study opened the realm of possibility that multiple types of cancer could be responsive to immunotherapy if appropriate population is selected even if heavily pre-

treated^[41].

A follow up report on above trial was presented and confirmed the durable response and encouraging OS across all histological subtypes in NSCLC subgroup^[54]. Currently a number of studies are ongoing to test the efficacy of Nivolumab and another investigational PD-1 inhibitor, Lambrolizumab (Table 1).

Data on MK-3475 (Lambrolizumab) from phase I study of 38 patients with advanced NSCLC who had received atleast 2 prior therapies was presented at the 15th World conference on Lung cancer by Garon *et al*^[61]. Early responses were seen in 24% of patients even at 9 wk assessment in both squamous and non squamous subtypes. One patient had PR after a single dose. Median duration of response had not been reached and at the time of abstract presentation, 7 of 9 responding patients were continuing therapy. Median OS was 51 wk. Common AEs were fatigue, rash, pruritus and diarrhea. One case each of grade 2 pneumonitis and grade 3 pulmonary edema were reported, no fatalities occurred.

PD-L1 pathway inhibitor

As described above, PD-L1 is one of the 2 ligands for PD-1 receptor. Presence of PD-L1 has been associated with poor prognosis^[62].

A high affinity, fully humazined PD-L1 IgG4 monoclonal antibody, BMS-936559 was studied in phase I trial in patients with advanced cancers^[42]. Total of 207 patients, 75 of whom had advanced NSCLC, were given escalating dose of BMS-936559. Objective responses were seen in 5 of 49 patients who were evaluable; with both squamous and non-squamous histologies. Also 6 other patients with NSCLC had stable disease at 6 mo.

In all tumor types, it was encouraging to see both durable tumor regression and prolonged disease stabilization. Grade 3 or 4 AEs were seen in up to 9% of patients, however as compared to some other immune therapies, such as anti CTLA-4; these were milder. Again, the response with this agent was promising, and further

Table 2 Ongoing clinical trials of programmed death ligand 1 mAbs in non-small cell lung cancer

Therapy	Trial	Study population	Study design	Ref.
MPDL3280A	NCT01846416	Advanced NSCLC, tumor positive for PD-L1 on IHC	Assess safety, efficacy and objective response rates in patients with PD-L1 positive NSCLC	[64]
MPDL3280A	NCT01903993	Advanced NSCLC, failed platinum based chemo	MPDL-3280A <i>vs</i> Docetaxel after failure of platinum based therapy in patients with advanced NSCLC	[65]

NSCLC: Non-small cell lung cancer; PD-L1: Programmed death ligand 1; IHC: Immunohistochemistry.

studies are needed to outline the patient population and tumor type that would derive benefit from this therapy.

Another anti PD-L1 agent, MPDL-3280A, was studied in a phase I clinical trial, the results of which were exciting as it shows remarkable and durable outcomes in patients with either squamous cell carcinoma or adenocarcinoma. More pronounced effect was seen in smokers, who typically have a poor response to other immunotherapies. This suggests an association between smoking and PD-1/PD-L1 pathway. In this phase I study, 85 patients with advanced NSCLC were evaluated for safety and 53 for efficacy. They received monotherapy with MPDL-3280A every 3 wk and then assessed after a median duration of 48 wk. Objective response rate was 21%, with higher rate observed in patients whose tumor stains positive for PD-L1. The responses were sustained and dramatic response was seen in the smoking cohort. AEs were mild and limited to cough and diarrhea^[63]. Ongoing clinical trials using PD-L1 mAbs are summarized in Table 2.

CONCLUSION

Improved understanding of cancer and its interplay with immune system has now rendered more insight into NSCLC which is being looked at as a “immunogenic” cancer. The application of immunotherapy to NSCLC is being brought back in a big way. It is exciting to see that preclinical success of some of the immunotherapeutic agents is being reflected onto actual clinical success as seen with PD-1 and PD-L1 inhibitors. Data from some major phase II / III clinical trials will be available soon for incorporation into our clinical practice. There are still many unanswered questions regarding the precise timing of these therapies, targeted population, patient selection and appropriate bio-immuno markers to assess response. Ultimately, it would be a high point in medical science if these agents are able to confer survival benefit and improve quality of life of patients who otherwise struggle with the disease. The hope is to identify the best effective “immunotherapeutic targeted agent or combination” and change the treatment paradigm of NSCLC.

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WJCO 5th Anniversary Special Issues (2): Breast cancer

MicroRNAs in pathogenesis of breast cancer: Implications in diagnosis and treatment

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Abstract

MicroRNAs (miRNAs) are small non-coding RNAs generated by a two-step complex process and are post transcriptional negative regulators of their target mRNAs. Dysregulation of many of these miRNAs has been associated with tumorigenesis in various cancers including breast cancer. Aberrantly high expression of specific miRNAs in breast cancer cells is demonstrated to be linked with inhibition of tumor suppressor genes and promote tumorigenesis. They are classified as oncogenic miRNAs. However, the tumor suppressor miRNAs are downregulated in breast cancer cells, since their major targets are oncogenic mRNAs. Understanding mechanism of action of specific miRNAs in breast cancer cells can be utilized to develop newer anti-cancer therapies. Recently, newer techniques are also developed to detect abundance of specific miRNA in the blood plasma samples and can be used in early diagnosis or prognosis in breast cancer. In this review article, we have discussed several miRNAs dysregulated in breast cancer and their therapeutic potential.

INTRODUCTION

MicroRNAs (miRNAs) were discovered two decades ago while studying the development of *Caenorhabditis elegans*. They are conserved, endogenous non-coding RNAs, which are crucial in post transcriptional regulation of several genes involved with various biological functions, such as apoptosis, differentiation, proliferation, *etc.* Many of these miRNAs have been implicated to take part in various pathological conditions including cancer^[1,2].

Mature miRNAs are 18-24 nucleotide long single-stranded RNA synthesized from precursor hairpin shaped double-stranded RNAs^[3]. Biosynthesis of miRNAs is an extremely complex process. The majority of miRNAs are being transcribed by RNA Pol II into primary miRNA (pri-miRNA) with long stem loop structure^[4]. These pri-miRNAs are then cleaved into approximately 70 nt long precursor microRNA (pre-miRNA) by RNase III endonuclease Drosha with DiGeorge syndrome critical region in gene 8 in humans^[5]. This pre-miRNA is transported to the cytoplasm from nucleus by Exportin-5. Once in

Table 1 List of major oncogenic microRNAs in breast cancer

MiRNA	Known target mRNA	Function	Ref.
MiR-10b	HOXD10	Promotes cell proliferation, metastasis and angiogenesis	[108]
MiR-126	IGFBP2, MERTK, PTPN1	Promotes angiogenesis	[18]
MiR-155	SOCS1, TP53INP1, FOXO3, RhoA	Promotes cell proliferation	[12,20,21,62]
MiR-21	PTEN, TPM1, PDCD4, Maspin	Promotes cell proliferation	[22-24,109]
MiR-375	RASD1	Epigenetic modification of tumor suppressor genes	[110,111]
MiR-221/222	TRPS1	Induce metastasis	[28,29,65]
MiR-373	CD-44	Induce metastasis	[14,26]
MiR-520c	CD-44	Induce metastasis	[14,26]
MiR-9	SOCS5, E-cadherin	Induce metastasis	[27,112,113]
MiR-632	DNAJB6	Induce metastasis	[30]
MiR-196b	HOXD10 (indirect)	Promotes angiogenesis	[15]
MiR-7	HOXB3	Epigenetic modification of tumor suppressor genes	[16]
MiR-218	HOXB3	Epigenetic modification of tumor suppressor genes	[16]
MiR-203	SOCS3	Promotes cell proliferation	[25]

HOXD10: Homeobox D10; IGFBP2: Insulin-like growth factor binding protein 2; MERTK: C-mer proto-oncogene tyrosine kinase; PTPN1: Phosphatidylinositol transfer protein, cytoplasmic 1; SOCS1: Suppressor of cytokine signaling 1; TP53INP1: Tumor protein p53 inducible nuclear protein 1; FOXO3: Forkhead box O3; RhoA: Ras homolog family member A; PTEN: Phosphatase and tensin homolog; TPM1: Tropomyosin 1 (alpha); PDCD4: Programmed cell death 4; RASD1: Rat Sarcoma dexamethasone-induced 1; TRPS1: Trichorhinophalangeal syndrome 1; DNAJB6: DnaJ (Hsp40) homolog, subfamily B, member 6; HOXB3: Homeobox B3; MiRNA: MicroRNA.

the cytoplasm, the pre-miRNAs are cleaved into mature 18-24 nucleotide long miRNA by RNase III type enzyme Dicer. Dicer is a highly specific enzyme which forms a complex with two other proteins TRBP and PACT to form miRNA induced silencing (RISC) complex. The RISC complex is responsible for the degradation of the complementary strand of miRNA and directs miRNA to its mRNA target^[4,6]. This results in mRNA degradation or destabilization and subsequently translation inhibition^[4,7]. This review summarizes the role of different miRNAs associated with breast cancer progression, breast cancer stem cells (BCSCs) and their implications in cancer diagnosis, prognosis and treatment.

ROLE OF miRNAs IN BREAST TUMORIGENESIS

Breast cancer is a leading cause of mortality due to cancer among women. Despite a decrease in mortality due to the advancement of scientific research, it is estimated that approximately 1.3 million females develop breast cancer each year with around 465000 expected to succumb to the disease^[8,9]. Early detection and newer treatments are urgently required to inhibit the cancer progression in breast cancer patients. In 2005, a role of miRNA dysregulation in breast cancer was first demonstrated. Since then many studies have found out several different miRNAs which are being deregulated in breast cancer^[10]. Some of these miRNAs act as oncogenic miRNAs by suppressing tumor suppressor genes. Whereas, some of the miRNAs exhibit tumor suppressor properties by down-regulating oncogenic genes^[7].

KNOWN ONCOGENIC MiRNAs IN BREAST CANCER

Usually, miRNAs promote oncogenesis by either destabi-

lizing or degrading tumor suppressor mRNAs in various types of human cancers^[11]. The miRNAs affecting oncogenesis and/or metastasis are classified as oncogenic miRNAs. Several miRNAs have been identified in breast cancer to have such oncogenic potential. Different oncogenic miRNAs exhibit their oncogenic potential by inducing cell proliferation and tumorigenesis and/or metastasis, promoting angiogenesis or inducing epigenetic changes^[12-16]. The Table 1 summarizes some of the most commonly deregulated oncogenic miRNA in breast cancer.

The miR-10b is shown to be upregulated in breast cancer cells and correlated with increased cell migration and metastasis^[11]. Overexpression of miR-10b disrupts the homeobox D10 (HOXD10) mRNA, which leads to the increased expression of RhoC [a Rat Sarcoma (RAS) family member] known to promote proliferation and metastasis^[17,18]. Several studies have suggested that miR-155 is an oncogenic miR by demonstrating that, its upregulation results in inhibition of tumor suppressor genes in breast cancer cells^[12,19-21]. The miR-155 exhibits its oncogenic ability by suppressing the expression of protein Suppressor of cytokine expression 1 (SOCS1) both *in vitro* and *in vivo*^[21]. Inhibition of SOCS1 is inversely correlated to pro-tumorigenesis. The miR-155 also represses tumor suppressor forkhead box O3a expression in breast cancer^[19,20]. Similarly, miR-21 is also demonstrated to be upregulated in breast cancer cells by multiple studies and also considered as oncogenic miRNA. Research thus far have shown that miR-21 inhibits the expression of various tumor suppressor proteins such as TIMP3, PDCD4 and tropomyosin 1 (alpha)^[22-24]. Recently, miR-203 also has been shown to inhibit SOCS3 expression in breast cancer cells^[25]. Inhibition of miR-203 leads to the activation of several tumor suppressor proteins including p53, Bax and p21^[25].

Oncogenic miRNAs such as miR-520c, miR-373, miR-221 and 222, miR-9 and others are known to promote metastasis in breast cancer and are sometimes referred

Table 2 List of major tumor suppressor microRNAs in breast cancer

MiRNA	Known target mRNA	Function	Ref.
Let-7 family	RAS, HMGA2	Inhibit cell proliferation and mammosphere formation	[11,114]
MiR-125	HuR, HER2, ETS1, Cyclin J, MEGF9	Inhibit cell proliferation and invasion	[38,39,115,116]
MiR-205	ZEB1 and ZEB2	Reduces EMT and metastasis	[1,40]
MiR-200 family	ZEB1/2	Reduces EMT and metastasis	[117]
MiR-206	Cyclin D2	Inhibits Cyclin D2 in MCF-7 cells	[118]
MiR-34a	Bcl2, SIRT1	Inhibits migration, invasion and metastasis	[69]
MiR-335	SOX-4, TNC	Inhibits metastasis	[41,42]
MiR-342	HER2	Increases cell proliferation	[119]
MiR-15a/16	HER2	Increases cell proliferation	[68]
MiR-302	RAD52 and AKT1	Affects DNA repair	[78]
MiR-31	RhoA, ITGA5, RDX	Reduces invasion and metastasis	[44]
MiR-519c	HIF-1 α	Inhibits angiogenesis	[45]

MiRNA: MicroRNAs; Let-7: Lethal-7; RAS: Rat Sarcoma; HMGA2: High mobility group AT-hook 2; HuR: ELAV like RNA binding protein 1; ETS1: V-ets avian erythroblastosis virus E26 oncogene homolog 1; MEGF9: Multiple EGF-like-domains 9; ZEB1/2: Zinc finger E-box binding homeobox 1/2; Bcl2: B-cell CLL/lymphoma 2; SIRT1: Sirtuin 1; SOX-4: SRY (sex determining region Y)-box 4; TNC: Tenascin C; HER2: Human epidermal growth factor receptor 2; RAD52: RAD52 homolog (S. cerevisiae); AKT1: V-akt murine thymoma viral oncogene homolog 1; RhoA: Ras homolog family member A; ITGA5: Integrin, alpha 5 (fibronectin receptor, alpha polypeptide); RDX: Radixin; HIF-1 α : Hypoxia inducible factor 1, alpha subunit; EMT: Epithelial to mesenchymal transition.

as MetastamiRs^[11,26,27]. MiRNAs 520c and 373 have been shown to increase migration and invasion both *in vitro* and *in vivo* by targeting the expression of CD44^[26]. miR-221 and 222 induce metastasis by targeting trichorhinal syndrome 1^[28,29]. The miR-9 is also shown to be significantly upregulated in breast cancer, by targeting E-cadherin to promote metastasis^[27]. A recent study has also revealed that miR-632 stimulates metastasis by down regulating the HSP40 family member: DNAJB6 in breast cancer^[30].

Several oncogenic miRNAs are also known to deregulate angiogenesis. The expression of miR-126 has been seen to be upregulated in many breast cancer cells^[31]. Recent studies suggests that miR-126 affects angiogenesis by inhibiting the protein synthesis of insulin-like growth factor binding protein 2, c-Mer tyrosine kinase and phosphatidylinositol transfer protein cytoplasmic 1. MiRNAs 10b and 196b have also been shown to regulate angiogenesis targeting vascular endothelial growth factor (VEGF) signaling through HOXD10^[16].

Some miRNAs are known to inhibit tumor suppressor genes by affecting epigenetic changes. In breast cancer cells MDA-MB-231 and MCF-7 miRNAs miR-7 and miR-218 affects histone modification and DNA methylation by targeting HOXB3. This results in inhibition of RASSF1A and Claudin 6 expression^[16].

KNOWN TUMOR SUPPRESSOR MiRNAs IN BREAST CANCER

Tumor suppressor miRNAs target mRNAs of various oncogenes and their dysregulation is critical in carcinogenesis^[11]. The most commonly deregulated tumor suppressor miRNAs in breast cancer are compiled in the Table 2. The Lethal-7 (let-7) family of miRNA due to their abundance was among first to be identified. This family of miRNA contains 12 members^[32,33]. Various studies

have shown that, expression of let-7 family members is downregulated in malignant breast cells, compared to the healthy tissues^[1,33,34]. Oncogenes RAS and High-Mobility Group AT-hook 2 (HMGA2) are found to be the direct targets of let-7^[33]. Increased expression of let-7 reduces cell proliferation and mammosphere formation by breast cancer initiating cells and also decreases metastasis *in vivo*^[33]. Another study revealed that let-7a directly binds to the 3'-Untranslated Regions (UTR) region of C-C chemokine receptor type-7 (CCR7) gene. Signaling of CCR7 and its ligand CCL21 has been demonstrated to play key role in cancer progression and metastasis^[35]. It was shown that expression of let-7a inhibits CCR7 expression and suppresses migration and metastasis in Zebrafish^[36].

In many breast cancer cell lines and breast cancer patient samples, the levels of miR-125a and miR-125b are often found to be greatly downregulated. miR-125b directly targets ETS: an oncogenic transcription factor and functions as a tumor suppressor miRNA^[37]. Further, both miR-125a and miR-125b are shown to be downregulated in human epidermal growth factor receptor (HER2) overexpressing cells. Expression of miR-125 in SKBR3 cells lead to the suppression of HER2 transcripts, which eventually lead to the slower cell growth and decreased invasiveness^[38]. Recently, several novel targets such as cyclin J (CCNJ) and multiple EGF-like-domains 9 (MEGF9) were found to be the direct targets of miR-125b^[39]. Both CCNJ and MEGF9 are potential oncogenes and their roles in tumorigenesis are only recently emerging^[39].

Another frequently down regulated tumor suppressor miRNA in breast cancer is miR-205, which is suggested to be the negative regulator of epithelial to mesenchymal transition (EMT) and metastasis^[1]. The miR-205 like the miR-200 family members targets Zeb1 and Zeb2 to prevent cells from undergoing EMT. Even though miR-200 and miR-205 share similar functionality, their expression levels differ in normal mammary gland and BCSCs. The expression of miR-205 is found to be elevated, whereas

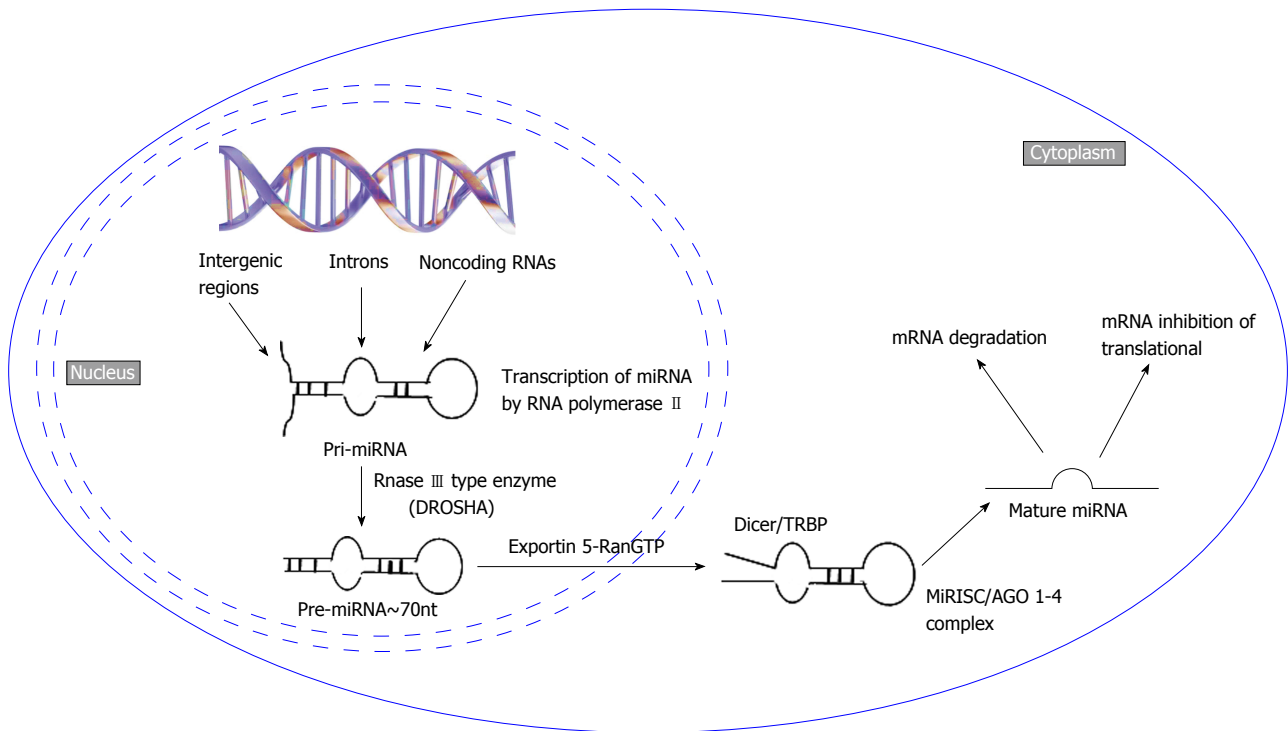


Figure 1 General mechanism of microRNA biosynthesis. Pri-miRNA: Primary microRNA; MiRNA: MicroRNA; Pre-miRNA: Precursor microRNA.

expression of miR-200 family members is decreased in normal mammary gland stem cells and BCSCs^[40]. This indicates the unique functionality of miR-205 in breast cancer.

The miR-335 act as a tumor suppressor miRNA in breast cancer cells because its expression suppressed the migration, invasion and metastasis^[41]. It was discovered that miR-335 targets transcription factor SOX4 and matrix protein Tenascin-C (TNC)^[42]. Both SOX4 and TNC were recently discovered to promote metastasis in pancreatic and liver cancers^[42].

miR-31 also recently came into light when it was demonstrated that multiple genes involved in the metastatic pathway of breast cancer were being targeted by it, including radixin, RhoA and integrin- α -5. When miR-31 is over-expressed expression of all three target genes is reduced and cells become less invasive and metastatic^[43]. In another study it was also seen that the ectopic expression of miR-31 in MDA-MB-231 and SUM-159 cell lines both *in vivo* and *in vitro* suppressed invasion and metastatic ability^[44].

The anti-tumor activity of miR-519c is attributed to its ability in regulating angiogenesis. In a study miR-519c directly targeted the hypoxia inducible factor 1 α (HIF-1 α), which regulates the angiogenesis by activating VEGF, interleukin-8 (IL-8) and basic fibroblast growth factor. Ectopic expression of miR-519c significantly suppressed HIF-1 α and reduced angiogenesis in a nude mouse model^[45]. Also, miR-519a/b/c expression is reduced in ovarian, kidney and lung tumor samples as compared to the healthy samples. Expression of miR-519 is found to be inversely correlated to the RNA binding protein HuR expression^[46].

MiRNAs AND CANCER STEM CELLS

Around a decade ago a concept was proposed that a small subset of cancer cells with stem-like characteristics might be the key factor in tumor development and metastasis in various types of cancers^[47]. Cancer stem cells (CSCs) gained more attention when its role was suggested in providing chemoresistance^[48]. In breast cancer, CD44⁺/CD24^{-/low} or high aldehyde dehydrogenase 1 (ALDH1) expression are typical characteristics of BCSCs. To enrich BCSCs, breast cancer cells are stained with fluorescently labelled antibodies for these markers and then sorted using Fluorescence-activated cell sorting^[49]. However, it should be noted that even after sorting, it is virtually impossible to get a pure cancer stem cell population. Recent researches have shown that the expression profile of specific miRNAs in BCSCs is distinct compared to the normal breast cells^[33,50]. The dysregulation of miRNA might contribute to the self-renewal of BCSCs and cancer progression^[33]. Here we have summarized the several miRNAs identified to be deregulated in BCSCs and their mechanism of action (Figure 1).

MiRNAs DOWNREGULATED IN BCSCS

One of the first group of miRNAs discovered to be dysregulated in BCSCs were the let-7 family members. It was noticed that expression of let-7 miRNA was significantly downregulated in SKBR-3 tumor-initiating cells than non-self-renewing population^[33]. These two population of cells were separated using CD44⁺ CD24^{-/low} phenotype. Let-7 miRNAs act as tumor suppressors mainly by

targeting RAS oncogene as described earlier^[33]. Upon induced expression of let-7 miRNAs led to the decreased BCSCs and decreased mammosphere formation^[33]. Another study have shown that decreased expression of let-7 is attributed to the RNA binding protein Lin28^[51]. It is also demonstrated that activation of STAT3 *via* inflammatory cytokines activates Lin28 expression and this results in let-7 downregulation. Consequently, target of let-7, HMGA2 is increased, which in turn enhances the EMT in CSCs.

The BCSCs can be enriched also by cultivating cancer cells as mammospheres. The 3D mammosphere is believed to be enriched with BCSCs. In a recent finding, miRNAs belong to miR-30 family are found to be downregulated in BCSCs enriched *via* mammosphere. The miR-30e downregulation increases the ubiquitin-conjugating enzyme 9 and integrin β 3 expression. Increasing the expression of miR-30e reduced self-renewal ability of CSCs and tumorigenesis^[52]. Overexpression of miR-30a reduces total number of mammospheres in MCF-7 cells and its downregulation leads to the increase in the number of mammospheres^[53].

MiR-200 family members (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) have been shown to regulate the BCSC population. The miRNAs belong to the miR-200 family are significantly downregulated in BCSCs (CD44⁺ CD24^{-/low}) when compared to non-cancerous cells. Expression of tumor suppressor miR-200c decreased the self-renewal ability of BCSCs *in vitro* and tumor formation ability *in vivo*^[40]. The decreased expression of miR-200b lead to the reduction of E-cadherin expression, which results in increase EMT^[54]. Another group has also demonstrated that re-expressing the miR-200 family members reprograms BCSCs to a more non-stem like cells and also promotes mesenchymal to epithelial transition^[55].

The miR-128 was also found to be downregulated in BCSCs (in both CD44⁺ CD24^{-/low} and mammospheres) compared to the non-cancerous cells. The miR-128 targets polycomb ring finger oncogene (Bmi-1) and ATP binding cassette sub-family C member 5 (ABCC5). Both of these genes are known to induce chemoresistance in breast tumor initiating cells. Induced expression of miR-128 reduces the levels of both Bmi-1 and ABCC5 and increases the drug efficacy^[56]. Also, similar to other miRNAs mentioned in this section, reduction in miR-128 expression results in the increase number of mammospheres^[57].

Another miRNA recently shown to be downregulated in BCSCs (high ALDH1) is miR-93. Ectopic expression of miR-93 prevents tumor growth in xenografts. In this study it was demonstrated that miR-93 regulates BCSCs by reducing the expression of several stem cell regulatory genes such as *SOX4*, *STAT3* and *AKT3*^[58].

BCSCs enriched from MCF-7 and SK-3rd (CD44⁺ CD24^{-/low}) cells were found to have lower levels of miR-34c. Decreased expression of miR-34c promotes self-renewal and EMT. When re-expressed, miR-34c inhib-

ited the expression of Notch4, reduced the number of mammospheres^[59].

Another tumor suppressor miRNA often down-regulated in BCSCs (enriched using mammosphere) is miR-16. It targets the oncogene Wip1 and ectopic expression of miR-16 inhibits Wip1 expression in MCF-7 cells and also increases the sensitivity to chemotherapeutic drugs^[60].

MiRNAs UPREGULATED IN BCSCS

In the mammospheres of human breast cancer cell lines MDA361, MCF-7 and BT474, miR-181 levels are found to be elevated compared to non-cancerous cells. Potential target of miR-181 seems to be the tumor suppressor gene: Ataxia telangiectasia mutated (ATM). ATM levels are usually reduced in mammospheres treated with transforming growth factor- β .

Another miR-found to be significantly upregulated in BCSCs is miR-495. The miR-495 is found to be upregulated in two distinct subpopulations of BCSCs based on two surface markers: (1) commonly used CD44⁺ CD24^{-/low}; and (2) novel surface marker PROCR⁺/ESA⁺ (PROCR stands for protein C receptor). Also, overexpression of miR-495 increased tumor formation *in vivo*. Moreover, expression of E-cadherin was lowered with overexpression of miR-495 which enhances the stem like phenotype in BCSCs^[61].

MiRNAs implicated to promote resistance against chemotherapy

So far it has been established that, miRNAs play critical role in regulation of several genes associated with various cancers including breast cancer^[4,11,62]. Many recent studies have implicated that several miRNAs can confer resistance against common chemotherapeutic drugs in breast cancer^[63,64]. The proposed mechanisms to explain this drug resistance in breast cancer are (1) intracellular drug depletion *via* transporters and enzymes; (2) impairing cellular functions through cell cycle arrest, DNA damage or apoptosis; (3) Inducing signaling cascade which promote transformation; and (4) Inducing epigenetic changes such as DNA methylation^[63-65].

The miR-7, miR-27, miR-326, miR-328, miR-451, miR-489 confer resistance to chemotherapeutic drugs such as Doxorubicin, Cisplatin or taxol *via* drug depletion by targeting transporters and enzymes in breast cancer cells^[63,66]. Some of these miRNAs target the ABC drug transporters and affect drug availability in the cell. The decrease in miR-451 leads to the increase in its target P-glycoprotein, a member of the ABC transporter family^[67]. Another family of genes associated with the drug resistance is multidrug resistance associated proteins (MRP-1). Various independent studies have revealed that miR-7 and miR-345 directly bind at the 3'-UTR region of MRP-1 mRNA and decrease their expression levels correlated with resistance to Cisplatin in MCF-7 cells^[64]. Similarly, miR-489 targets MRP-2 and affects the drug ef-

flux in breast cancer cells^[63].

Dysregulation of tumor suppressor miRNAs miR-342 and miR-15a/16 are related to the tamoxifen resistance in HER2 overexpressing tumors. HER2 overexpression is found in approximately 30% of breast cancers and is one of the major factors responsible for the chemoresistance^[11]. A new study revealed that a splice variant of HER2, HER2Δ16 is linked to metastatic breast cancer and chemoresistance^[68]. It was demonstrated that decreased levels of miR-342 and miR-15a/16 expression contribute to tamoxifen resistance by increasing anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) expression in cell lines overexpressing HER2Δ16^[68].

Similarly, expression of miR-34a is also found to be downregulated in breast cancer. It is suggested that the miR-34a is regulated by p53. Breast cancer cell line MDA-MB-231 with low levels of miR-34a was more resistant to radiation therapy compared to cells with elevated levels of miR-34a^[69]. In a separate report, miR-34a was found to be downregulated in multiple breast cancer cell lines. Here it was shown that miR-34a suppresses proliferation and migration by inhibiting Bcl-2 and sirtuin^[70].

Several chemotherapeutic drugs used in the treatment of breast cancer interfere with cellular functions such as DNA repair, cell cycle and apoptosis. Over activation of DNA repair pathways are associated with resistance to chemotherapy^[71]. MiR-373 down regulation is shown to increase DNA repair efficiency and resistance to drugs^[72]. Similarly, miR-21 and miR-141 are also associated with drug resistance, which target the mismatch repair pathway^[73,74]. One of the most studied genes in breast cancer, BRCA1 also achieves drug resistance by inducing repair in double strand DNA breaks generated by anticancer drugs^[75]. The miR-17, miR-182, miR-146 and miR-28 have a potential binding site at the 3'-UTR of BRCA1 transcript^[63,76,77]. Another, miRNA affecting dsDNA repair is miR-302. The expression of miR-302 is associated with radioresistant breast cancer cells. The miR-302 directly inhibits the expression of RAD52 and AKT that are known to provide radioresistance in many cancers including breast cancer^[78] to provide radioresistance in many cancers including breast cancer. The PI3K/AKT is found to be one of the major pathways in various mechanisms to confer radioresistance in breast cancer. RAD52 is important in dsDNA repair mechanism and its activation leads to radioresistance upon down regulation of miR-302^[79].

As mentioned above, miRNA dysregulation can trigger several signaling pathways to alter the levels of various receptors or hormones. The estrogen receptor (ER) is expressed in the vast majority of breast cancers and induces cell proliferation. Anticancer drugs like Tamoxifen inhibit the expression of steroid receptors to prevent ER-driven cell proliferation. Increased expression of miR-221/222 and miR-101 decreases the sensitivity to Tamoxifen in ER-positive tumors^[65]. Overexpression of HER2 (or HER2) is found in approximately 30% of malignant breast cancer patients and known for poor prognosis^[80]. The downregulation of miR-125 and

miR-331 is suggested to be responsible for reduced efficacy of HER2 targeted therapy such as trastuzumab. Both miR-125 and miR-331 can decrease the expression of HER2^[63].

Several genes responsible for induction of breast cancer can be regulated epigenetically, and several of them are known to induce drug resistance in breast cancer cells. Abnormal DNA methylation is one of the major characteristic of cancer cells. DNA methyl transferases DNMT1, 2 and 3 are crucial for DNA methylation. Often levels of DNMTs have been found to be elevated in breast cancer cells. Several recent studies have suggested that miR-148, miR-152, miR-29, miR-194 and miR-143 regulate the expression of various DNMTs in breast cancer, thereby inducing drug resistance^[81-84]. Moreover, it is shown that upregulation of DNMTs leads to the hypermethylation of miRNAs such as miR-148 and represses their expression. Increasing the miR-148 expression resulted in decreased tumor growth and metastasis^[85]. Recent studies clearly demonstrated that miR-143 is downregulated in breast cancer cells and regulates the expression of DNMT3A. Restoring the expression of miR-143 can decrease the phosphatase and tensin homolog (PTEN) hypermethylation^[81]. Other than DNA methylation, aberrant change in chromatin structure can induce drug resistance in cancer cells^[86]. The miR-101 and miR-221/222 upregulation leads to aberrant histone H3 modification and is associated with chemoresistance^[87,88]. Similarly, miR-342 downregulation affects histone demethylation and is associated with Cisplatin resistance^[64].

MiRNA as new diagnostic and prognostic markers

Chances of survival of a breast cancer patient is significantly more when detected at an early stage over late detection^[89]. Moreover, biomarkers which can predict the treatment outcome are also very important in managing breast cancer therapy. Various approaches have been used to identify different biomarkers to diagnose breast cancer at an early stage. In recent years miRNAs have emerged as important diagnostic and prognostic tool in breast cancer research.

Dysregulation of miRNA in breast cancer cells as novel prognostic biomarkers

Other than early detection of breast cancer, prediction of treatment response is also very critical in better outcome of the treatment and patient survival. It was hypothesized that dysregulation of specific miRNAs in breast tumors can be used in determining prognosis of drug treatment in breast cancer patients. Data from various findings with breast tumor samples have supported this hypothesis. In this section role of different miRNAs in breast cancer prognosis is briefly discussed.

A recent study showed that expression levels of miR-10B, miR-21, and miR-335 were higher in 112 breast tumor samples compared to the healthy tissues and directly correlated with disease free survival^[13]. Also, upregulation of miR-21 can be used as prognostic tool for the

lung metastasis of breast cancer^[90]. In this study it was also reported that down regulation of tumor suppressor miR-205 correlates with disease free interval and overall survival^[13]. Recent computational analysis indicated that association of miR-330-3p with MAF mRNA directly correlates with lower survival rates of breast cancer patients^[91]. Further validation of these miRNAs can be useful in identifying reliable prognostic biomarker of breast cancer.

Among all breast cancer patients around 30% patients are HER2 positive and found to have poor prognosis than PR⁺ or ER⁺^[92]. Moreover, around 10% breast cancer patients acquire triple negative (PR⁻/ER⁻/HER2⁻) phenotype with worse prognosis^[77,92]. Finding a better prognostic marker is urgently required for these types of breast cancers. In a study RNA samples of 49 HER2 positive and 48 triple negative breast cancer (TNBC) patients were subjected to the deep sequencing to identify potential prognostic markers of these relatively resistant breast cancers to chemotherapy. This study revealed that patients who developed metastasis, had increased levels of miR-184 and decreased levels of miR-375 and miR-423^[10]. This data suggest that these miRNAs can be used as prognostic markers for HER2⁺ or TNBC breast cancer. Further, research is required to confirm these findings with bigger sample size.

Circulating miRNA as a diagnostic and/or prognostic tool for breast cancer

Specific miRNAs are very stable in serum of various breast cancer patients, making them very valuable targets in early diagnosis of the disease. Recent advances in high-throughput techniques provided an instrumental platform to detect dysregulation of miRNA in serum samples of breast cancer patients. Various studies have identified several miRNA specifically deregulated in the blood plasma of breast cancer patients compared to a healthy individual^[6,9,32]. Moreover, these findings also demonstrated that dysregulation of miRNA in plasma is attributed to the primary tumor site^[6,9,32]. In this section, several of these miRNAs identified as biomarkers of breast cancer diagnosis are discussed.

A recent study has identified miR-571, miR-139-3p, miR-206, miR-193a, miR-526b, miR-519 to be more than 1.5 fold downregulated in breast cancer patients of age 50-53^[9]. In the same study they also found elevated levels of miR-376c, miR-801, miR-148b, miR-424, miR-184, miR-409, miR-376a, miR-190 and miR-127-3p in the blood plasma of breast cancer patients^[9]. It is important to determine that the dysregulation of miRNA found in the serum samples of patients is tumor-derived. To test this, Ng *et al.*^[93] developed a method, in which miRNA levels of serum samples of patients are compared with their tumor samples. During the profiling they found that 8 miRNAs (miR-16, miR-21, miR-27a, miR-150, miR-191, miR-200c, miR-210, miR-451) up-regulated and miR-145 down-regulated in both plasma and tumor tissues in breast cancer patients. Further it was demon-

strated that miR-451, miR-21 and miR-16 levels were significantly elevated in the serum of breast cancer patients compared to the healthy individuals^[93]. Interestingly miRNA levels measured in the postoperative samples were drastically lowered when compared with preoperative samples. These findings indicate that miRNAs from serum samples can be used as diagnostic tool in breast cancer^[9,93].

Another recent study found that miR-18a, miR-181a and miR-222 showed the highest percentage difference in the serum samples of 205 women who eventually developed cancer and 205 women who remained cancer free^[94]. This study shed light on detecting miRNAs which can predict increased risk of getting breast cancer. Together all these results clearly demonstrates that using the high-throughput methods, dysregulation of miRNA can be used as a diagnostic tool in various cancers including breast cancer to predict disease progression, risk of metastasis and/or treatment outcome^[9,93,94].

Few reports have suggested that a limited number of specific miRNAs detected in blood plasma can also be used as both diagnostic and prognostic marker of breast cancer. One study demonstrated that miR-155 levels are significantly higher in 81.9% breast cancer patient samples and is an excellent diagnostic marker. After surgical removal of breast tumor and four rounds of chemotherapy, 79% of patients exhibited reduced levels of miR-155 and sustained treatment response^[95]. In the blood plasma elevated levels of miR-200a/b/c, miR-203 and miR-210 were also shown to have prognostic significance in breast cancer patients^[96].

TARGETING MiRNAs FOR BREAST CANCER TREATMENT

So far we established that several miRNAs are play critical role in breast cancer initiation, progression and metastasis, and thus become attractive targets for therapy^[31,49,79]. Various research groups have demonstrated different approaches to regulate these target miRNA expression to improve therapy. Two distinct ways miRNA based therapy can be utilized as anticancer treatment: (1) by antagonizing oncogenic miRNA; or (2) by enhancing expression of tumor suppressor miRNAs.

Antagonizing oncogenic miRNA in breast cancer

There are various approaches being utilized to inhibit oncogenic and metastatic functions of miRNA. One of the most obvious strategies is to silence these oncogenic miRNAs using an anti-microRNA oligonucleotide (AMO) to prevent interaction with their target proteins. The AMO competes with the target mRNA of oncogenic miRNAs and inhibits their function^[17]. These anti-sense miRNAs are chemically modified to increase their stability in the system. These chemical modifications also make them more stable in the hybridization state^[7].

Use of anti-sense RNA of miR-10b in mouse reduced mammary tumor metastasis^[17]. It was clearly observed that

upon treatment with AMO against miR-10b decreased the levels of miR-10b significantly and by simultaneously increased the levels of its target, HOXD10. Also, it was observed that anti-sense miR-10b did not reduce the size of primary tumor but prevented lung metastasis. These results suggest that anti-miR-10b can be a good candidate as an anti-metastatic agent but might not be useful in reducing primary tumor burden in breast cancer^[17].

Another strategy used in miR-therapeutics is modification of 2'-hydroxyl to 2'-O-methyl in ribonucleotide. This chemical modification also prevents the degradation of these miRNAs and improves their stability. The anti-sense of oncogenic miR-21 is used in the xenograft carcinoma model using MCF7 breast cancer cells. It was observed that mice treated with anti-miR-21 had half the size of tumors when compared to the control group^[97]. The miR-21 is an oncogenic miRNA and known to induce cell proliferation by targeting PTEN. Using 2'-O-methyl antisense oligonucleotide only destabilizes the mRNA and does not degrade them. Therefore, any change in their levels can be measured only by measuring the levels of their targets^[98].

Another novel method to inhibit miRNAs was introduced as an alternative to the chemically modified AMO. Here, miRNA-inhibitors are directly expressed in cells under the strong promoter which contains multiple sites complementary to the target miRNA and are known as "miRNA sponges"^[99]. Utilizing this novel approach a very recent investigation have revealed that inhibition of miR-22 by providing complementary binding site *via* reporter gene in 3'UTR reduced the cell migration and metastatic phenotype of breast cancer cell line LM2. Oncogenic miR-22 is known to increase breast cancer metastasis and stemness. Moreover, using same approach inhibition of miR-22 also led to the inhibition of breast cancer metastasis *in vivo*^[100].

Several studies have made it apparent that dysregulation of multiple miRNAs seems to be responsible for oncogenesis and/or metastasis. Thus targeting single miRNA might not be sufficient to achieve the optimal result in inhibiting cancer progression. To address this issue, a novel method has been proposed where AMO is designed to target more than one miRNA. In a study it was shown that a longer multiple target AMO (MTg-AMO_{21/155/17}) decreased the cell viability of MCF-7 cancer cells to 18% at 10 nmol/L concentration. To achieve the similar levels of cell death by individual AMOs against miR-21, miR-155 or miR-17 required 10 times higher concentration of AMOs. These results indicated that targeting multiple miRNAs by longer AMOs can be a better approach to inhibit the disease progression^[101].

Recently, libraries of active clinical small molecule compounds have been tested for their ability to inhibit specific miRNA of interest^[102,103]. A recent finding demonstrated that levels of miR-21 were effectively reduced by the small molecule inhibitor "azobenzene"^[102]. Similarly, another study identified two small molecule inhibitors polylysine and tryptaflavine as effective inhibitors of various onco-

genic miRNAs and attenuation of tumorigenesis^[103].

Another novel approach in miRNA antagonism is by using peptide nucleic acid (PNA) as a miRNA inhibitor. Chemically in PNA the sugar-phosphate backbone is replaced by N-(2-aminoethyl) glycine, which makes them efficient hybridization agent resistant to DNases and proteases. In aggressive breast cancer cells PNAs targeting miR-221 and miR-210 reduced the levels of miR-221 and miR-210 respectively. Also, inhibition of these miRNAs resulted in the elevated levels of a major apoptosis player p27Kip1^[104]. Treatment with PNA targeting miR-21 showed inhibition of tumorigenesis of MCF-7 breast cancer cells in female nude mice compared to the control group of mice^[105].

Restoration of tumor suppressor miRNAs

Several tumor suppressor miRNAs are shown to be downregulated in various types of cancers. It is proposed that restoring their levels should be beneficial in inhibiting cancer progression. One approach to restore the tumor suppressor miRNA in cancer cells is by using the miRNA mimics. A study demonstrated that in aggressive breast cancer cell MDA-MB-231 restoring the levels of miR-200c using the miR-200c mimic results in reduced cell proliferation, invasion and migration^[106]. Similarly, in breast cancer cells increasing the levels of let-7 miRNA by let-7-lentivirus infection reduced the cell proliferation and mammospheres formation^[33].

CONCLUSION

Accumulating evidences supports that dysregulation of specific miRNAs is crucial in breast cancer progression. Targeting these miRNAs can have tremendous potential in breast cancer diagnosis and treatment. This review summarizes the impact of various deregulated miRNAs in breast cancer progression, metastasis and angiogenesis. Understanding the molecular mechanisms of these miRNAs with their target genes is critical in utilizing them as a therapeutic tool. To manage more aggressive types of breast cancers, other than development of novel therapies, early diagnosis and prognosis markers are urgently required. Recently, various online databases of miRNA targets are made available to predict the role of specific miRNA in regulating biological pathways. Better use of these databases can save a lot of time and efforts for clinical research in finding better target miRNA for cancer therapeutics or diagnosis. Also, for future a better collaboration among clinicians and researchers is required to develop novel miRNA based anti-cancer therapies.

Several publications have indicated that differences in specific miRNA levels among blood plasma samples of breast cancer patients compare to the healthy individuals can be used as diagnostic markers of breast cancer. However, it is not fully understood that, what is the source of these miRNAs? And how are they released in the blood stream? Future research is necessary to find this mechanism to develop better diagnostic tools using miRNAs.

Despite of all the advances, miRNA based therapy faces some stiff challenges. One of them is specificity. Also, therapeutic miRNAs are subjected to degradation. This results in inefficient treatment in cancer cells. Further research is required to address these issues to improve their specificity and enhancing efficiency of treatment. Moreover, future research warranted to improve delivery of these miRNA to prevent their degradation^[1,80].

Studying the function of various miRNAs in different biological pathways have improved our understanding of their role in cancer development. Also, several studies have shown very encouraging data in breast cancer treatment using miRNA both *in vitro* and *in vivo*. Despite of these successes it is believed that, it will take several years for miRNA based therapy to enter clinics to treat human cancers because of the limitations we discussed above^[1,107].

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Magnetic resonance imaging in breast cancer: A literature review and future perspectives

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Abstract

Early detection and diagnosis of breast cancer are essential for successful treatment. Currently mammography and ultrasound are the basic imaging techniques for the detection and localization of breast tumors. The low sensitivity and specificity of these imaging tools resulted in a demand for new imaging modalities and breast magnetic resonance imaging (MRI) has become increasingly important in the detection and delineation of breast cancer in daily practice. However, the clinical benefits of the use of pre-operative MRI in women with newly diagnosed breast cancer is still a matter of debate. The main additional diagnostic value of MRI relies on specific situations such as detecting multifocal, multicentric or contralateral disease unrecognized on conventional assessment (particularly in patients diagnosed with invasive lobular carcinoma), assessing the response to neoadjuvant chemotherapy, detection of cancer in dense breast tissue, recognition of an occult primary breast cancer in patients presenting with cancer metastasis in axillary lymph nodes, among others. Nevertheless, the development of new MRI technolo-

gies such as diffusion-weighted imaging, proton spectroscopy and higher field strength 7.0 T imaging offer a new perspective in providing additional information in breast abnormalities. We conducted an expert literature review on the value of breast MRI in diagnosing and staging breast cancer, as well as the future potentials of new MRI technologies.

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Key words: Breast magnetic resonance imaging; Cancer; Diffusion-weighted imaging; Spectroscopy; 7.0 tesla

Core tip: Early detection and diagnosis of breast cancer are essential for successful treatment. Magnetic resonance imaging (MRI) has become increasingly important in the detection and delineation of breast cancer in daily practice. However, the clinical benefits of the use of pre-operative MRI in women with newly diagnosed breast cancer is still a matter of debate. We conducted a literature review on the value of breast MRI in diagnosing and staging breast cancer, as well as the future potentials of new MRI technologies, such as MR spectroscopy, diffusion-weighted imaging and higher field strength 7.0 tesla imaging.

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INTRODUCTION

Breast cancer is the most common malignant disease

occurring in women worldwide with a lifetime risk of 12.4%^[1,2]. Early detection and diagnosis of breast cancer are prerequisites for successful treatment selection. Although mammography and ultrasound are the most commonly imaging tools used for the detection and characterization of breast abnormalities, the relatively low sensitivity and specificity of these techniques (especially in patients with dense breast tissue, with breast implants or postsurgical scar distortions)^[3-6] resulted in a demand for new imaging modalities. Contrast enhanced magnetic resonance imaging (CE-MRI) with its high soft tissue contrast, multiplanar sectioning and three dimensional representation of the breast provides high sensitivity (over 90%) in the detection of breast cancer. However, the specificity for lesion characterization is still low to moderate (72%)^[7-19], turning the discrimination between cancer and benign lesions into a challenge. The main additional diagnostic value of MRI relies on (1) detecting foci of multifocal, multicentric or contralateral disease unrecognized on conventional assessment (physical examination, mammography and ultrasound); (2) recognition of invasive components in ductal carcinoma in situ (DCIS); (3) assessing the response to neoadjuvant chemotherapy (NAC); (4) detecting an occult primary breast cancer in patients presenting with metastatic cancer in axillary nodes; and (5) detection of cancer in dense breast tissue^[14,20-26]. The development of new technologies has also resulted in information gain concerning breast lesions. We reviewed the recent literature to clarify the role of MRI in diagnosing and staging breast cancer with focus on the implementation of new techniques, such as MR spectroscopy, diffusion-weighted imaging (DWI) and higher field strength 7.0 T imaging.

SEARCH

In this expert literature review, we conducted a literature search on Pubmed in papers published between 1990 and 2013 using the keywords “breast”, “MRI”, “staging”, “spectroscopy”, “diffusion-weighted imaging” and “high field breast MRI”. Articles published in English pertaining to adult humans with available abstracts were included. References of articles were also included. First we present main guidelines on the use of MRI in diagnosing and staging of breast cancer and, subsequently, we will discuss the new technologies currently available for research.

RESULTS

Detection of additional disease

The main evidence in favor of MRI is based on the superior capability of this technique in detecting ipsilateral and contralateral disease, when compared to mammography and ultrasound (Figure 1)^[21,26,27]. In a prospective trial, Schelfout *et al.*^[26,27] found that MRI detected 96% of multifocal/multicentric disease, while mammography and ultrasound depicted only 28.6% and 26.5%, respec-



Figure 1 Magnetic resonance imaging scanner with closed bore magnet and a dedicated 8 channel phased-array breast coil (top right). Technically any magnetic resonance imaging scanner could be used in breast image acquisition. However, in daily practice, field strengths of 1.5 T and 3 T are often used due to higher spatial resolution at similar temporal resolution, providing better diagnostic efficacy.

tively. Taking the histological types of breast cancer into account, invasive lobular carcinoma (responsible for 5% to 15% of all cases of invasive breast cancers)^[28-30] is well known to have a higher incidence of multifocal, multicentric and contralateral disease when compared to invasive ductal carcinoma. MRI is, therefore, particularly important in the preoperative work-up and staging of these patients^[7,11,12,30-32]. In a recent retrospective study, Menezes *et al.*^[33] also found a high incidence of multifocal, multicentric and contralateral disease in patients with mixed tumors containing different percentages of lobular component. This might corroborate the hypothesis that MRI would also be valuable in the work-up of patients with mixed breast tumors (Figure 2). MRI also has shown higher accuracy in determining tumor size (correlated to histopathology) than ultrasound or mammography^[26]. However, some studies emphasize that MRI tends to overestimate lesion size, particularly in patients diagnosed with invasive lobular carcinoma and DCIS^[34-38].

An additional value of MRI is the detection of invasive component in DCIS lesions. In a retrospective study, Kim *et al.*^[38] concluded that MRI was more accurate for the detection and assessment of the size of DCIS than mammography. In a prospective cohort, Hwang *et al.*^[22] demonstrated MRI to be superior to mammography in detecting invasive components in patients diagnosed with DCIS. MRI also showed a higher sensitivity and superior negative predictive value for detection of residual DCIS. However, the small number of patients in this study (51) might not be representative of a large population with DCIS. Further research might be necessary in order to confirm the use of MRI in detecting invasive component in DCIS.

Patients with an increased risk

MRI has an important role in screening high-risk patients. The American Cancer Society Guidelines for the Early Detection of Cancer advises annual breast MRI beginning at the age of 25-30 years in patients carrying

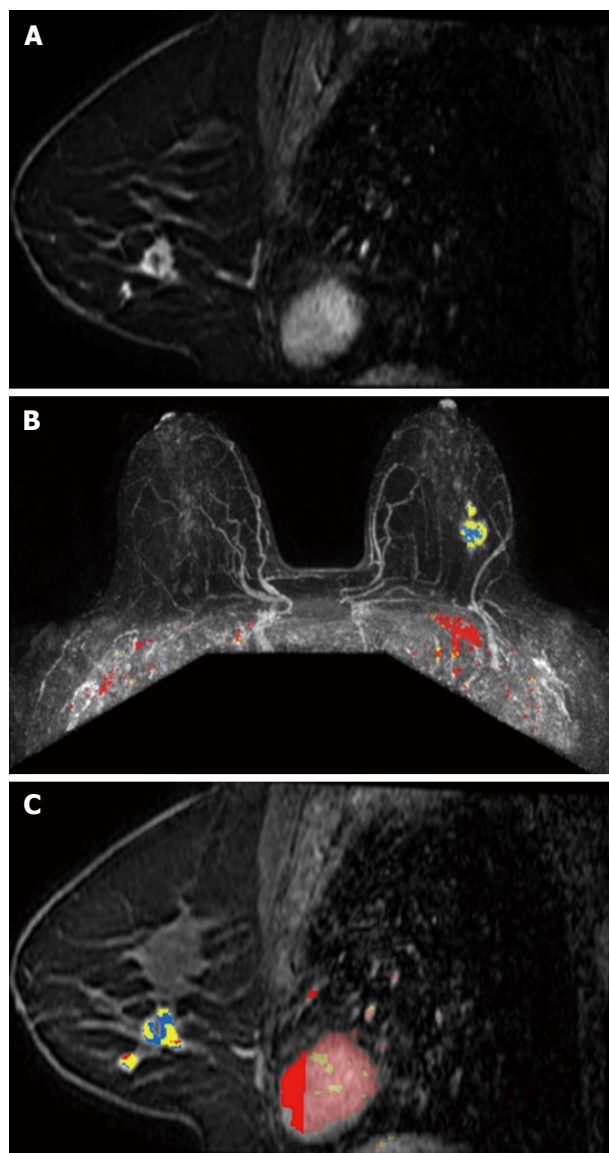


Figure 2 A 48-year-old woman, with positive family history for breast cancer, presented with a palpable lump on the left breast, finally diagnosed as invasive ductolobular carcinoma. A: Sagittal contrast-enhanced fat-suppressed T1-weighted gradient echo images obtained at 3 T shows a spiculated mass, with rim enhancement and small satellite lesion (multifocal disease); B and C: Color parametric enhancement map in axial postcontrast maximum intensity projection and sagittal projection indicates predominantly a plateau enhancement behavior, with some areas of washout.

a *BRCA* gene mutation, in women who are untested but have a first-degree relative with a *BRCA* mutation and women with an approximately 20% to 25% or greater lifetime risk of breast cancer^[39-41]. In a cohort of 496 women, Passaperuma *et al.*^[42] concluded that MRI surveillance of women with *BRCA* mutations detects most breast cancers at an early stage. Although the results of this study are promising, data on longer-term follow up is needed in order to encourage MRI surveillance as a safe alternative to prophylactic mastectomy. Likewise, patients diagnosed with breast cancer under the age of 50 have a 20% lifetime risk of recurrence (even after radiotherapy to the tumor bed following surgical approach)^[43,44]. In

these particular cases, the European Society of Breast Imaging also recommends annual MRI screening^[45].

Patients with dense breast parenchyma

Additional MRI can be beneficial in patients with dense breast parenchyma. Mammography has a high false negative rate in patients with dense breast tissue^[46-49], and the sensitivity remains low in dense breasts even when computer-aided detection is applied to digital mammography^[50]. In a large multicenter study, Schnall *et al.*^[51] proved that MRI has superior capability to detect additional occult cancer foci when compared to mammography, particularly in women with radiographically dense breasts and larger index cancers (18% *vs* 7.2%). Many other studies confirm that MRI has the highest diagnostic value when used in patients with heterogeneous or extremely dense breast parenchyma^[21,26,27]. The European Breast Imaging Society also advises the use of pre-operative MRI in staging malignant lesions in patients with dense breast tissue^[45]. MRI also has a substantial advantage in detecting breast lesions in scattered fibroglandular breast parenchyma^[20,52,53].

Impact on treatment

Despite all advantages of MRI, there is no consistent evidence supporting the clinical benefit of pre-operative MRI for all patients with breast cancer. In the MONET trial, 418 patients with non-palpable BI-RADS 3-5 lesions were randomized to undergo routine clinical care (211 patients) or standard clinical care associated to contrast enhanced MRI (207 patients) prior to large core needle biopsy^[54,55]. In total 74 patients had 83 malignant lesions in the MRI group and 75 patients had 80 malignant lesions in the control group. The choice of prioritizing non-palpable breast tumors was based on the difficulty in determining the margins of a lesion that cannot be seen or palpable during surgery. Thus, additional surgical intervention is often required in those cases. The authors hypothesized that the use of CE-MRI of the breast would reduce the need of additional surgical procedure, once MRI would add important three-dimensional information about the lesion, would be an important tool in defining tumor margins and in detecting multifocal and multicentric disease.

Surprisingly the re-excision rate due to positive resection margins after breast conserving therapy was even higher in the MRI group (34%) than in the control group (12%). Also the rate of additional surgical interventions after initial breast conserving therapy was higher in the MRI group than in the control group (45% *vs* 28%), although significance was not reached ($P = 0.069$). The COMICE trial attempted to determine whether the addition of breast MRI in 1623 breast cancer patients proven by triple assessment (clinical, radiological and pathological) would aid tumor localization and reduce re-operation rates. Patients were randomized to undergo or not MRI. The results showed no difference in the re-operation rates between the study arms (18.8% in patients who un-

derwent MRI *vs* 19.3% in patients who did not undergo MRI). The economic analysis of this trial showed no significant difference in cost-effectiveness between the research arms. The addition of MRI to triple assessment did not reduce the re-operation rates, and the use of MRI consumed extra resource with few benefits^[56]. Differently from the MONET study, the COMICE trial included patients with breast cancer proved by biopsy and most patients presented with palpable tumors. Both trials have a high level of evidence and, in both studies, the authors admonished the use of pre-operative breast MRI as a routine clinical care in patients with non-palpable breast cancer^[55]. Up to now, the results of these randomized controlled trials discourage the standard use of pre-operative MRI in all patients with breast cancer.

TECHNOLOGICAL DEVELOPMENTS AND ONGOING RESEARCH

Morphologic assessment can be a subjective task. It is strongly related to the experience of the radiologist and it is vulnerable to interobserver variations (especially in small lesions and nonmass-like lesions). An adjunct method which could provide a higher specificity would be of value. The use of new available technologies, such as breast MR spectroscopy and DWI is being verified in order to improve the accuracy and specificity of CE-MRI^[57-60].

Diffusion weighted imaging

DWI is a non-invasive MRI technique that measures the mobility of water molecules in tissue, providing information such as cellular density, viscosity, membrane integrity, and tissue microstructure, without the need of contrast injection^[59,61]. DWI is able to differentiate between tissue types based on the use of the apparent diffusion coefficient (ADC). Malignant breast tumors usually have a higher cellularity and generally present with restricted water diffusion and lower ADC values when compared to benign lesions^[62,63]. CE-MRI enables the assessment of morphological and kinetic patterns of benign and malignant breast tumors, but has a low specificity and high false-negative rates^[17,64,65]. The use of DWI is being considered as a new approach in order to improve the sensitivity and specificity of breast lesion characterization and may be incorporated into routine breast MRI assessment of breast lesions. In a retrospective study, El Khoully *et al*^[66] selected 93 women with 101 lesions (68 malignant tumors and 33 benign tumors) who underwent MRI using a 3.0 T magnet and both CE-MRI and DWI were performed. The association of DWI with ADC significantly improved the diagnostic performance and lesion characterization when compared to conventional 3D T1-weighted and CE-MRI at 3.0 T. In a study with 70 patients, Partridge *et al*^[67] showed that CE-MRI added to ADC criteria providing a superior positive predictive value than contrast enhanced MRI alone (47% *vs* 37%). Tan *et al*^[68] analyzed 44 breast lesions (31 malignant and 13 benign) on 3.0 T MRI. The cut-off ADC values

for benign and malignant lesions were $1.21 \times 10^{-3} \text{ mm}^2/\text{s}$ for $b = 500 \text{ s/mm}^2$ and $1.22 \times 10^{-3} \text{ mm}^2/\text{s}$ for $b = 1000 \text{ s/mm}^2$, respectively. Although the authors had a small sample size, the values obtained between ADC values of benign and malignant lesions were significant ($P < 0.001$). The sensitivity of CE-MRI was 100% with a specificity of 66.7%. CE-MRI combined with $b = 1000 \text{ s/mm}^2$, showed a specificity of 100% and a sensitivity of 90.6%. There was no significant between ADC values and prognostic factors^[68]. Marini *et al*^[69] investigated 81 breast lesions. Considering a mean diffusivity threshold value of $1.1 \times 10^{-3} \text{ mm}^2/\text{s}$, malignant lesions were differentiated from benign lesions with a sensitivity of 80% and specificity of 81%. A meta-analysis from Chen *et al*^[70] described 964 lesions (maximum $b = 1000 \text{ s/mm}^2$, 95%CI, area under curve of summary receiver operating characteristic 0.9085). ADC measurement of DWI showed a sensitivity and specificity of both 84% to differentiate between benign and malignant lesions. DWI also has the advantage of not requiring the use of intravenous contrast and the use of this technique could be an alternative to CE-MRI. For example, in a retrospective study with 118 breast lesions (12 DCIS, 15 invasive carcinomas and 91 benign lesions), 89% of malignant breast tumors were found to be clearly hyperintense on DWI. ADC values helped in differentiating malignant from benign lesions. In a study of Yabuuchi *et al*^[71], the authors compared the detection of non-palpable breast cancers in mammography, DWI and CE-MRI. DWI was significantly more accurate than mammography, although it has shown to be not as accurate as CE-MRI.

The use of DWI in patients with DCIS has also been described. Partridge *et al*^[72] reported that ADC values of DCIS were lower when compared to benign lesions and invasive carcinoma. Rahbar *et al*^[73] found 96% of pure DCIS lesions to be hyperintense in DWI.

Considering pre-treatment prediction of response to NAC in breast cancer patients, results suggest DWI associated to ADC to be useful for predicting tumor response. Park *et al*^[74] performed DW-MRI (1.5 T, b values 0 and 750 s/mm^2) and CE-MRI of 53 invasive breast cancers before and after chemotherapy prior to surgery. The percentage of ADC increase in responders was bigger than in non-responders ($P < 0.001$), the best cutoff to differentiate responders from non-responders was $1.17 \times 10^{-3} \text{ mm}^2/\text{s}$ (sensitivity of 94% and a specificity of 71%).

Sharma *et al*^[75] assessed the response of 56 patients with breast malignant lesions at four different times, before and after three cycles of NAC. ADC has shown a statistically significant change in volume and diameter in responders (sensitivity 68% and specificity 100%) and the authors suggested ADC would be useful in predicting early tumor response. According to Pickles *et al*^[76], significant alterations on ADC could be observed even before changes in tumor size in patients undergoing chemotherapy. Therefore the authors suggested that DWI might have the ability to provide indication of response to treatment, preceding changes in tumor size.

Proton spectroscopy

Spectroscopy is an additional non-invasive method that can provide chemical information from a selected region in the body. In clinical practice, spectroscopy is used mainly for brain applications and prostate cancer^[77,78]. Breast cancer spectroscopy is slightly behind that of prostate in development and in determining the suitability of this technique for clinical practice.

In mammary gland area, total choline (tCho), or just Cho, is considered the most important metabolite in proton MR spectroscopy. Many different metabolites overlap and contribute to the Cho peak, such as choline, phosphocholine, glycerophosphocoline, taurine and myo-inositol, among others^[79-81]. The Cho peak is centered at 3.2 ppm.

Cholines are precursors of phospholipids which are components of cell membranes and increased Cho signals are associated with increased cellular turnover^[82-84].

The use of breast MR spectroscopy to distinguish between benign and malignant lesions (using elevated tCho level as an indicator of malignancy) can potentially improve the accuracy of an MRI scan by offering increased specificity. In a recent systematic review and meta-analysis, Baltzer *et al.*^[85] included 19 studies with 1183 patients in order to evaluate the diagnostic performance of spectroscopy in differentiating breast lesions in field strengths of 1.5 and 3.0 T. They found a high pooled specificity (88%) and sensitivity (73%). Higher field strength, post contrast acquisition or qualitative *vs* quantitative MR spectroscopy had no significant influence on the results. Katz-Brull *et al.*^[86] performed a similar meta-analysis and found a combined sensitivity and specificity of 83% and 85%, respectively.

In a study with 184 patients with breast cancer, Shin *et al.*^[87] have shown that the use of absolute tCho-containing compound peak integral, normalized tCho-containing compound integral, and signal-to-noise-ratio determined by spectroscopy could be valuable in differentiating between IDC and DCIS. These same parameters could also be useful in determining tumor aggressiveness.

Many researchers suggest measurements of tCho with breast spectroscopy to be useful to monitor the response to NAC.

In a recent study, Tozaki *et al.*^[81] concluded that changes in Cho after NAC determined by ¹H MR spectroscopy are more sensitive to predict the pathological response than changes in the tumor size. Meisamy *et al.*^[88] used a 4 T strength field to evaluate the concentration of tCho in patients diagnosed with breast cancer before NAC, within 24 h after the first dose and after the fourth dose. Twenty-four hours after the first dose there was a significant variation in concentration of tCho (compared to baseline) and this change had a significant positive correlation with the change in lesion size ($P = 0.001$). The change observed in tCho concentration after first dose of NAC was significantly different between responders and non-responders ($P = 0.007$).

Jacobs *et al.*^[89] evaluated NAC response using magnetic

resonance spectroscopy, and ²³Na magnetic resonance. According to the authors, multiparametric and multi-nuclear imaging parameters were reduced after the first cycle of NAC in responders, specifically, Cho signal-to-noise ratio and sodium ($P \leq 0.01$).

To evaluate if applying DWI and spectroscopy together would help to improve the differentiation of breast lesions at 3.0 T, Tsougos *et al.*^[90] selected 51 women with known breast abnormalities (18 benign lesions and 33 malignant lesions). DWI and spectroscopy together provided higher accuracy and higher specificity for the differentiation between malignant and benign lesions when compared to these techniques used separately.

High field breast MRI at 7.0 tesla

Recently, high-field MRI (7.0 T) has become available for research. 7.0 T MRI has an inherent advantage over lower field's strengths and is, therefore, able to provide better signal to noise ratio, improve morphology assessment of breast lesions and increase the modality's sensitivity and specificity (Figure 3)^[57,58,60]. However, there are limitations in the use of 7.0 T. Higher magnetic field results in longer T1 relaxation time, shorter T2* decay time, greater radiofrequency, specific absorption rate, and increased B1 + field inhomogeneity. Nevertheless, some studies indicate that these disadvantages can be overcome.

Stehouwer *et al.*^[57] observed 7.0 T images in 20 patients with 23 suspicious breast lesions. The radiologist correctly identified all malignant tumors (BI-RADS 4 or 5) and in most cases image quality was considered good or excellent by both radiologists.

Field strength is considered an important factor affecting sensibility of spectroscopy. Particularly, 7.0 T is expected to provide increased signal to noise ratio and achieve more accurate information between closely overlapping resonances in the spectral domain^[57,58,60,91,92]. In a recent study, Korteweg *et al.*^[92] selected 3 patients who received NAC and tried to establish if DWI and spectroscopy could provide diagnostic information in breast cancer patients. One of the patients had nonspecific reaction to NAC and, during the whole NAC course, an increment of values of ADC was observed, suggesting either tumor responsiveness or cystic development/tumoral necrosis. In addition a decrease in Cho concentration during NAC cycles was reported, which could also mean responsiveness of the tumor. After the last NAC course, Cho concentrations and tumor size increased, suggesting acquired resistance to treatment. Pathology assessment confirmed this hypothesis. A second patient had no visible index lesion on 3.0 T or at 7.0 T and Cho was undetectable on both examinations. No tumor was observed in pathologic analysis, which is suggestive that NAC was effective. Even low Cho levels (0.77 mmol/kg-water) were detected, suggesting high sensitivity of 7.0 T in detecting alterations in Cho Metabolism.

7.0 T is still in its early stages and studies with larger number of patients are required in order to confirm these results and check clinical applications.

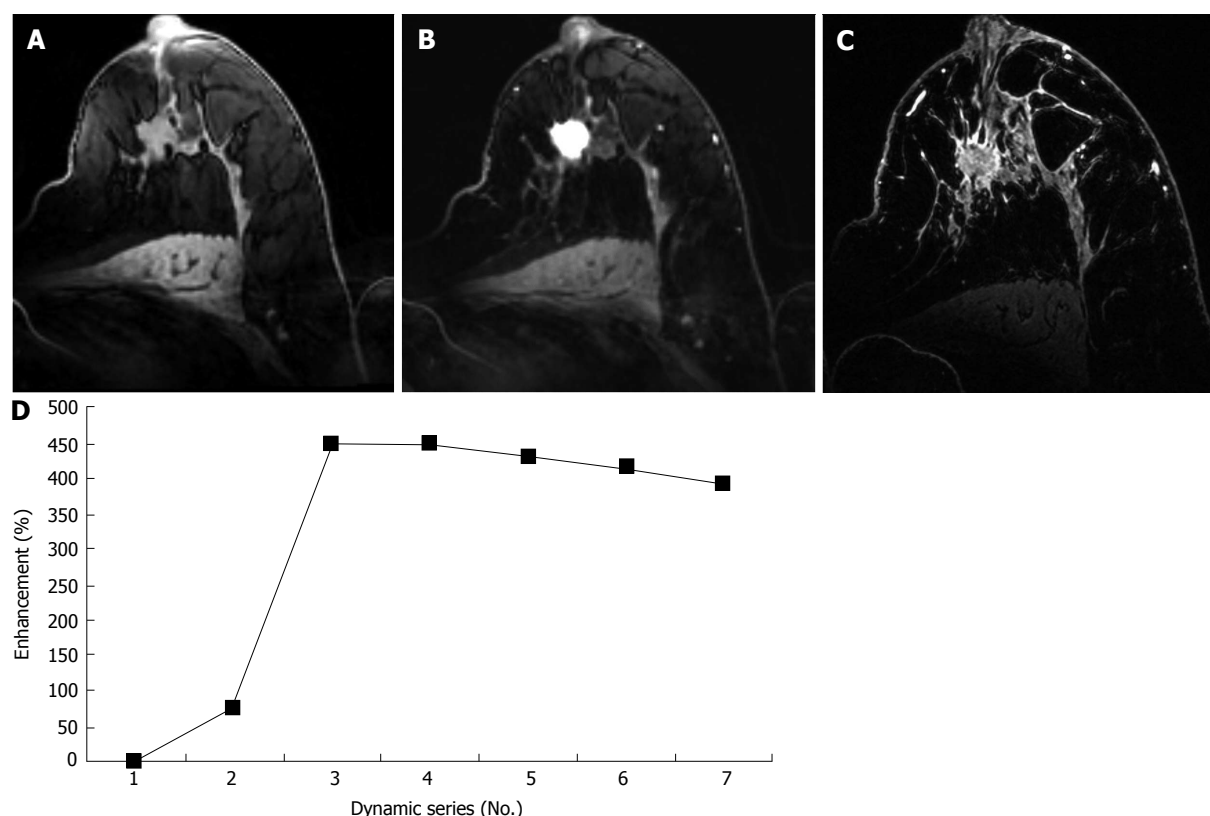


Figure 3 A 62-year-old patient with nipple withdrawal, finally diagnosed as ductolobular carcinoma. A, B and C: Axial T1-weighted gradient-echo images obtained at 7 T before and after contrast injection. An irregular mass with spiculated margins can be observed on pré-contrast imaging (A). An intense homogeneous enhancement (B) and a rapid wash-out kinetic curve (D) can be observed following contrast administration. In Figure 3C, an ultra-high-resolution T1-weighted gradient-echo sequence with fat suppression was performed, and the morphological aspects of the lesion can be more clearly seen.

CONCLUSION

To date, pre-operative MRI is indicated in defined groups of patients in which a potential benefit of local staging is expected, *i.e.*, women with mammographically heterogeneous or extremely dense breasts, at high risk for breast cancer, diagnosed with invasive lobular carcinoma and/or with multifocal, multicentric or contralateral disease^[93-95]. These recommendations are based on high-quality randomized controlled trials with narrow confidence intervals and on The American Cancer Society Guidelines for the Early Detection of Cancer and on the Guidelines from the European Breast Imaging Society^[45,55,56,95]. MR spectroscopy, DWI and 7.0 T MRI of the breast are promising, but the clinical value of these techniques still remains unclear mostly due to the fact that the number of studies investigating these techniques is small and they are still in early stage. Larger studies with more statistical power are necessary to confirm the clinical value and the cost-benefit of these new modalities.

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WJCO 5th Anniversary Special Issues (2): Breast cancer

Micro-RNAs as clinical biomarkers and therapeutic targets in breast cancer: Quo vadis?

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at the same time, accumulating evidence has underscored the possible contribution of miRNAs as valuable biomarkers to predict response to chemotherapy or radiotherapy. Data from *in vitro* and *in vivo* studies on BC have revealed promising therapeutic approaches *via* miRNA delivery and miRNA inhibition. The purpose of this review is to explore the ontological role of miRNAs in BC etiopathogenesis as well as to highlight their potential, not only as non-invasive circulating biomarkers with diagnostic and prognostic significance, but also as treatment response predictors and therapeutic targets aiding BC management.

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Key words: Biomarker; Breast cancer; Cancer diagnosis; Micro-RNA; Oncogene; Therapy; Tumor suppressor

Abstract

Breast cancer (BC) is the most frequent type of non skin cancer among women and a major leading cause of cancer-related deaths in Western countries. It is substantial to discover novel biomarkers with diagnostic, prognostic or predictive usefulness as well as therapeutic value for BC. Micro-RNAs (miRNAs) belong to a novel class of endogenous interfering RNAs that play a crucial role in post transcriptional gene silencing through mRNA targeting and, thus, are involved in many biological processes encompassing apoptosis, cell-cycle control, cell proliferation, DNA repair, immunity, metabolism, stress, aging, *etc.* MiRNAs exert their action mainly in a tumor suppressive or oncogenic manner. The specific aberrant expression patterns of miRNAs in BC that are detected with the use of high-throughput technologies reflect their key role in cancer initiation, progression, migration, invasion and metastasis. The detection of circulating extracellular miRNAs in plasma of BC patients may provide novel, non-invasive biomarkers in favor of BC diagnosis and prognosis and,

Core tip: The specific aberrant expression patterns of micro-RNAs (miRNAs) in breast cancer (BC) that are detected with the use of high-throughput technologies reflect their key role in cancer initiation, progression, migration, invasion and metastasis. The detection of circulating extracellular miRNAs in plasma of BC patients may provide novel, non-invasive biomarkers in favor of BC diagnosis and prognosis and, at the same time, accumulating evidence has underscored the possible contribution of miRNAs as valuable biomarkers to predict response to chemotherapy or radiotherapy. Data from *in vitro* and *in vivo* studies on BC have revealed promising therapeutic approaches *via* miRNA delivery and miRNA inhibition.

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INTRODUCTION

Breast cancer (BC) is the most frequent type of non skin cancer among women and a major leading cause of cancer-related deaths in women in Western countries^[1-3]. One in eight women has a chance of developing BC once in her lifetime^[4,5]. Early diagnosis and a followed patient-monitored therapy can lead to successful treatment of BC. However, due to the lack of sensitivity and specificity of known biomarkers, especially in early stage disease, there is no available efficient biomarker up to now for screening or early detection of BC^[6,7].

Micro-RNAs (miRNAs or miRs) belong to a novel class of endogenous interfering RNAs that play a crucial role in post transcriptional gene silencing through messenger RNA (mRNA) targeting and are thus involved in many biological processes encompassing apoptosis, cell-cycle control, cell proliferation, DNA repair, immunity, metabolism, stress, aging, *etc*^[8].

Since their initial discovery in 1993 during a study of the gene *lin-4* in *Caenorhabditis elegans*^[9], more than 2000 molecules have been determined in humans so far, regulating the expression of almost 30% of genes^[10]. MiRNAs are short, non-coding RNAs of approximately 20-25 nucleotides in length that are transcribed either from independent genes or from exons or introns of protein-coding genes^[8]. MiRNAs present unique nucleotide sequences that are strongly conserved among species^[11] and possess a specific critical region of 7 nucleotides long, known as the seed sequence, which is responsible for mRNA base pairing^[8]. Their abundant repertoire and the fact that the seed is so short may explain their combinatorial character in regulation: a given miRNA may target different mRNAs and a given mRNA could similarly be targeted by multiple miRNAs^[12].

MiRNA biogenesis is initiated in the cell nucleus with the generation of primary miRNA (pri-miRNA), usually by RNA polymerase II. The next step is the conversion of pri-miRNA into a smaller hairpin precursor miRNA (pre-miRNA) which is arbitrated by the microprocessor complex^[8]. The latter consists of the ribonuclease Drosha and the DiGeorge syndrome critical region in gene 8 protein that recognizes the hairpin loop of the pri-miRNA, ensuring unfaulty cleavage by Drosha. Pre-miRNAs are transported to the cytoplasm by the receptor exportin 5, whereas new cleavage occurs by the RNase III enzyme Dicer along with the transactivation response RNA-binding protein, resulting in a double stranded miRNA about 22 nucleotides long^[8,13]. One strand of the miRNA/miRNA duplex represents the mature miRNA which in conjunction with the Argonaute (Ago) protein and other proteins form the RNA-induced silencing complex (RISC)^[13]. Within RISC, mature miRNA is guided mainly to complementary sequences in 3' or 5' untranslated region of mRNA targets, open reading frames and promoter regions^[14]. Depending on the perfect or partial base pairing between miRNA and mRNAs molecules, the consequence is mRNA degradation or translational repression at both pre-initiation and post-

initiation stages, respectively; leading, thus, to a negative regulation of gene expression^[15]. However, studies have also shown a possible miRNAs involvement in positive regulation of their target genes (transcriptional and translational activation)^[16].

MiRNAs, as major gene-expression regulators, are implicated in the pathogenesis of many diseases, including cancer^[17]. The specific aberrant expression patterns of miRNAs in several cancer types, such as BC, that are detected with the use of high-throughput technologies reflect their key role in cancer initiation, progression, migration, invasion and metastasis^[18]. MiRNAs exert their action mainly in a tumor suppressive or oncogenic manner^[19].

The purpose of this review is to explore the ontological role of miRNAs in BC etiopathogenesis as well as to highlight their potential, not only as non-invasive circulating biomarkers with diagnostic and prognostic significance, but also as treatment response predictors and therapeutic targets aiding BC management.

ROLE OF MiRNAs IN BC ETIOPATHOGENESIS

Aberrant expression of miRNAs in BC

MiRNA expression profiling studies have detected aberrations with specific signatures of miRNA expression in breast carcinoma^[20]. Certain miRNA expression signatures have been associated with tumor classification, stage and prognosis, while others have been useful in detecting the primary site of tumors of unknown origin^[21].

Accumulating evidence has revealed that miRNAs may act either as oncogenes, commonly named oncomirs, by suppressing the expression of tumor suppressor genes or genes responsible for apoptosis; or as tumor suppressors or oncosuppressors by inhibiting genes that promote carcinogenesis and therefore controlling apoptosis and differentiation. Nevertheless, this miRNA categorization in oncogenes with an upregulated profile and tumor suppressors with a downregulated profile may be inaccurate, as many studies have shown that miRNAs may present a dual function with oncogenic or tumor suppressive properties based on tumor type and cellular context^[22].

In Figure 1, we have included a list of the most important miRNAs acting as oncogenes or tumor suppressors in BC. MiR-21 appears as a significant BC-related intracellular and extra-cellular biomarker and a therapeutic target with upregulated expression detected in human BC tissues and cell lines, playing a key role in all phases of BC pathogenesis^[23,24]. In BC clinical specimens, miR-21 increased expression was associated with advanced clinical stage, lymph-node positivity and shorter survival^[20]. Another example of a miRNA associated with an oncogenic potential in BC is miR-155, whereas the upregulation of miR-155 was correlated with advanced grade, clinical stage, estrogen receptor (ER) negative tumors, lymph node invasion, metastasis and poor prognosis^[25]. However, miR-21 and miR-155 increased expression do not characterize only BC but also colorectal and lung

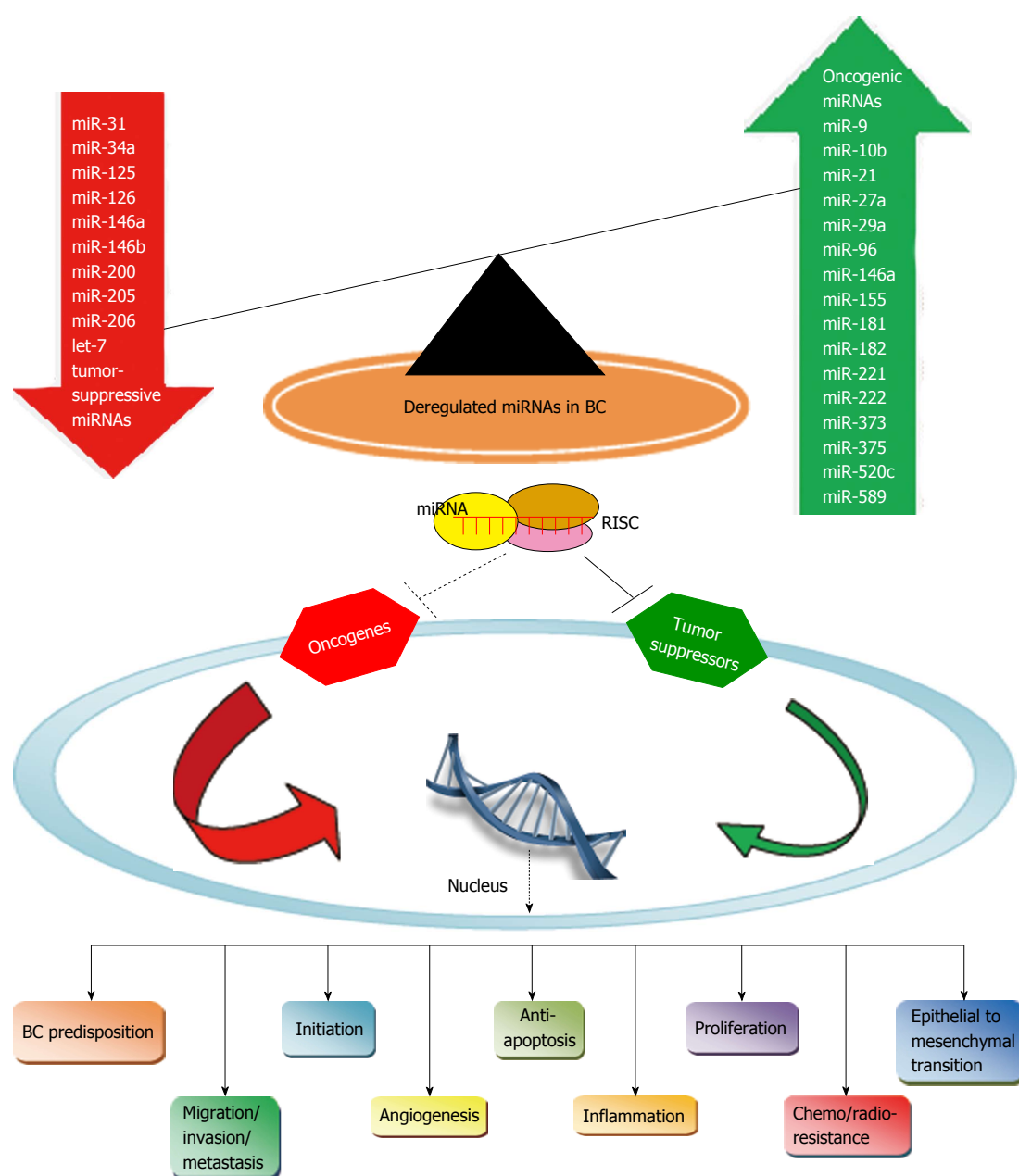


Figure 1 Dysregulation of micro-RNAs in breast cancer pathogenesis. The downregulated/tumor suppressive micro-RNAs (miRNAs) exert decreased inhibition on putative oncogenes in breast cancer (BC). The upregulated/oncogenic miRNAs show enhanced inhibition on tumor suppressors. These mechanisms may lead to increased oncogene-induced gene and decreased tumor suppressor-mediated transcription respectively. Both mechanisms lead to aberrant gene expression that play a significant role in BC predisposition, initiation, cell proliferation, resistance to apoptosis, invasion, angiogenesis, inflammation and metastasis in BC cells. RISC: RNA-induced silencing complex.

cancer as well as leukemia^[25,26].

Examples of overexpressed miRNAs in BC include miR-9/10b/21/27a/29a/96/146a/155/181/221/222/373/375/520c/589, where some of these have been validated in BC clinical specimens, highlighting their potential role in BC diagnosis, prognosis and therapeutics^[18,23]. Concerning downregulated miRNAs, miR-30a/31/34a/125/126/146a/146b/200/205/206 and let-7 emerge in BC pathogenesis through the loss of their tumor suppressor properties^[18,23]. Generally, it is important to mention that miRNA downregulation represents a more frequent event in BC pathogenesis, allowing oncogenes to be activated during BC development.

Dysregulation of miRNAs in BC predisposition, initiation, progression and metastasis

Aberrant expression of miRNAs in cancer-associated genomic regions: MiRNA genes are often situated in cancer-associated genomic regions and are subject to deletions, rearrangements, breakpoints and loss of heterozygosity^[27]. Approximately half of all annotated human miRNA genes are situated in fragile sites or genomic areas that have been associated with cancer^[27,28]. For example, miR-15a and miR-16-1, which are often down-regulated in cancer, occupy the most frequently deleted genomic region and may harbor germline mutations in familial cases of B-cell chronic lymphocytic leukemia and

BC^[22,28].

Single nucleotide polymorphisms in miRNA genes or miRNA target genes and genetic susceptibility to BC: Single nucleotide polymorphisms (SNPs) in miRNA genes, their processing machinery and their target binding sites could also increase the susceptibility to BC and affect patient prognosis and treatment efficacy^[29]. Despite the fact that SNPs are rare in miRNA genes, they may alter miRNA biogenesis and function as well as miRNA binding sites^[30]. Many SNPs in miRNA genes or in miRNA target genes in germline cells, independently from their possible/putative functional effects, have been analyzed in association (case-control) studies. For example, the SNP rs11614913 in pre-miR-196a-2 has been linked to an elevated BC risk^[31] and the SNP rs895819 in pre-miR-27a has been associated with a decreased BC risk^[32]. Therefore, miRNAs may be used as potential biomarkers for BC predisposition in populations at risk.

Aberrant expression of miRNAs in BC initiation:

Cancer stem cells (CSCs) or tumor-initiating cells lead tumor promotion, progression and heterogeneity by proliferating and forming some differentiated tumor cells. CSCs present a self-renewal (symmetric and asymmetric division) potential and the capacity to generate all types of cancer cells within the tumor^[33]. Targeting these initial CSCs may be an effective therapeutic strategy as CSCs are responsible for tumor growth and propagation of cancer and are considered more resistant to chemotherapy and radiotherapy. Breast CSCs were first reported by Al-Hajj *et al.*^[34] and are characterized by the expression of the surface biomarkers CD44⁺, CD24^{-/low}, the epithelial specific antigen and aldehyde dehydrogenase 1^[33]. Under non-adherent conditions for human mammary epithelial cells, only breast CSCs are capable of surviving, proliferating and building mammospheres, which are multicellular formations containing a large number of mammary stem cells^[33]. The Hedgehog signaling pathway activates the self-renewal of breast CSCs *via* the polycomb ring finger oncogene B lymphoma Mo-MLV insertion region 1 homolog (Bmi-1)^[35]. The aberrant expression of miRNAs may play a role in carcinogenesis and breast CSCs self-renewal by acting as oncogenic or tumor suppressive miRNAs and also regulating the stem cell-like phenotype of breast CSCs^[33]. The miRNA triad let-7/miR-200c/miR-30 suppresses the self-renewal of breast CSCs and the spontaneous conversion of immortalized mammary epithelial cells to a stem-like phenotype with less differentiated and mesenchymal properties by targeting Ras, Bmi-1, ubiquitin-conjugating enzyme 9 and integrin b3 respectively^[24,33]. On the contrary, the upregulation of miR-181 family members and miR-495 plays a significant role in modulating breast CSCs and maintaining a stem cell-like phenotype in BC by targeting respectively the tumor suppressor serine/threonine kinase ataxia telangiectasia mutated and E-cadherin and short for regulated in development and DNA damage responses. In particular, over-expression of miR-495 in human BC cells enhances

colony formation *in vitro* and carcinogenesis *in vivo*^[36].

Aberrant expression of miRNAs in BC progression:

MiRNAs may be involved in the cell cycle by controlling critical components of the regulatory pathways. In particular, miRNAs could regulate the cyclin/cyclin dependent kinase (CDK) pathway which constitutes a significant pathway in the cell cycle control. The pair miR-17-5p/miR-20a has been shown to attenuate the synthesis of cyclin D1 encoded by the gene *CCND1* in BC MCF-7 cell line, blocking S-phase entry and inhibiting cell proliferation^[37]. MiR-27a, which is also associated with the cyclin/CDK pathway, targets zinc finger and BTB domain containing 10 and Myt-1 which halt BC cell proliferation by suppressing cyclin D1 and cyclin B respectively^[38].

The estradiol (E2)/ER α /Sp1 is another important cell cycle regulatory pathway in BC that aids proliferation by activating cyclin D1, leading to the G1/S phase transition. In ER positive BC, E2 increases the expression of miR-21 and let-7 family members^[24]. The upregulation of let-7 family members in ER positive BC may result in diminished ER α activity and cell proliferation^[18]. BC cells at the stage of ductal carcinoma *in situ* and invasive ductal carcinoma are characterized by let-7 downregulation in comparison to benign lesions^[18]. In contrast to ER positive BC, the E2/ER signaling pathway is inhibited and miR-21 is downregulated in ER negative BC, whereas miR-18a, miR-18b, miR-206, miR-221 and miR-222 are upregulated leading to inhibition of ER expression and induction of other signaling pathways regulating cell growth and proliferation^[39]. The upregulation of the pair miR-221/222 in ER negative BC may lead to reduced p27kip1 levels and continuous BC proliferation^[40].

In BC, miR-31 acts as a tumor suppressor targeting the human frizzled transmembrane receptor frizzled-3 and is downregulated in all interactions with the Wnt signaling transduction pathway^[41]. The oncosuppressor miR-34a, which is downregulated in triple negative and mesenchymal-type BC cell lines, has been shown to inhibit BC proliferation and migration *via* downregulation of B-cell lymphoma 2 (Bcl-2) and sirtuin 1^[18]. The tumor suppressor miR-205 targets human epidermal growth factor receptor 3 (HER3) directly, a receptor tyrosine kinase of the epidermal growth factor receptor (EGFR) family, inactivating thereby the downstream mediator Akt, suppressing the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway and inhibiting BC proliferation with improved response to targeted therapies^[42]. Finally, the overexpression of the oncogenic miR-146a may be responsible for the altered expression of the breast cancer type 1 susceptibility protein (BRCA1), which is a negative regulator of BC growth associated with the hypophosphorylated form of Rb, and the BC metastasis suppressor 1 (BRMS1), which reduces the metastatic potential of BC cells^[43].

MiRNAs also interfere with the apoptotic process in BC cells. MiR-21 has been shown to play an anti-apoptotic role by indirectly targeting Bcl-2 in MCF-7 BC cells^[44]. On the contrary, the oncosuppressor miR-145 targets

Rhotekin (RTKN), the gene coding for the Rho effector, which activates B-cell lymphoma extra large *via* the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signaling pathway^[45]; hence, promoting apoptosis. The oncogenic miR-155 is an effective suppressor of the execution phase of apoptosis *via* its actions in caspase 3 and its negative regulation of the tumor suppressor gene suppressor of cytokine signaling 1^[18]. The members of forkhead box protein O family (FOXO) which are often the target of miRNAs, represent transcription factors characterized by a distinctive forkhead DNA binding domain and play an important role in promoting the cell cycle arrest at the G₁/S checkpoint, apoptotic responses *via* the pro-apoptotic factor Bcl-2 homology domain 3-only molecule Bcl-2-interacting mediator (Bim) of cell death and cellular metabolism^[43]. In BC, the up-regulation of the oncogenic triad of miR-27a, miR-96 and miR-182 which targets FOXO1 may contribute to BC progression and maintenance of the oncogenic state^[46]. In addition, the oncogenic miR-155 may induce BC cell survival and anti-apoptosis by blocking FOXO3a^[47].

Aberrant expression of miRNAs in BC migration, invasion and metastasis: After initiation and progression, tumor cells proceed to invasion and metastasis, which are enabled by the epithelial to mesenchymal transition (EMT). BC cells in carcinoma *in situ* lose cell adhesion mediated by E-cadherin repression, become more motile and break through the basement membrane with increased invasive properties, progressing to invasive BC. EMT, which is essential in cancer invasion, metastatic dissemination and resistance acquisition to cancer therapy, is characterized by the loss of the epithelial phenotype marker E-cadherin^[18,43]. EMT is activated by tumor necrosis factor- α , hepatocyte growth factor and transforming growth factor- β (TGF- β), while many transcription factors, including zinc finger E-box binding homeobox 1 (ZEB1) and ZEB2, can repress E-cadherin directly or indirectly^[43]. In BC, the expression of the miR-200 family is downregulated, resulting in the overexpression of ZEB1 and ZEB2, which are crucial EMT activators by inhibiting E-cadherin expression^[48]. MiR-9 directly targets the E-cadherin coding gene CDH1, leading to an enhanced cell motility and invasiveness of SUM149 human BC cells^[49]. Ras homolog gene family member (Rho) A, a prometastatic gene regulating EMT in a multiphasic manner, is the target of both oncogenic miRNAs such as miR-155, which mediates the TGF- β induced EMT, and tumor suppressor miRNAs such as miR-31^[41].

For the invasion and metastatic processes, attachment of BC cells to matrix components must take place. Metalloproteinases (MMP) degrade the extra-cellular matrix (ECM) which mediates cell attachment, while the tissue inhibitor of metalloproteinases suppresses the MMP activities^[50]. MiRNAs may induce cell migration and invasion through ECM destruction or disruption of recognition between ECM and cells. Later, circulating BC cells exit the bloodstream to form micro-metastases, undergoing mesenchymal to epithelial transition for clonal

outgrowth at the metastatic sites.

MiR-21 inhibits TIMP3 expression in BC, promoting ECM disruption, BC invasion in multiple cell lines *in vitro* and metastasis^[51]. Furthermore, the overexpressed miR-21 directly blocks the tumor suppressor programmed cell death protein 4 (Pdc4), tropomyosin 1 and maspin, enhancing the promotion of invasion and metastasis^[52]. The prometastatic miRNAs miR-373 and miR-520c may promote cell migration and invasion *in vitro* and *in vivo* by inhibiting the expression of CD44, which is a cell surface receptor for ECM components and cell to cell interactions with ECM^[53]. The basal-like subtype-specific miRNAs miR-221 and miR-222 are associated with increased cell migration and invasion, aiding in the progression of the clinically aggressive basal-like BC^[39]. Interestingly, miR-223 transferred from macrophages to BC cells through exosomes may lead to enhanced invasiveness of BC cells; highlighting the important role of exosomal communication between BC cells and macrophages^[54]. The oncogenic miR-10b, which is overexpressed in metastatic BC, may initiate tumor invasion and metastasis *in vivo* and *in vitro* by interrupting the homeobox D10 (HOXD10) expression (a transcription factor that maintains a differentiated phenotype in epithelial cells), resulting in an increased expression of Ras homolog gene family member C which leads to BC cell invasion and metastasis^[55]. The upregulation of miR-375 contributes to breast lobular neoplasia and invasive lobular breast carcinoma progression^[18].

The miRNA pair miR-126 and miR-335 has been associated with the capacity of BC cells to metastasize to bones and lungs through blocking the expression of tenascin c, a ECM component^[56]. The downregulation of the tumor suppressive let-7 contributes to BC metastasis. Let-7 modulates the repressive action of Raf kinase inhibitory protein, a BC suppressor gene inhibiting NF- κ B, mitogen-activated protein kinases and G protein-coupled receptor kinase-2 signaling pathways in BC metastatic cells^[57]. The tumor suppressive miR-31, which is undetectable in metastatic BC cells, has been shown to inhibit the expression of multiple prometastatic genes blocking BC metastasis^[18]. The tumor suppressor miR-146a/b diminishes the expression of EGFR, inhibiting metastasis^[58].

Distant BC metastases need tumor-induced formation of new blood vessels (angiogenesis) in order to allow expansion of the primary breast tumor and to obtain sufficient oxygen and nutrients. The angiogenic factor vascular endothelial growth factor (VEGF) represents the most important inducer of angiogenesis and may be regulated by several miRNAs. In particular, miR-126 has been shown to target VEGF expression in BC whereas the VEGF/PI3K/Akt signaling cascade is activated^[59]. On the contrary, miR-9 may promote angiogenesis by enhancing VEGF-A expression in BC and downregulating E-cadherin^[49]. In hypoxic conditions within the tumor microenvironment, hypoxia inducible factor-1 may also mediate the expression of VEGF in BC cells in a miR-20b-dependent way^[60]. Also, the downregulation of the oncosup-

pressor miR-125a is associated with the overexpression of a stress-induced HuR protein in the cytoplasm, which in turn could increase the invasiveness of BC cells and angiogenesis *via* VEGF-A expression^[61]. In addition to angiogenesis, miRNAs may constitute feedback mechanisms that link inflammation to BC. In particular, the upregulation of miR-155 in BC could lead to stimulation of signal transducer and activator of transcription 3 *via* the Janus kinase pathway and activation of BC cells by interleukin-6, interferon γ and lipopolysaccharide^[62].

Finally, miRNAs may also represent key regulators of the epigenetic interaction that takes place in BC cells with DNA methylation and histone modifications. The expression of the oncosuppressor miR-200 was shown to be epigenetically modulated by DNA promoter methylation and histone modifications^[33]. The downregulation of the pro-apoptotic and tumor suppressor miR-34c, which inhibits invasion, occurs through hypermethylation of the promoter region and may lead to enhanced self-renewal and EMT of breast CSCs^[63].

MI RNAs AS EXTRACELLULAR CIRCULATING BIOMARKERS IN BC

Several blood-based profiling studies have tried to elucidate the role of extracellular miRNAs in BC biology and pathogenesis^[64]. It should be also noted that miRNAs have been detected in breast milk^[22]. MiRNAs could be used as promising diagnostic, prognostic and predictive biomarkers in BC, presenting the following advantages: (1) their remarkable stability in plasma due to their association not only with RNA binding proteins [Ago2 protein, high density lipoprotein or nucleophosmin 1 (NPM1)] but also exosomal vesicular transportation^[65,66]; (2) miRNAs represent a non-invasive diagnostic approach as a liquid biopsy in contrast to the existing tissue-dependent biopsy; and (3) miRNAs may be regarded as tumor-derived molecules that have been present early into circulation, therefore reflecting tumor status.

Genome-wide expression profiling studies of extracellular miRNAs have investigated whether serum samples could be used to identify differentiated miRNA expression levels between BC patients and healthy individuals; thus, distinguishing normal from diseased state. Wu *et al*^[67] showed that serum miR-29a and miR-21 levels were significantly increased in 20 BC patients compared to healthy controls. Kumar *et al*^[68] also demonstrated an overexpression of miR-21 and miR-146a in plasma samples of BC patients. Using microarray-based expression profiling followed by real time quantitative polymerase chain reaction (RT-qPCR), Zhao *et al*^[69] found deregulated expression levels of 49 miRNAs in plasma from 20 women with early stage BC compared to 20 matched controls. Furthermore, the authors showed that both up-regulated ($n = 26$) and downregulated ($n = 23$) miRNAs could discriminate patients from controls with acceptable specificity and sensitivity scores. Let-7c and miR-589 were significantly decreased and increased respectively in

BC patients. In a study by Chan *et al*^[70], 4 (miR-1, miR-92a, miR-133a and miR-133b) of the 7 miRNAs that were differentially expressed in a set of serum samples from a cohort of 132 Asian BC patients and 101 healthy controls were validated and identified as the most significant diagnostic markers. Interestingly, only 7 miRNAs out of the total 20 were overexpressed in both tumor and serum of BC patients, indicating that miRNAs could be released into serum selectively. Profiling results of another study have indicated that the combination of circulating miR-145 and miR-451 seems capable of predicting BC patients from normal individuals^[71].

Other investigations have shown a correlation between systemic miRNA levels and various clinicopathological features of BC. MiR-10b and miR-373 were related to lymph node status^[72], while the upregulated miR-21 was associated significantly with visceral metastasis^[73]. MiR-21, miR-126, miR-155, miR-199a and miR-335 levels were closely correlated with histological grade and hormone receptor expression. A significantly higher relationship of miRNA expression levels between BC tumor tissues and sera was also found^[74]. Roth *et al*^[75] revealed that circulating miR-10b, miR-34a and miR-155 levels were significantly related to the presence of overt metastases. Serum levels of miR-182 in ER and progesterone receptor (PR) positive BC patients were lower when compared with patients suffering from ER-PR negative BC^[76]. On the other hand, miR-155 expression levels were higher in serum of women with hormone sensitive BC (PR positive)^[77]. Similarly, in a prospective study, levels of miR-195 and let-7a were significantly correlated with ER and lymph nodal status and decreased interestingly in BC patients postoperatively following curative tumor resection^[78]. Cuk *et al*^[79] showed that a panel of 7 circulating miRNAs, including miR-127-3p, miR-148b, miR-409-3p, miR-652 and miR-801, presents a substantial diagnostic potential, not only as a screening method for benign and malignant breast tumors, but also for the detection of early BC stage. Notably, another study has linked exosomal miRNAs to poor prognosis in BC through the maintenance of dormant BC cells in the bone marrow stroma^[80]. Sieuwerts *et al*^[81] have highlighted the diagnostic potential of detecting tumor specific miRNAs in circulating tumor cells (CTCs) in the bloodstream in an attempt to discriminate BC patients with CTCs from patients with no detectable CTCs and healthy volunteers. Accumulating evidence has underscored the possible contribution of miRNAs as valuable biomarkers to predict response to chemotherapy or radiotherapy. For example, downregulated miR-34, miR-17 and let-7a were associated with chemosensitivity to fluorouracil, adriamycin and cyclophosphamide, respectively^[82]. Studies in BC cell lines have also related targeted miR-21 downregulation with increased sensitivity to topotecan and taxol^[44,83], whereas other investigations have indicated that restoration of the oncosuppressor miR-205 expression levels was associated with improved response to tyrosine-kinase inhibitors gefitinib and lapatinib through abrogating the HER3-

mediated resistance^[42]. A growing number of studies have demonstrated a correlation between circulating miRNA expression levels and patterns of chemoresistance or chemosensitivity. Zhao *et al*^[84] have found that plasma miR-221 could be a predictive biomarker for neoadjuvant chemotherapy sensitivity in BC patients. In other studies, circulating miR-210 and miR-125b were associated with sensitivity to trastuzumab and neoadjuvant chemotherapeutic resistance respectively; underlining the possibility of using them as indicators of treatment response^[85,86].

MI RNAs AS PROMISING THERAPEUTIC TARGETS IN BC

The pivotal role of miRNA as oncogenes or tumor suppressors has stimulated scientists to manipulate their expression; an effort that indicates their emerging role as therapeutic targets and replacement therapies for BC treatment. Depending on a given miRNA that is up or downregulated, various methods exist in order to inhibit or increase its expression, including miRNA inhibition *via* antisense targeting with oligonucleotides (anti-miRs) or miRNA replacement *via* viral or liposomal delivery (miRNA mimics), respectively^[87,88]. Functional analyses using knockdown mouse models and BC cell lines have revealed great therapeutic potential for the studied miRNA molecules. Potentially, every miRNA could serve as a possible therapeutic target. Si *et al*^[44] showed that inhibition of miR-21 expression using anti-miR-21 oligonucleotides resulted in reduced MCF-7 BC cell growth and tumor growth in the xenograft mouse model due to decreased proliferation and increased apoptosis. In agreement with these findings, Yan *et al*^[89] showed that knockdown of miR-21 inhibited growth and migration of MCF-7 and MDA-MB-231 BC cell lines *in vitro* and tumor growth in nude mice *in vivo*. MiR-21's potential therapeutic relevance is also supported by its capacity to sensitize BC cells to anticancer therapy. MiR-21 suppression has been reported to increase sensitivity of BC cells to topotecan and taxol^[44,83], whereas its tumor suppressive gene target phosphatase and tensin homolog has been shown to be a regulator of sensitivity to trastuzumab^[90]. These findings suggest that the combination of anti-miR-21 with classical chemotherapy may result in overcoming drug resistance and in individualizing therapy in BC patients. Furthermore, Kong *et al*^[47] demonstrated that knockdown of miR-155 led to apoptosis and increased chemosensitivity by upregulation of its direct target FOXO3a, suggesting that miR-155 inhibition could present a promising therapeutic potential for BC. Additionally, let-7 could contribute to cancer therapeutics due to its association with self-renewal ability and tumorigenicity of BC cells^[91]. Let-7 also regulates apoptosis and CSC differentiation^[92]; thus, targeting let-7 in BC could serve as an effective treatment option.

Recent studies have targeted miR-205 for inhibiting the metastatic nature of BC. Wu *et al*^[93] demonstrated that ectopic expression of the downregulated miR-205 effec-

tively hinders cell proliferation, anchorage-independent growth and cell invasion, supporting its use as a possible therapeutic target. MiRNA delivery *via* nanoparticles is also a promising technique. Jin *et al*^[94] have recently used nanoparticles to deliver anti-miR-10b for targeting the overexpressed miR-10b which is related to BC cell migration and invasion through inhibition of HOXD10 target synthesis. A RNA poly L-lysine complex has been developed; it releases concentrations of anti-miR-10b into the cytoplasm of BC cells with sustainable effectiveness.

Further therapeutic potential is likely *via* targeting breast CSCs with miRNA manipulation. Certain miRNAs seem to be responsible for breast CSCs behavior, including self-renewal characteristics, increased chemotherapeutic resistance and EMT^[33]. Thus, anti-CSC therapy with miRNAs could combat breast CSCs' positive effect on tumorigenesis. Additionally, a potential mesenchymal stem cell-mediated anti-miR delivery directly to the tumor area was proposed based on mesenchymal stem cells ability to migrate^[95].

Unmasking the precise role of miRNAs in the regulation of breast CSC renewal and the potential for combination of stem cell and novel miRNA-associated targeted therapies may represent effective therapeutic strategies of significant clinical benefit, probably when combined with the classic anticancer agents.

CONCLUSION

MiRNAs constitute a novel class of dysregulated molecules that could provide new avenues for diagnosing and classifying tumor-specific malignancies such as BC but also different phases of BC development from initiation to progression, migration, invasion and metastasis. The scientific interest is mainly concentrated on two basic aspects in regard to the clinical utility of miRNAs: their extracellular presence in body fluids, particularly blood, and their potential therapeutic applications either by miRNA replacement or miRNA inhibition. In BC, both aspects appear promising, with miRNAs being used as potential circulating non-invasive biomarkers detected in serum and plasma samples, and as therapeutic targets for cancer under current investigation, respectively. Although the available recent data are almost exclusively pre-clinical evidence, the application of miRNAs in BC therapy as adjuvant tools or targets appears exciting and very promising. A better understanding of the complex network of genes and cellular signaling transduction pathways regulated by miRNAs would enrich our knowledge of BC etiopathogenesis and hence would improve the therapeutic outcome of BC patients.

The diagnostic potential of circulating miRNAs as BC biomarkers is based mainly on their non-invasive detection in serum and plasma and on their high resistance and stability under difficult environmental conditions that could degrade the majority of RNAs, such as extended storage, frequent freeze-thaw cycles, extreme pH variations, boiling, preservation in archived human blood samples

for several years, transport, *etc.* Several methodologies are available for establishing miRNA signatures in BC, such as RT-PCR, miRNA microarrays and next-generation sequencing, with several limitations regarding their cross-comparison, various reference genes used to normalize miRNA levels and differences in blood collection.

Nonetheless, crucial issues need to be resolved before establishing extracellular miRNAs as biomarkers and therapeutic tools for BC. The lack of larger prospective clinical trials with robust and standardized analyzing methods, the necessity for clarifying the real origin of circulating miRNAs as they may be confused with by-products of normal tissues or dead cells, and the validation of a well-characterized BC-specific signature of circulating miRNAs are important limitations that need to be overcome when bringing miRNAs from bench to bedside. However, taking into account the important pace of evolution in understanding the main ways of miRNA effects on BC pathogenesis, these small molecules will amaze the scientific world with more revelations in the near future.

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WJCO 5th Anniversary Special Issues (2): Breast cancer

Resection of the primary tumor in stage IV breast cancer

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Abstract

Stage IV breast cancer refers to breast cancer that has already metastasized to distant regions when initially diagnosed. Treatment for stage IV is intended to "prolong survival and palliate symptoms". Resection of a primary tumor is considered to be "effective only at alleviating chest symptoms and providing local control" in spite of the advances of imaging examination and medication for breast cancer. Molecular target and endocrine drugs are very effective and useful to tailor-make a treatment strategy according to breast cancer subtypes. Positron emission tomography-computed tomography can detect and diagnose the very small metastases and recurrences which can potentially be cured even if they are distant metastases. Recently, many retrospective studies have reported the survival benefit of surgery for breast cancer patients with metastases and some clinical trials which confirm the surgical prognostic benefit for them have started to enrol patients. The goal of treatment has to be clearly identified: increase the patient's survival time, provide local control or perform histology to determine the cancer's properties. The best evidence is absolutely essential to treat patients who need surgery at the right time. We need to evaluate the treatment strategy, including primary resection for stage IV breast

cancer particularly, and find new evidence by prospective analysis.

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Key words: Breast cancer; Metastasis; Surgery; Survival; Stage IV; Clinical trial

Core tip: Resection of a primary tumor of stage IV breast cancer was considered to be "effective only at alleviating chest symptoms and providing local control" in spite of the advances of imaging examination and medication for breast cancer. Recently, many retrospective studies have reported the survival benefit of surgery for breast cancer patients with metastases and some clinical trials which confirm the surgical prognostic benefit for them have started to enrol patients. We need to evaluate the treatment strategy, including primary resection for stage IV breast cancer particularly, and find new evidence by prospective analysis.

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INTRODUCTION

Stage IV breast cancer refers to breast cancer that has already metastasized to distant regions when initially diagnosed. Even if such cancer were to be treated, complete cure would not be expected. Treatment is intended to "prolong survival and palliate symptoms". Medication has made advances and treatments that are anticipated to be efficacious are administered. This situation has changed little as new drugs are coming out every year. In an increasing number of patients, appropriate use of

those drugs allows long-term control of symptoms and a longer life with disease.

In addition, marked advances in diagnostic imaging equipment have been made. Over the past few years, the prevalence of positron emission tomography-computed tomography has led to the early diagnosis of extremely small metastases that were not previously noted^[1]. Stage IV breast cancer with these small metastases is referred to as “minimal stage IV disease^[2]” and patients with this more limited form are expected to have a better prognosis than patients with full-blown stage IV breast cancer. Although it has yet to be precisely defined, the concept of “oligometastasis” is being debated^[3]. According to this concept, metastases can potentially be cured, even if they are distant metastases, depending on their location and number.

Resection of a primary tumor was previously considered to be “effective only at alleviating chest symptoms and providing local control”, but some studies have reported that resection increases survival time^[4,5]. Breast-conserving surgery is a widely used form of surgery for breast cancer. Anesthesia has also made advances and is safe. At the current point in time, surgery for breast cancer is extremely simple, depending on tumor size, and minimally invasive. A longer survival time seldom results from drug administration but it can result from surgery. Surgery for stage IV breast cancer is an important topic that may substantially alter future treatment strategies.

SIGNIFICANCE OF RESECTION OF THE PRIMARY TUMOR IN STAGE IV BREAST CANCER: STUDIES REPORTING INCREASED SURVIVAL TIMES AND RELATED ISSUES

As mentioned earlier, a number of recent studies have reported that surgery for stage IV breast cancer affects a patient's survival time. Many of these retrospective studies indicated that surgery prolonged survival time. Several systematic reviews have reported significant differences in survival time (HR of about 0.6)^[4,5]. A look at subgroups indicates that factors facilitating surgery include “complete excision of the primary tumor”, “metastasis only to bone and/or soft tissue”, “few metastases” and “being younger”^[6,7]. A study reported differences in the effectiveness of surgery for different subtypes of tumors^[8]. However, all of the findings cited were the result of retrospective analysis so they are presumed to be highly biased. “Patients who undergo surgery” are invariably “patients in good enough condition to undergo surgery” while “patients who do not undergo surgery” are possibly “patients who are unable to undergo surgery because of their worsening condition”. In addition, medication has not been studied in detail and patients who undergo surgery are likely to include a number of patients whose condition could have been satisfactorily controlled with medication. The timing of surgery is also unclear. There

is no clear answer as to whether surgery should be done during initial treatment or whether it should be a final option that is used after medication proves inefficacious.

WHY DOES RESECTION OF ONLY THE PRIMARY TUMOR HELP WHEN CANCER CELLS HAVE SPREAD THROUGHOUT THE BODY?

According to the seed and soil theory by Paget^[9], the distant metastasis is not local disease. Cancer cells have already spread to whole body circulation. So, local therapies do not affect overall survival, whereas there are several theories on the basic rationale for resection of the primary tumor increasing the survival time for patients with stage IV breast cancer. The first is a “reduction in total tumor volume”. Circulating tumor cells (CTCs) are a major indicator of tumor volume. A reduction in CTCs is reported to be correlated with prognosis^[10]. Resection of the primary tumor reduces the tumor volume and thus reactivates autoimmunity and increases the efficacy of medication^[11]. A study prospectively demonstrated that resection of the primary tumor is useful when kidney cancer is in stage IV (this is the only other solid tumor besides breast cancer for which this holds true)^[12]. According to the study, resection of the primary tumor is a theoretical basis for the effectiveness of surgery.

Another theory as to why resection of the primary tumor increases the survival time concerns the particular action of the primary tumor. “Cancer stem cells” that are prevalent in the primary tumor are resistant to medication^[13]. In addition, the concept of “cell seeding” indicates that cells released into the blood by the primary tumor return to the primary tumor, so the primary tumor activates those cancer cells^[14]. Both of these mechanisms are based on results of basic experiments and no studies have described results from actual patients. If, however, they are true, then they are sure to be key to devising cancer treatment strategies in the future. These mechanisms should be verified in the future.

LOCAL CONTROL

As mentioned at the very beginning, resection of the primary tumor has been useful in alleviating chest symptoms, such as bleeding and ulceration as well as pain due to invasion of the chest wall. However, no studies or prospective trials have determined whether or not earlier surgery is useful to achieve local control. At the current time, there are absolutely no data corroborating the contention that “earlier surgery is useful since it improves local control, even if it does not increase survival time”. When local control alone was envisioned, radiation therapy was considered in addition to surgery. Although sample sizes are small, studies have described an improvement in the prognosis for the primary tumor in stage IV breast cancer as a result of radiation therapy (like the improvement in

Table 1 Ongoing randomized trials testing the worth of local therapy for an intact primary in women with stage IV breast cancer
xvii

Country	Trial number	Accrual period	n	Initial therapy	Radiotherapy	Primary endpoint
India	NCT00193778	2005-12	350	Adriamycin-cytoxan	If indicated	Time to progression
Turkey	NCT00557986	2008-12	281	Surgery	For breast conservation	Survival
United States and Canada	NCT01242800	2011-16	368	Appropriate systemic therapy	Per standards for stage I -III	Survival
Netherlands	NCT01392586	2011-16	516	Surgery	For positive margins or palliation	2-yr survival
Austria	NCT01015625	2010-19	254	Surgery	Per standards for stage I -III	Survival
Japan	JCOG 1017	2011-16	410	Appropriate systemic therapy	No	Survival

JCOG: Japan Clinical Oncology Group; NCT number: A unique identification code given to each clinical study registered on ClinicalTrials.gov.

prognosis as a result of surgery)^[15]. In addition, a study has reported a satisfactory prognosis for asymptomatic rather than symptomatic patients, regardless of whether treatment was administered and regardless of the type of treatment^[16]. Results suggest that local control itself may act beneficially on prognosis, irrespective of whether treatment is classified as surgery, radiation, *etc.*

TRIALS CURRENTLY UNDERWAY TO DETERMINE THE USEFULNESS OF RESECTION OF THE PRIMARY TUMOR IN STAGE IV BREAST CANCER

As noted previously, there are absolutely no prospective data at the current time to corroborate the usefulness of resection of the primary tumor in stage IV breast cancer in terms of increasing survival time or improving local control. At the current time, there is no evidence actively in favor of such a resection. That said, many results of retrospective studies continue to be discussed in various fora. In the absence of robust evidence, this meta-analysis provides an evidence base for primary resection in the setting of stage IV breast cancer for appropriately selected patients^[17]. Resection of the primary tumor could greatly affect breast cancer care so this clinical question needs to be answered in prospective trials. Given this potential, 6 groups are currently enrolling patients^[18-20] (Table 1). The first reports of two prospective studies were indicated in the San Antonio Breast Cancer Symposium 2013^[21,22]. Both studies did not demonstrate a significant survival benefit of primary surgery. From the Indian trial, the distant disease free survival in the patients with surgery was significantly worse than that of the patients without surgery. One of the reasons was the insufficient systemic chemotherapy after surgery. They did not continue systemic chemotherapy after randomization and appropriate systemic therapies according to breast cancer subtypes were not selected in these protocols. So, the median survival time was shorter than that of retrospective European and American data. In particular, they did not use molecular target therapy for patients with human epidermal growth factor receptor type 2 positive breast cancer. Moreover, the diagnosis of metastasis was uncertain. They only used bone scintigraphy to diagnose a solitary bone metastasis. The Breast Cancer Study Group of

the Japan Clinical Oncology Group (1017) and Eastern Clinical Oncology Group (2108) began enrolling patients for a phase 3 trial in June 2011^[23]. Patients receive current standard systemic therapy before and after randomization and the latest imaging examination before treatment in these trials. A trial by the current authors is determining the significance of early resection of the primary tumor in stage IV breast cancer when that tumor can be controlled by medication. Items being assessed include the total survival time as well as the significance of local control; the results of the trial are sure to provide clinically significant evidence.

CONCLUSION

At the current point in time, one cannot say whether or not resection of the primary tumor provides a clear benefit in the management of stage IV breast cancer. Basic studies have revealed the biology of breast cancer in detail and the role of surgery is changing as treatment is better tailored to the individual in accordance with the individual's biology. The goal of treatment has to be clearly identified: increase the patient's survival time, provide local control or perform histology to determine the cancer's properties. Without a doubt, the best evidence is absolutely essential to treat patients who need surgery at the right time. Announcement of the results of clinical trials that are currently underway and examination of those results in detail are the first steps to obtain that evidence. However, obtaining results takes time and other strategies to treat breast cancer are constantly changing. In addition, the drugs used and patient attributes differ completely in different countries. An effective strategy to treat stage IV breast cancer must be devised in accordance with medication in light of the patient's symptoms while remaining mindful of the significance of surgery.

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WJCO 5th Anniversary Special Issues (3): Cervical cancer

Different strategies of treatment for uterine cervical carcinoma stage I B2- II B

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Core tip: There is no currently demonstrated the best option of treatment for women with locally advanced cervical cancer Federation of Gynecology and Obstetrics stage I B2- II B. This article describe the evidence as well as the advantages and disadvantages of the main strategies of treatment.

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Abstract

Uterine cervical cancer is the second most common gynecological malignancy. It is estimated that over 35% of tumors are diagnosed at locally advanced disease, stage I B2- II B with an estimated 5-year overall survival of 60%. During the last decades, the initial treatment for these women has been debated and largely varies through different countries. Thus, radical concurrent chemoradiation is the standard of care in United States and Canada, and neoadjuvant chemotherapy followed by radical surgery is the first line of treatment in some institutions of Europe, Asia and Latin America. Until today, there is no evidence of which strategy is better over the other. This article describe the evidence as well as the advantages and disadvantages of the main strategies of treatment for women affected by uterine cervical cancer stage I B2- II B.

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INTRODUCTION

Uterine cervical cancer is the second most common gynecological malignancy^[1]. In developing countries, it is estimated that over 70% of cases are diagnosed at an advanced stage of disease, thus being a major cause of morbidity and mortality^[2]. Based on the fact that cervical cancer tends to grow locally involving the cervix and the paracervical structures, the International Federation of Gynecology and Obstetrics (FIGO) staging system is clinical. It is based primarily on pelvic examination to estimate tumor size and local extension toward the vagina, parametria and pelvic sidewalls. Thus, cervical cancer can be divided in three groups: (1) early stage of disease, tumors up to 4 cm without involvement of extracervical structures. (FIGO stage I A- I b1); (2) locally advanced cervical cancer (LACC), tumors growing locally bigger than 4 cm or with initial involvement of paracervical tissue (FIGO stage I B2- II B); and (3) advanced stage

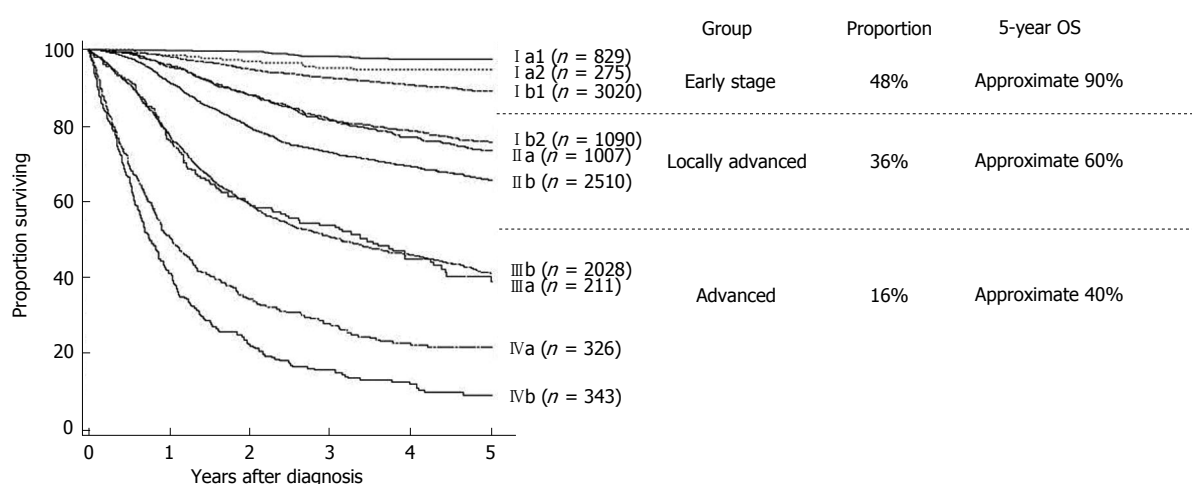


Figure 1 Distribution and 5-year overall survival of women with uterine cervical cancer divided by International Federation of Gynecology and Obstetrics stage disease according with International Federation of Gynecology and Obstetrics annual report^[4]. OS: Overall survival.

disease, tumors that largely involve pelvic structures or tumors with distant metastasis (FIGO stage IIIA-IVB).

Despite the fact that radiotherapy and radical surgery (RS) are equally effective for early stage disease^[3], the latter strategy is generally accepted as a standard of care with a 5-year overall survival (OS) of 90%^[4]. On the other hand, concurrent chemo-radiotherapy is used as first line treatment for patients with advanced stage disease (FIGO stage IIIA-IVB) with a 5-year OS of 40%^[4]. (Figure 1) However, there is a group of patients in the middle with locally advanced disease (FIGO stage I B2-II B) with a 5-year OS of 60%^[4] for whom there are great controversies regarding the appropriate initial treatment approach. Traditionally, these tumors were treated by radiotherapy alone.

In 1999, based on the results of five large randomized controlled trials, the National Cancer Institute of United States launched an alert recommending concurrent chemoradiation for treating women with LACC becoming the standard of care^[5]. Nevertheless, other treatment modalities with similar efficacy were developed in other regions such as Europe, Japan, South Korea, and Latin America. These include: platinum-based neoadjuvant chemotherapy (NACT) followed by radical hysterectomy^[6-8]; chemoradiotherapy followed by adjuvant chemotherapy^[9] or followed by RS^[10]. Therefore, the treatment of women with LACC seems to be multidisciplinary and the standard of treatment seems to be far from being elucidated. Thus, this article describes the different options of treatment, with their advantages and disadvantages, for women with uterine cervical cancer IFGO stage I B2- II B.

CONCURRENT CHEMORADIATION

Radiotherapy has been the standard of care for treating women affected by LACC for the past 100 years. In 1999, after publication of five randomized controlled trials (RCT)^[11-15], the National Cancer Institute issued an alert recommending that “concomitant (cisplatin-based) chemo-radiotherapy be considered instead of radiotherapy alone

in women with cervical cancer”. This led to a change in the treatment of many women with cervical cancer^[5]. Latter on, a meta-analysis evaluated 15 RCTs comparing chemo-radiotherapy *vs* radiotherapy alone in 3452 women with cervical cancer FIGO stage I B2-IV^[16]. Eleven trials used platinum-based chemoradiotherapy, either as a single agent (eight trials) or in combination regimens (three trials). Three trials used nonplatinum regimens comprising of fluorouracil, mitomycin, or a combination of the two. Each of the trials aimed to prescribe external-beam radiation at a dose to a tumor of between 40 and 61.2 Gy, and 14 trials used brachytherapy. The total planned duration of radiation treatment (external-beam plus brachytherapy) was from 40 to 70 d across all trials. The median follow-up for surviving patients across all 15 trials was 5.2 years. The study noted a 6% improvement in 5-year survival with chemoradiotherapy in comparison with radiotherapy alone (HR = 0.81, $P < 0.001$). However, a great majority of the trials evaluated in this meta-analysis included patients with cervical cancer FIGO stage I B2 to IVB and, as Figure 1 shows, there is a great disparity regarding OS between them.

It is interesting to note that only one study compared chemoradiation *vs* radiation alone in patients with cervical cancer FIGO stage I B bulky^[11]. This study included 374 patients with FIGO stage I B2 excluding high-risk patients with evidence of radiologic enlarged lymph nodes. Women were allocated to receive radiotherapy alone or radiotherapy plus weekly cisplatin intravenously at a dose of 40 mg/m². They observed a risk of progression of the disease and death of 0.51 (95% confidence interval, 0.34 to 0.75) and 0.54 (95% confidence interval, 0.34 to 0.86), respectively. The rates of both progression-free survival ($P < 0.001$) and OS ($P = 0.008$) were significantly higher in the combined-therapy group at four years. With this treatment modality, 80% of patients did not relapsed and 85% were alive at four year after initial treatment.

Current recommendations state that the total paracervical tumor dose (sum of external-beam radiotherapy and brachytherapy) be between 85 and 90 Gy, that total pelvic

sidewall dose be between 55 and 65 Gy, and that overall treatment time not exceed 8 wk^[17]. The greater majority of trials comparing radiotherapy *vs* chemoradiation were conducted in United States and Canada, under strict criteria of treatment administration and in well-equipped institutions. In this sense, radiotherapy is administered by using conventional equipment in the majority of women with cervical cancer and mainly in developing countries where this disease is more frequent^[18]. Nevertheless, by integrating computed tomographic imaging into the radiotherapy planning process, allow the dose of the radiation to match or conform to the outline of the target. The recently introduced intensity-modulated radiotherapy (IMRT) is an extension of this principle. The aim is to produce a highly shaped high-dose volume that maximizes normal tissue sparing with the goals of decreasing toxicity and possibly increasing tumor dose. Thus, the dose distribution with IMRT fits more precisely to the target volume, reducing the dose to the rectum, the central bladder and bowel. Several clinical studies have demonstrated a reduction in the mean volume received on the bladder, the rectum, and the bowel^[19], decreasing bowel adverse events and lymphedema^[20]. A pilot study compared 58 women with cervical cancer treated with radiotherapy ($n = 35$ in four-field box group, $n = 33$ in IMRT group) reporting similar local control of the disease with less toxicity in IMRT group^[21]. Other authors recently confirmed the same results^[22]. Thus, the availability of modern equipment of radiotherapy such as IMRT can help to reduce treatment's morbidity. Radiation therapy, in addition, should be taken with caution if an ideal schedule of care is not possible for some patients.

In this sense, it has been demonstrated that a prolonged treatment time, beyond 50 to 56 d, is associated with a 1% loss of local control for every additional day of treatment with radiotherapy^[23]. In addition brachytherapy, as an integral part of radiation treatment for cervical cancer, is critical for obtaining a cure. Unfortunately, 5% to 10% of patients are unable to receive brachytherapy^[24] because of technical difficulties with the insertion of the devices (*e.g.*, stenotic cervix).

NACT FOLLOWED BY RS

Cervical cancer was traditionally interpreted as chemoresistant cancer. Since 1983, where the first study reporting a response to combined systemic therapy was reported^[25], chemotherapy was evaluated in patients with cervical carcinoma. Thus, in regions such as Europe, Asia or Latin America, NACT followed by radical hysterectomy has been suggested for treating patients with LACC. The main objective of NACT is, to reduce the volume of the tumor, to achieve radical operability; and to reduce the number of patients who finally require adjuvant radiation treatment^[8]. This strategy is based on the strong surgical tradition in some centers; and the lack of accessibility of patients to the radiotherapy centers in some countries. To this regard, one Italian multicenter RCT compared NACT followed by RS *vs* radiotherapy alone in patients

with LACC^[26]. The authors found that only 23% of patients allocated to radiotherapy arm received the adequate treatment in terms of total dose and/or time of delivery of radiotherapy^[26].

The efficacy of NACT for treating LACC has been largely tested by several authors over the last 30 years^[7,8,27]. An Italian multicenter RCT compared 441 women with LACC FIGO stage I B2-III who randomly received radiotherapy alone or NACT followed by RS. The 5-year survival in patients FIGO stage I B2 to II B showed significantly longer progression-free survival (59.7%, 95%CI: 51.3%-68.1% *vs* 46.7%, 95%CI: 38.1%-55.3%, $P < 0.02$) and OS (64.7%, 95%CI: 56.5%-72.9% *vs* 46.4%, 95%CI: 37.2%-55.6%, $P < 0.005$) for patients in NACT + RS arm^[26]. Additionally, RS might play an important role in patients with stable disease after 3 courses of NACT. One study^[28] evaluated 32 patients with cervical carcinoma FIGO stage I B2-II A with stable disease after receiving 3 cycles of cisplatin and 5-fluorouracil-based NACT. The 5-year OS in patients who received RS after NACT was 76.4%, while those patients who received NACT followed by adjuvant radiation treatment experienced a 5-year OS of 37.5%, $P = 0.01$.

A meta-analysis^[16] compared the results of five studies evaluating NACT followed by RS *vs* radical radiotherapy. A total of 872 women were included, mostly with FIGO stage I B-II A tumors. The number of courses of NACT ranged between two and seven cycles of cisplatin based, chemotherapy before radical hysterectomy. Together these trials gave a highly significant (HR = 0.65, 95%CI: 0.53-0.80, $P < 0.0004$) 35% reduction in the relative risk of death, with NACT. These results translate into a 14% absolute improvement in 5-year survival and this effect did not seem to vary according to age, stage, histology, grade or performance status. Local, distant and overall disease-free survival (DFS) was similar between groups.

The optimal drug and schedule is also to be determined. During the last three decades, several institutional experiences (with disparity on the drugs combinations and schedules), few phase II trials^[29] and RCT have been reported^[30,31]. Despite some differences in design and results between trials, the reported response rate achieved a range between 70% and 100%^[32]. The main tested drugs include cisplatin, taxanes, irinotecan, vinorelbine, and gemcitabine. The combined regimen, however, have been also investigated with encouraging results^[30,31]. In 2005, the first RCT comparing different schemes of NACT was published^[30]. An Italian Multicenter study compared 219 patients with LACC FIGO stage I B2-IV who received ifosfamide 5 g/m² during 24 h plus cisplatin 75 mg/m², plus paclitaxel 175 mg/m² (TIP scheme) or ifosfamide 5 g/m² during 24 h plus cisplatin 75 mg/m² every 3 wk for three courses (SNAP-01). The authors observed grade 3 to 4 neutropenia, anemia, and thrombocytopenia to be more frequent with TIP; and a higher optimal pathologic response rate, defined as residual disease < 3 mm of stromal invasion, with TIP (48% *vs* 23%; OR = 3.22; 95%CI: 1.69-5.88; $P < 0.0003$). In the median follow-up of 43.4

Table 1 Institutional capabilities, advantages and disadvantages of the main strategies of treatment for treating locally advanced cervical cancer International Federation of Gynecology and Obstetrics stage I B2- II B

	Institutional capabilities	Advantages	Disadvantages
CT-RT	Well radiotherapy equipment (ideally, intensity-modulated radiotherapy IMRT) Availability of schedule for radiotherapy	Well-documented oncologic benefit over radiotherapy alone Standardized treatment	Limited benefit in case of delay of treatment for toxicity or difficult access to radiation treatment (schedule, few equipment, <i>etc.</i>) Possible permanent local toxicity of radiotherapy, mainly in young and sexually active women Delay local treatment such as RT or RS selection of resistant cells clones
NACT + RS	Welltrained surgeons Institutional support for complex surgical procedure (Intensive care units, Urologists, Internist, <i>etc.</i>)	Reduce the tumor size Control of metastasis Select chemosensitive patients (prognostic factor) Allow to spare RT for relapsed disease or chemorefractory patients	chemotherapy-induced immunosuppression Negative lymph nodes at RS Cumulative toxicity of multimodal treatment, mainly in case of adding postoperative RT
Chemoradiation + adjuvant chemotherapy	Similar to CT-RT strategy	Possible but not welldocumented benefit yet (limited trials)	Similar to chemoradiation cumulative toxicity of multimodal treatment

NACT: Neoadjuvant chemotherapy; RS: Radical surgery; IMRT: Intensity-modulated radiotherapy; CT-RT: Chemoradiation.

mo there were no significant differences in terms of OS between both groups of treatment^[30]. A subsequent Italian Multicenter study (SNAP-02) investigated 154 patients who were randomized to receive TIP as it was previously studied or paclitaxel 175 mg/m² + cisplatin 75 mg/m² for three cycles, followed by RS. Grades 3-4 leukopenia (6%/53%) and neutropenia (26%/76%) were significantly more frequent with TIP. The overall optimal response showed a significant benefit by using TIP OR = 2.3 (95%CI: 1.1-4.7, *P* = 0.027). No significant differences in survival were noted between groups^[31].

The possible limitation of this strategy is the fact that over 30% of patients will require adjuvant radiotherapy after surgery due to pathologic risk factors on the specimen^[33]. Thus, patients could have the adverse effect of a RS plus radical pelvic/abdominal radiotherapy (Table 1).

The question of whether NACT or chemoradiation is a more efficacious treatment for patients with LACC (FIGO stage I B2- II B) remains to be answered. The current RCT being conducted by the European Organization for Research in Cancer Therapy (EORTC) compares these two treatment modalities in patients with LACC (EORTC-55994, NCT00193739) and is expected to reveal important information for determining the most effective treatment protocol.

ADJUVANT CHEMOTHERAPY AFTER INITIAL TREATMENT

Chemotherapy could be given after initial treatment in patients with a higher risk of systemic relapse. Peters *et al.*^[13] evaluated 268 patients who were randomly treated with surgery plus radiotherapy or surgery plus chemoradiation plus adjuvant chemotherapy based on four cycles of 5 fluorouracil every 3 wk in patients with early stage disease (FIGO stage I A2- II A). Progression-free and OS was significantly improved in patients receiving chemotherapy. The hazard ratios for progression-free survival and OS in the radiation only arm *vs* the chemoradiation

arm were 2.01 (*P* < 0.003) and 1.96 (*P* < 0.007), respectively. This advantage was more pronounced in patients that received three or four cycles of adjuvant chemotherapy. In addition, a recent analysis of more mature data^[34] showed maximal benefit for patients with spread to two or more lymph nodes after postoperative radiotherapy and adjuvant chemotherapy compared with patients receiving postoperative radiotherapy alone (5-year survival 55% *vs* 75%). However, the effect was minimal in low-risk patients, such as only one metastatic lymph node or a tumor size of 2 cm or less in diameter^[34].

Adjuvant chemotherapy was also investigated after NACT followed by RS. In 1998, Sananes *et al.*^[35] evaluated the efficacy of adding 3 courses every three weeks of cis-platinum 50 mg/m², methotrexate 30 mg/m², and cyclophosphamide 500 mg/m² in 56 women with LACC FIGO stage I B- III B after NACT followed by RS. After a median follow-up of 75 mo the authors noted an OS for stage I B of 88%, Stage II B 78%, and 50% for III B. Angioli *et al.*^[36] reported a case series of 246 women affected by a LACC FIGO stage I B2- II B who had undergone NACT followed by RS and postoperative adjuvant chemotherapy based on 4 cycles of Cisplatin 100 mg/m² and Paclitaxel 175 mg/m². The study showed a 5-year OS and DFS are 77% and 61%, respectively.

In summary, adding chemotherapy after initial treatment for treating women with LACC requires further investigation. Despite the fact that this strategy should be used under clinical trials, it seems reasonable its indication in high-risk patients, such as node metastasis and lymphovascular space involvement.

AORTIC LYMPH NODE TREATMENT

Lymph node involvement and tumor size are the most important prognostic factors in women with LACC^[37,38]. Based on the results of positron emission tomography-computed tomography (PET-CT), over 20% of patients with LACC have aortic node metastasis, and 93% of them have concomitant pelvic node involvement^[39]. De-

termination of nodal involvement before treatment has been suggested as an important factor for disease control and for obtaining better oncologic outcomes^[40]. Surgical staging has been demonstrated to be the best option for establishing the status of aortic node in women with LACC. A prospective study on 60 women with LACC without evidence of aortic involvement on CT scan underwent PET-CT and aortic surgical staging. A total of three out of 26 patients (21%) with negative pelvic and aortic node on PET-CT had histological positive aortic nodes (false negative rate). In addition, 6 (22%) patients with positive pelvic but negative aortic nodes on PET-CT had positive aortic node at final histology report^[41]. Other similar study on 125 patients performed by Leblanc *et al.*^[42] found similar results. Standard treatment for patients with aortic nodal involvement is based on the extension of radiation field to the aortic area up to the level of the renal veins. As it was previously mentioned, that using IMRT the intestinal toxicity can be reduce without effect on survival^[43].

Despite the fact that aortic lymph node dissection can be performed by laparoscopy or robotic surgery, arguments against lymphadenectomy include increased morbidity, delay in starting first line treatment and the questionable advantages on the patients' OS^[40]. To this regard, the oncological outcomes of aortic lymph node dissection have been evaluated in several studies with controversial results. A sub-analysis of three gynecologic oncology group (GOG) studies (GOG 85, GOG 120, and GOG 165) analyzed the impact of surgical staging ($n = 555$) or radiographic determination (CT or magnetic resonance imaging) ($n = 130$) on survival in women with LACC after chemoradiation treatment^[44]. Despite the fact that the multivariate analysis demonstrated a positive benefits of surgical staging on progression free survival and OS, the study had some limitations. Inclusion criteria for this sub-analysis were heterogeneous and the population was unbalanced not only in the sample size, but also regarding the pre-surgical nodal evaluation^[40]. The effect on survival of aortic node dissection in women with LACC and negative aortic nodes on PET-CT is currently being investigated in two phase III RCT. (NCT01049100 and NCT01365156).

CONCLUSION

During the last years, several strategies of treatment have been evaluated for treating LACC FIGO stage I B2- II B. After reviewing the literature, there is clear evidence that chemoradiotherapy is better than radiotherapy alone but there is no evidence for using chemoradiotherapy in lieu of NACT followed RS as standard of care. Until definitive trials currently ongoing are published, the strategy of treatment should be individualized after adequate patient counseling and based on specific institution capability.

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WJCO 5th Anniversary Special Issues (4): Head and neck cancer

Relationship between head and neck cancer therapy and some genetic endpoints

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Abstract

Head and neck cancer (HNC) is the sixth most common human malignancy worldwide. The main forms of treatment for HNC are surgery, radiotherapy (RT) and chemotherapy (CT). However, the choice of therapy depends on the tumor staging and approaches, which are aimed at organ preservation. Because of systemic RT and CT genotoxicity, one of the important side effects is a secondary cancer that can result from the activity of radiation and antineoplastic drugs on healthy cells. Ionizing radiation can affect the DNA, causing single and double-strand breaks, DNA-protein crosslinks and oxidative damage. The severity of radiotoxicity can be directly associated with the radiation dosimetry and the dose-volume differences. Regarding CT, cisplatin is still the standard protocol for the treatment of squamous cell carcinoma, the most common cancer located in the

oral cavity. However, simultaneous treatment with cisplatin, bleomycin and 5-fluorouracil or treatment with paclitaxel and cisplatin are also used. These drugs can interact with the DNA, causing DNA crosslinks, double and single-strand breaks and changes in gene expression. Currently, the late effects of therapy have become a recurring problem, mainly due to the increased survival of HNC patients. Herein, we present an update of the systemic activity of RT and CT for HNC, with a focus on their toxicogenetic and toxicogenomic effects.

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Key words: Chemotherapy; Head and neck cancer; Radiotherapy; Toxicogenetic; Toxicogenomic

Core tip: The main therapies for head and neck cancer (HNC) are surgery, radiotherapy (RT) and chemotherapy. Considering that both RT and chemotherapeutic drugs can interact with the DNA, one of the important, late-occurring complications is a therapy-related secondary tumor resulting from the genotoxic effects of the therapy on the healthy cells. This review presents an update of the toxicogenetic and toxicogenomic effects of HNC treatments, highlighting the main mechanisms evolved in the secondary damage caused by RT and chemotherapies.

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INTRODUCTION

Head and neck cancer (HNC) is the sixth most common

human malignancy^[1], representing 3% of all types of malignant tumors. The head and neck are anatomically complex regions where a wide variety of cancers with different phenotypes, histologies and invasiveness may occur^[2]. Approximately 48% of the cases are located in the oral cavity, and 90% of these cases are squamous cell carcinoma (SCC), which affects the lips, mouth, tongue, nasopharynx, oropharynx, hypopharynx, larynx and paranasal sinuses^[2,3]. Annually, more than 500000 new cases of SCC are diagnosed^[4]. High rates of morbidity and mortality are observed^[5], mainly because of the advanced clinical stage at the time of diagnosis^[6]. However, the use of concurrent chemotherapy (CT) and radiation demonstrates that survival has substantially improved over the past decades for patients with most of the forms of HNC^[7]. Data from some studies have suggested that SCC develops through two mechanisms: directly from the normal mucosa, called “*de novo*”, and following the sequence “dysplasia-carcinoma”^[8-10].

Tobacco, alcohol consumption and human papillomavirus (subtypes 16, 18 and 33) are responsible for, at least, 75% of HNC^[11]. However, other factors, such as diet^[12], mechanical trauma^[13], occupational factors, oral hygiene, inflammation^[14,15] and some gene polymorphisms, are also associated^[12,16]. Little is known about the molecular mechanism of HNC. Some studies have shown that alterations in the PI3K pathways, such as mutations in the *PIK3A* gene, have been reported in HNC^[17,18]. The PI3K/AKT/mTOR pathway is activated in 57%-81% of SCC patients, and AKT is usually upregulated^[19]. Moreover, it has been reported that *TP53* mutations are frequently detected in the tissues of young adult SCC patients^[20], and *NOTCH* mutations have also been found in head and neck carcinomas^[21]. Furthermore, EGFR expression has been described in up to 90% of cases, and it is associated with poor prognosis^[22].

Several therapies and treatment protocols are used for HNC, including surgery, CT^[2] and radiotherapy (RT) and more recently, immunotherapy^[23], gene therapy^[24] and photodynamic therapy^[25]. While treatment for loco-regionally recurrent HNC may include surgery or RT, systemic CT is used in locally advanced tumor, recurrence or metastasis^[26]. CT may, or may not, be associated with surgery and/or RT^[27]. The choice of HNC therapy depends on the tumor staging and the approaches for organ preservation^[28]. Currently, the late effects of therapies have become a recurring problem, mainly due to the increased survival of HNC patients.

In the following paragraphs, we highlight some toxicogenetic (stable and heritable alterations in the genome that are able to influence the relative susceptibility of an individual to the adverse effects that may result from exposure to an exogenous material) and toxicogenomic (relationship between the genome and the biological response of the body after exposure to toxic agents or stressors) effects of HNC therapies.

RT

In recent years, RT has technically and biologically im-

proved, aiming at including only the target, with minimal unnecessary irradiation to normal tissue^[29,30]. Actually, RT fractionation schedules includes more fractions per day in order to reduce the overall treatment time (accelerated fractionation) and/or the use of multiple small fraction doses (hyperfractionation), which allows a higher total dose to be given without enhancing the risk of morbidity induced by radiation^[31].

Radioresistance is one of the main determinants of treatment outcome in HNC patients, and it can be related to tumor hypoxia and changes in gene expression^[32,33]. A meta-analysis study showed that when hypoxic modification is given in conjunction with curative intended RT result in a significant improvement in loco-regional control, disease specific control and overall survival^[34]. However, another study showed that RT (5 Gy radiation) had no effect on hypoxia-inducible factor-1 α (*HIF-1 α*) gene expression in human oral SCC cell lines (SAS, Ca9-22, TT, BSC-OF and IS-FOM). Conversely, SCC cells expressing high levels of *HIF-1 α* were resistant to radiation^[35].

Several genes, such as sensors, transducers and effectors of DNA damage, have been associated with the ionizing radiation-induced cellular response^[36]. One of the main molecules is the tumor-suppressor protein p53, which acts through the transcriptional control of target genes, influences multiple response pathways and leads to a diverse response to ionizing radiation in mammalian cells^[37]. Additionally, one study demonstrated that the radiation-resistant capacity of nasopharyngeal tumors was mostly due to changes in the expression of genes related to cell Ca²⁺ homeostasis. This same study also showed that cell proliferation induced by extracellular and intracellular factors may maintain the tumor size during RT, leading to recurrence after treatment^[38]. Bohn *et al.*^[39] showed that RT induces a systemic stress response, as revealed by the induction of stress relevant gene expression in blood cells. However, the authors discuss that further studies are still needed to confirm that these changes reflect a systemic effect or are biomarkers of the tumor microenvironment. Moreover, a genomic (transcriptomic and proteomic) study of the response to radioresistance showed 265 up-regulated and 268 down-regulated genes, 30 of which were cancer-related genes. The proteomic analysis identified 51 proteins with altered expression in the radioresistant cell lines, 18 of which were cancer-related proteins. In both methodologies, the over-expression of NM23-H1 and PA2G4 were identified^[33]. The influence of GP96 protein in radioresistance has been also described^[40-43]. GP96 is a multifunctional protein^[44] that acts as a chaperonin for peptides and modulates the innate and adaptive immune responses that are mediated by antigen-presenting cells and other immune cells^[40-43]. Furthermore, GP96 is important for protein maturation and protein homeostasis^[45]. In patients undergoing RT, it was shown that GP96 may serve as a novel prognostic marker of RT and may play important role in radioresistance, favoring tumor invasiveness^[46].

In addition to gene expression changes, it is well

known that ionizing radiation damages the DNA, causing single (SSB) and double-strand breaks due to direct action or by the generation of free radicals^[47], resulting in oxidative stress^[48], base damage and DNA-protein cross-links^[49-51]. The oxidative DNA damage arising from RT can be responsible for both therapeutic and adverse effects^[52]. In other words, RT may kill the tumor cells, but it can also damage normal tissues^[53]. A second tumor may develop immediately or years after the primary tumor treatment^[54]. Therefore, the most important dose-limiting factor is the tolerance of the adjacent normal tissue, which depends on the stage and location of the primary tumor^[55]. Quantification of chromosome aberrations in circulating lymphocytes is used to estimate the effects of the RT dose^[56].

RT mutagenicity on tumor and healthy tissues depends on the DNA repair capacity^[57]. Genomic integrity following irradiation is maintained by specific DNA repair pathways that are initiated based on the type of DNA damage^[58]. Double-strand DNA breaks are repaired *via* homologous recombination repair and/or non-homologous end joining repair^[58]. In HNC, variants of the *XRCC2*, *XRCC3* and *RAD-51*^[59] genes were found to be associated with acute mucositis^[60,61]. The alkaline comet assay has become a popular technique for detecting a range of DNA damage types during the last decade^[62], including the effects of RT. In this sense, DNA damage and repair efficiency were evaluated by comparing the peripheral blood lymphocytes, which were isolated from tissue biopsies and from metastases biopsies of SCC patients after treatment with gamma radiation (Cobalt 60). It was observed that the repair mechanisms were less effective in patients with metastasis than in the healthy controls. Thus, the differences in radiation sensitivity of the cancer and control cells suggests that DNA repair might be critical for the treatment of SCC^[57]. On the other hand, the 8-oxoGua is one of the most common DNA lesions that results from reactive oxygen species (ROS)^[63], and this lesion can lead to mismatched pairing^[64]. The repair hOGG1 glycosylase removes 8-oxoGua from the cellular DNA and repairs the base excision^[52,65,66]. Thus, Cooke *et al.*^[65] showed that urinary excretion of 8-oxoGua and 8-oxodG can be used to measure the activity of the enzymes that are involved in the removal of oxidative DNA damage. In this sense, Roszkowski *et al.*^[52] demonstrated that fractionated RT of HNC patients resulted in elevated urinary excretion of 8-oxodG and a significant reduction of uric acid in the blood. The authors suggested that the RT is responsible for oxidative stress/oxidative DNA damage throughout the whole body and, therefore, may be responsible for a significant increase in the level of 8-oxodG in a distinct subpopulation of HNC patients^[52]. The lack of increase of urinary 8-oxoGua in irradiated patients may reflect a reduction in the activity of hOGG1. Recently, it has been observed that HNC patients have a significantly reduced ability to repair the 8-oxoGua lesion that is generated by oxidative stress^[67]. However, the decreased activity

of the main enzyme responsible for 8-oxoGua removal should result in the accumulation of the lesion in cellular DNA^[52]. Some authors have also shown different inter-individual responses in patients after RT. Kadam *et al.*^[68] studied gamma radiation-induced SSBs in peripheral blood leukocytes of SCC HNC patients with different lifestyles, both during and after RT. The results indicated that gamma radiation caused considerable DNA damage in all of the dose intervals of the treatment. However, a comparison between the smokers and non-smokers revealed the significantly greater DNA damage in the smoking patients at the pre-therapy level (10 Gy and 60 Gy of irradiation), indicating a higher sensitivity of the smokers to gamma radiation at these doses. At doses of 20-50 Gy, gamma irradiation failed to cause increased DNA damage in smokers, indicating the radio-protective or shielding effect of the tobacco (nicotine) that is possibly related to the antiapoptotic property of nicotine in the targeted/non-targeted cells. This may have important implications for RT that could indicate less effective treatment in smokers. Prolonged exposure to gamma radiation (40-60 Gy) led to a gradual decline in the intensity of the DNA damage, suggesting saturation of DNA damage in the peripheral blood leukocytes^[68].

One of the cytogenetic biomarkers that is widely used to predict cancer risk in humans is the presence of micronuclei (MN)^[69,70]. MN are chromosome fragments or whole chromosomes that are not incorporated into the main nucleus during mitosis. Therefore, they only appear in cells that have undergone a nuclear division^[71]. The MN test is being applied to cytokinesis-blocked peripheral blood lymphocytes and exfoliated cells to monitor human exposure to mutagens^[72-74]. The evaluation of cytogenetic damage by measuring the frequency of micronucleated cells (MNC) in peripheral blood and buccal mucosa of HNC patients undergoing RT showed a significant increase in the number of MNC during RT. The number of micronucleated lymphocytes remained high 30 to 140 d after the end of treatment. These data confirmed the clastogenic potential of RT in the circulating lymphocytes and buccal cells of HNC patients^[75] (Figures 1 and 2).

The severity of radiotoxicity can be directly associated with the radiation dosimetry and the dose-volume differences^[76]. While epidemiological studies have demonstrated the correlation between the formation of a secondary tumor with exposure to moderate-to-high doses of ionizing radiation, a statistically significant increase has hardly been described with low doses of radiation^[77]. The principal RT effects in normal tissue are acute radiotoxicity (mucositis, dysphagia and dermatitis) that occurs in tissues with rapid turnover rates and late radiotoxicity [subcutaneous skin fibrosis and osteoradionecrosis (ORN)] in tissues with slower turnover rates; these effects may become evident months or years after therapy^[78]. There are variable normal tissue responses to RT^[79], and this may be due to the stochastic or deterministic variation effects in radioresponsiveness^[76]. Mucositis, for in-

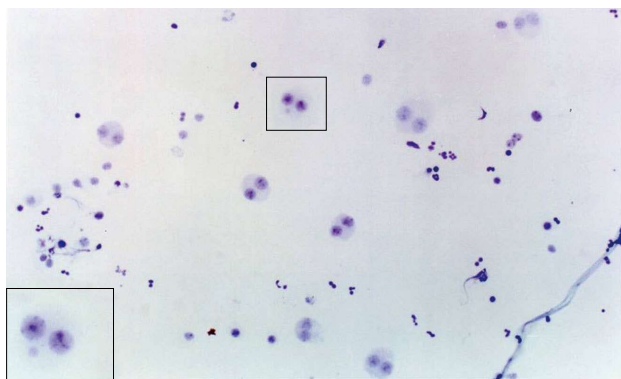


Figure 1 Micronucleated lymphocytes. Stained with 5% Giemsa (increased $\times 400$).

stance, is characterized by mucosal ulceration in the oropharyngeal and gastrointestinal tract, resulting in pain, dysphagia and diarrhea, depending on the dysfunction of the affected tissue^[80]. Some authors have demonstrated that intestinal mucositis is the consequence of a complex cascade of biological events, rather than solely due to direct clonogenic cell death of the epithelial cells^[81]. However, evidence suggests that EGF and its receptor do not have a critical role in prevention or repair of fluorouracil CT-induced intestinal damage^[82]. In addition to cell death events that are associated with the pathogenesis of mucositis, the activation of a wide variety of transcription factors, the production of proinflammatory cytokines, matrix metalloproteinases, leukotrienes, and ceramide^[81], caspase activation and the generation of oxidative stress and ROS by chemotherapeutic agents or radiation appear to be primary events in most pathways leading to mucositis. ROS causes DNA damage and subsequent clonogenic cell death in the epithelial layer^[81]. Of the transcriptional factors that may be significant, nuclear factor κ B (NF- κ B), which is activated by either RT or CT, has many of the characteristics that suggest it may be a key element in the genesis of mucositis. Once activated, NF- κ B leads to the up-regulation of many genes, including those that result in the production of the pro-inflammatory cytokines *TNF- α* , *IL-1 β* and *IL-6*. This leads to tissue injury and apoptosis^[81].

Moreover, evidence of genetic mutations and the role of single nucleotide polymorphisms (SNPs) have been shown to underlie the inter-individual differences in the adverse responses of normal tissues to radiation^[83-85]. Interindividual variations in radiotoxicity responses exist despite the uniform treatment protocols. It is speculated that normal genetic variations, particularly SNPs, may influence normal head and neck tissue radiotoxicity^[78]. The first systematic review of the association of SNPs with the occurrence of HNC radiotoxicity evaluated the association of 11 polymorphisms in 8 genes with acute radiotoxicity and 6 polymorphisms in 4 genes for late radiotoxicity^[78]. The risk of severe acute mucositis was associated with the G allele of *XRCC1* (1196 A > G) in patients treated with RT alone or CT. Severe acute dysphagia was associated with the T allele of *XRCC3* (722

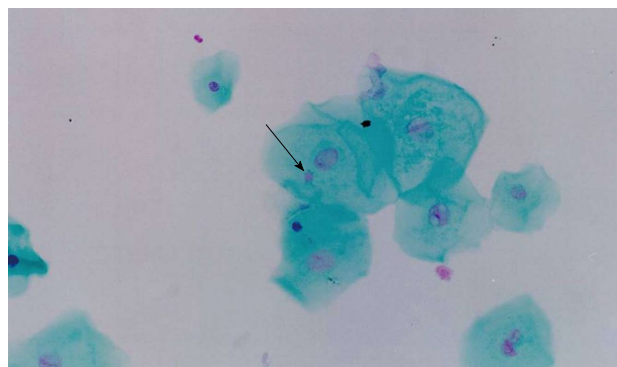


Figure 2 Micronucleated buccal mucosa cells. Stained with feulgen/fast-green (increased $\times 400$).

C > T) and the G allele of *XRCC6* (1310 C > G), and severe acute dermatitis was associated with the T allele of *RAD51* (3392 G > T). The G allele of *XRCC1* (1196 A > G) was associated with a lower grade of subcutaneous fibrosis, suggesting that the wild-type alleles were the risk alleles^[86]. ORN was found to be associated with the T allele of the *TGF β 1* (509 C > T) polymorphism, while the CC genotype was significantly associated with post-extraction related ORN^[78] (Figure 3).

In addition to gene expression changes and DNA damage, microRNAs have been associated with the RT response. In oral SCC cells, it was observed that *ICAM2* gene inhibition by miR-125b expression downregulation induces radiosensitization, suggesting that this miRNA was associated with proliferation and radioresistance mechanisms. Therefore, the control of the expression or activity of miR-125b might contribute to the suppression of proliferation and overcoming radioresistance in OSCC^[87].

CT

Cisplatin-based CT is still the standard protocol for treating SCC^[26,88]. However, simultaneous treatment with cisplatin, bleomycin (radiomimetic antitumoral drug) and 5-fluorouracil (anti-metabolite drug) has also been used^[89]. Other protocols have added paclitaxel to the traditional cisplatin regimen^[90,91]. Combinations with methotrexate and docetaxel may be also used. The administration of the combination therapies can be curative or palliative^[92].

Considering that these drugs can induce DNA damage, one of the important late-occurring complications from treatment of the primary tumor is therapy-related secondary cancer that can result from the genotoxic activity of the drugs on healthy cells^[89,93]. Desai *et al*^[94] showed that head and neck SCC patients were predisposed to chromosomal rearrangements by anticancer drug treatment, which may promote secondary tumorigenesis. Furthermore, Minicucci *et al*^[95] observed higher frequencies of micronucleated lymphocytes in children with malignant tumors before therapy than in healthy children. The authors suggest that the presence of malig-

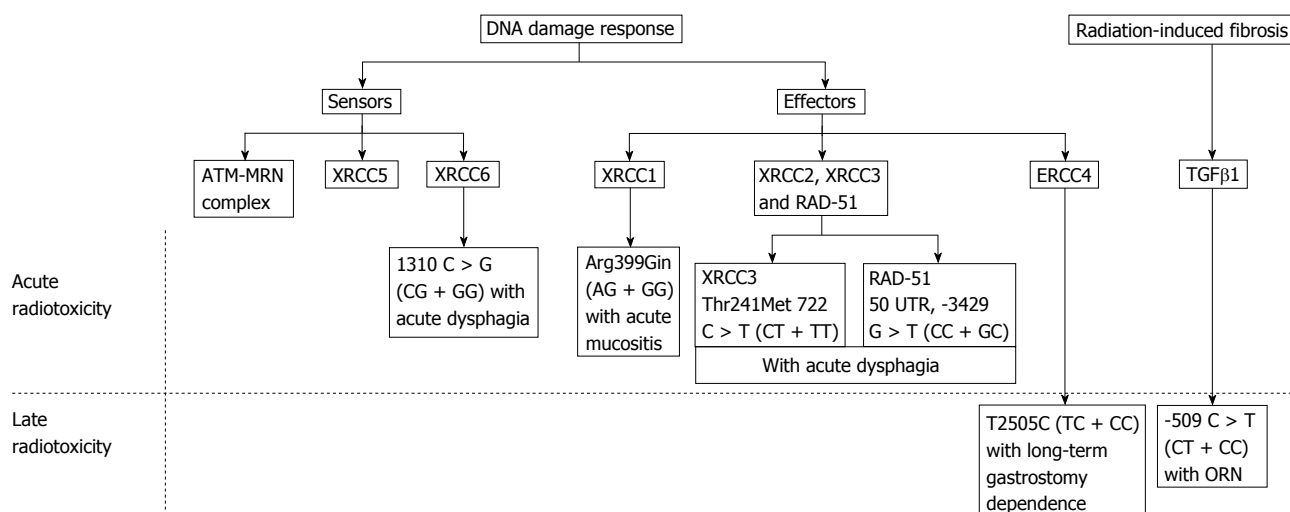


Figure 3 Diagram summarizing the genetic variants associated with radiotoxicity. Extracted from Ghazali *et al.*^[78].

nant tumors may increase the frequency of DNA damage in circulating lymphocytes. Other authors have shown that adjacent tumor tissues shared common genetic changes, and it appears that multiple tumors can arise from a single transforming event that spreads throughout the mucosa surface. On the other hand, some authors have demonstrated the presence of a second tumor that is not clonally-related to the first, which supports the hypothesis of widespread genetic changes after exposure to carcinogen^[96]. In this sense, Ronchetti *et al.*^[97] studied patients with HNC and found that the pattern of microsatellite changes observed in the primary cancer exhibited a completely different genetic arrangement than the second tumor, indicating an independent origin in about three-quarters of the cases.

Cisplatin activity is based on the formation of DNA adducts that block DNA replication and transcription^[98]. These crosslinks represent about 90% of the total DNA damage induced by this drug and are the major contributing factor to its cytotoxic effects^[99]. Carboplatin is another CT drug used for HNC. It belongs to the same group as cisplatin; thus, it is a platinum-based antineoplastic agent. However, when compared to cisplatin, a higher concentration of carboplatin is required to reach equivalent DNA binding because it forms intrastrand DNA crosslinks at a slower rate, and the elimination of free platinum is 10-fold lower than cisplatin^[100]. In relation to treatment with cisplatin, bleomycin and 5-fluorouracil, some authors using the wing somatic mutation and recombination test (Smart) in *Drosophila melanogaster* (*D. melanogaster*) have shown that the combination of these drugs produced synergistic and antagonistic genotoxic effects depending on the concentrations used, and these studies suggest that secondary effects associated with their genotoxic effects could exist, emphasizing the importance of long-term monitoring in these patients^[101]. The anti-metabolite 5-fluorouracil was developed by Heidelberger *et al.*^[102] and is based on the observation that, during DNA synthesis, the uracil base was used more

effectively by tumor cells than normal cells. 5-fluoracil is considered to be an S-phase active chemotherapeutic agent and causes DNA damage, such as double and single-strand breaks^[103]. The genotoxicity of treatment with paclitaxel and cisplatin was also studied, as their effects are not restricted to malignant cells. Using the wing Smart method in *D. melanogaster*, the authors suggested that the combination of paclitaxel and cisplatin did not appear to increase the risk of secondary cancer development; however, the aneugenic activity of paclitaxel could be responsible for the reduced genotoxicity of cisplatin^[104]. Methotrexate blocks the formation of tetrahydrofolic acid because of its high affinity for dihydrofolic acid reductase and suppresses protein synthesis in the G1 phase. Thus, high dose methotrexate could be possible in combination with leucovorin rescue, which prevents normal cells from being affected by the methotrexate-induced folic acid deficiency^[92].

Effects of the genotype are also observed. In patients with prior HNC, 13-cis-retinoic acid (13-cRA) has been shown to prevent second primary tumors (SPT)^[105]. However, other authors demonstrated that low dose 13-cRA treatment did not significantly reduce the occurrence of SPT. The results seem to be influenced by the genotype of the patient. The GST-M1 genotype is an influential risk factor for the development of SPTs in patients who were successfully treated for HNC, and the absence of the GST-T1 enzyme demonstrated a protective effect against SPT^[106].

To avoid late effects of systemic CT, targeted therapy has been also used for treating HNC patients. Bevacizumab, an antiangiogenic monoclonal antibody that targets vascular endothelial growth factor^[107]; trastuzumab, a monoclonal antibody that targets human epidermal growth factor receptor 2 (HER-2)^[108]; lapatinib, a dual EGFR and HER-2 receptor tyrosine kinase inhibitor^[109]; rapamycin, an mTOR inhibitor with antiproliferative effects^[110]; sorafenib, an ERCC1 protein expression DNA repair inhibitor^[111] are some of the drugs currently used

in target therapy.

Among the chemoradiation-induced toxicities, mucosal barrier injury has been well studied. In this sense, peripheral blood cells of patients treated with carboplatinum, paclitaxel and radiation demonstrated the potential impact of the chemoradiation on healthy tissues. Of potential relevance to the development of mucosal injury, it has been observed that Dkk-1, a specific Wnt inhibitor, is upregulated in the tumors^[112]. Some authors suggest that the presence of the Wnt inhibitor provides a mechanism for a reduction in epithelial proliferation, as studies have demonstrated that the presence of Dkk-1 is associated with crypt loss in mice^[113].

CONCLUSION

Nowadays, due to advances in cancer therapies, a significant increase of survival has been observed for cancer patients. Reduction of the toxicogenetic and toxicogenomic side effects has been one of the major goals in the search for new anticancer drugs and therapy protocols.

Rapid innovations in RT have resulted in an urgent need for methods to predict cancer risks from RT, as direct observation of the late effects of newer treatments will require patient follow-up for a decade or more^[114]. Cancer RT involves the eradication of the cancer cells while sparing the surrounding normal tissues. Currently, global tissue responses to RT appear to be directed towards limiting the damage, inducing repair processes and restoring tissue homeostasis^[115]. These newer treatments aim to reduce the amount of healthy tissue exposed to high doses of radiation, but this may occur by increasing the amount of normal tissue exposed to lower doses of radiation^[114]. Combined chemotherapeutic protocols have also been used aiming synergistic effects and decreased toxicity. Furthermore, the concurrent use of chemo and RT has shown a substantial improvement of survival over the past decades^[7]. Alternative therapies, as well as target therapy have also been developed for treating HNC patients. Prevention of second primary tumors is also a field of research that has increased considerably, because of its impact on long-term survival.

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Maintaining clarity: Review of maintenance therapy in non-small cell lung cancer

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Abstract

The purpose of this article is to review the role of maintenance therapy in the treatment of advanced non-small cell lung cancer (NSCLC). A brief overview about induction chemotherapy and its primary function in NSCLC is provided to address the basis of maintenance therapies foundation. The development of how maintenance therapy is utilized in this population is discussed and current guidelines for maintenance therapy are reviewed. Benefits and potential pitfalls of maintenance therapy are addressed, allowing a comprehensive review of the achieved clinical benefit that maintenance therapy may or may not have on NSCLC patient population. A review of current literature was conducted and a table is provided comparing the results of various maintenance therapy clinical trials. The table includes geographical location of each study, the number of patients enrolled, progression free survival and overall survival statistics, post-treatment regimens and if molecular testing was conducted. The role of molecular testing in relation to therapeutic treatment options for

advanced NSCLC patients is discussed. A treatment algorithm clearly depicts first line and second line treatment for management of NSCLC and includes molecular testing, maintenance therapy and the role clinical trials have in treatment of NSCLC. This treatment algorithm has been specifically tailored and developed to assist clinicians in the management of advanced NSCLC.

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Key words: Maintenance therapy; Clinical trials; Non-small cell lung cancer; Molecular aberrations; Progression-free survival; Overall survival

Core tip: This review article addresses the role of maintenance therapy in the treatment of advanced non-small cell lung cancer (NSCLC). Maintenance therapy utilization in NSCLC patient population and review of current guidelines for maintenance therapy are discussed. A treatment algorithm was created to depict first line and second line treatment for managing NSCLC and includes molecular testing, maintenance therapy, and the role of clinical trials in the treatment of NSCLC. A comprehensive review of the achieved clinical benefit that maintenance therapy may or may not have on the NSCLC patient population is presented.

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CURRENT FIRST-LINE THERAPY MANAGEMENT OF ADVANCED NSCLC

Lung cancer remains one of the leading causes of cancer-

related death in men and women worldwide and attributes approximately 1.37 million deaths per year worldwide^[1]. Non-small cell lung cancer (NSCLC) is the most common form of lung cancer and approximately 2/3 of patients with NSCLC present with advanced disease^[2]. This advanced disease state leads to limited treatment options^[3], primarily systemic therapy. According to National Comprehensive Cancer Network guidelines, 4-6 cycles of platinum-based doublet chemotherapy is recommended as first-line treatment in patients without a driver mutation, such as, epidermal growth factor receptor (*EGFR*) mutation or anaplastic lymphoma kinase (*ALK*) rearrangement^[4]. For those patients with an *EGFR* mutation or *ALK* rearrangement, use of a specific inhibitor directed at that target is indicated either as the initial treatment or as therapy when progressive disease develops.

The platinum doublet generally consists of cisplatin or carboplatin with another cytotoxic agent, sometimes in combination with a biologic agent such as bevacizumab (B). Multiple cytotoxic agents in addition to cisplatin and carboplatin have antitumor activity in NSCLC. These include pemetrexed, taxanes (docetaxel, paclitaxel, nanoparticle albumin bound paclitaxel), gemcitabine, vinorelbine, and camptothecins (irinotecan, topotecan). The use of cytotoxic chemotherapy as the initial treatment for patients not selected based upon *EGFR* mutation status and for those whose tumors do not contain an *EGFR* mutation is supported by the results of the tarceva or chemotherapy trial^[5]. In that trial, 760 patients were randomly assigned to either first-line erlotinib followed by chemotherapy (cisplatin plus gemcitabine) upon progression or the same first-line chemotherapy followed by erlotinib upon progression. Overall survival (OS) was significantly longer in unselected patients assigned to initial chemotherapy followed by second-line erlotinib (median 11.6 mo *vs* 8.7 mo, HR = 1.24, 95%CI: 1.04-1.47). For patients known to be *EGFR* mutation negative, OS was significantly longer with initial chemotherapy (median 9.6 mo *vs* 6.5 mo). Combination chemotherapy regimens using a platinum doublet result in median OS of 8-11 mo^[5].

EVALUATION OF THE ROLE OF MAINTENANCE THERAPY

Extending the duration of treatment with the initial platinum based chemotherapy beyond four to six cycles has been evaluated. Currently, there is little evidence to support continuous doublet cytotoxic chemotherapy after 4-6 cycles being given until disease progression^[6], although longer treatment duration increases progression-free survival (PFS), it has at most only a modest effect on OS^[7]. Maintenance therapy is an extension of induction chemotherapy and is continued for a determined period of time unless there is disease progression or significant toxicities develop^[8]. The goal is to extend a favorable patient response from first-line platinum based combination chemotherapy^[9]. There are two types of maintenance therapy, known as continuation and switch maintenance

therapy. Continuation maintenance therapy is the administration of one chemotherapy agent that was part of the initial chemotherapy regimen. Continuation maintenance therapy can involve either a non-platinum cytotoxic drug or a molecular targeted agent. Switch maintenance therapy, involves administration of a new chemotherapy agent that was not part of the original chemotherapy regimen and a potentially non-cross-resistant agent that is started immediately after completion of first-line induction chemotherapy^[9]. Currently, switch-maintenance therapy with pemetrexed or erlotinib is food and drug administration (FDA)-approved. With the standard 4-6 cycles of platinum based chemotherapy, patients may have a response within the first 2-4 cycles; however, many patients cannot tolerate long-term treatment^[10]. Disease progression and co-morbidities that arise due to disease progression contribute to the intolerance of long-term treatment.

Historically, treatment for advanced NSCLC involved waiting until disease progression before a second-line therapy was started^[8]. After first-line therapy, “drug holidays” rarely lasting more than 3 mo in duration can pose a risk for rapid clinical deterioration leading to ineligibility for second-line treatment^[11,12]. This led to clinical trials investigating the role for maintenance therapy using 3rd generation cytotoxic agents and targeted therapy^[8]. Many of these studies either did not have adequate power to detect statistical significance for survival benefits or did not have a placebo control arm^[8].

Advocates of maintenance therapy point to potential merits including: higher probability that tumor will be exposed to effective therapies, decreased development of chemotherapy resistance, maximizing the efficacy of chemotherapy, potentiating the anti-angiogenic effects of chemotherapy, and enhancing anti-tumor immunostimulation^[9]. Many patients do not go on to receive second-line therapy due to rapid progression of disease, decrease in their performance status, or increase cancer-related symptoms. By treating patients with maintenance therapy, the window of opportunity for treatment may be extended. Those patients that benefit from maintenance therapy have better performance status and responded to first-line therapy^[9].

Critics of maintenance therapy argue that the trials evaluating maintenance therapy have: inconsistent clinical trial endpoints, impose a detrimental effect on quality of life, prevent some patients from having a drug holiday, add increased associated costs^[9], and eliminate from the armamentarium standard second-line chemotherapy agents if they are used as maintenance therapy. Patients on maintenance chemotherapy with stable disease may also be exposed to additional toxicities^[6] although some maintenance therapies like pemetrexed have limited grade 3-4 toxicities, such as fatigue and neutropenia^[8], and may be better tolerated.

There are currently five medications that are United States FDA approved for maintenance therapy in NSCLC (B, cetuximab, pemetrexed, gemcitabine, and erlotinib)^[4]. Data exist on some agents that perform better or worse based on tumor histology. For example, regimens con-

taining pemetrexed are more effective in patients with adenocarcinoma and have not demonstrated a meaningful clinical benefit for patients with squamous cell carcinoma. The impact of histology was illustrated by a phase III trial in which cisplatin plus pemetrexed was compared with cisplatin plus gemcitabine as initial therapy^[13,14]. Survival in the 847 patients with adenocarcinoma was significantly prolonged with cisplatin plus pemetrexed compared to cisplatin plus gemcitabine (median 12.6 mo *vs* 10.9 mo, $P = 0.03$). Conversely, cisplatin plus gemcitabine was superior to cisplatin plus pemetrexed in the 473 patients with squamous cell carcinoma (median 10.8 mo *vs* 9.4 mo, $P = 0.05$). Ultimately, the outcome from this study and review of previous trial data led to the re-labeling of pemetrexed for use in non-squamous NSCLC.

REVIEW OF MAINTENANCE THERAPY TRIALS

A list with pertinent details of large randomized maintenance therapy trials in NSCLC is provided in Table 1.

In a study published in 2005, vinorelbine 25 mg/m² was evaluated as a maintenance therapy given weekly for 6 mo until disease progression compared with observation alone in stage IIIB/IV NSCLC patients after induction with MIC treatment (mitomycin 6 mg/m², ifosfamide 1.5 mg/m², cisplatin 30 mg/m² given every four wk \times 2-4 cycles \pm radiotherapy)^[15]. A total of 91 patients were randomized to vinorelbine maintenance therapy. Median PFS for vinorelbine was 5 mo *vs* 3 mo with observation, but the difference was not statistically significant. Median OS for both groups were the same at 12.3 mo and evaluation of molecular subtypes were not performed.

A phase III trial evaluating continuation maintenance therapy with gemcitabine 1250 mg/m² every 3 wk until disease progression or request for removal *vs* best supportive care (BSC) was reported^[2]. Advanced NSCLC patients were given gemcitabine 1250 mg/m² and cisplatin 80 mg/m² every 3 wk for 4 cycles as an induction regimen. Two hundred six patients were given gemcitabine while 138 patients received BSC alone. Median time to progression (TTP) from induction was measured and was a median of 6.6 mo with gemcitabine *vs* 5 mo with BSC ($P < 0.001$, HR = 0.7, 95%CI: 0.5-0.9). Median OS from induction for gemcitabine was 13 mo compared to 11 mo, but not significantly different ($P = 0.195$). Karnofsky performance status (KPS) was taken into consideration with OS and patients were split into KPS > 80 *vs* KPS \leq 80. Patients with KPS > 80 had a HR = 2.1 of dying while on gemcitabine and patients with KPS \leq 80 had HR = 0.8. Using continuation maintenance therapy with gemcitabine after induction with gemcitabine and cisplatin did demonstrate a longer TTP *vs* BSC for patients with advanced NSCLC. No molecular testing was conducted in this study.

The Eastern Cooperative Group (ECOG) 4599 study evaluated the effectiveness of B maintenance therapy in

patients with advanced NSCLC nonsquamous histology only^[16]. Patients completed carboplatin 6 mg/mL AUC and paclitaxel 200 mg/m² induction chemotherapy every three weeks for six cycles or carboplatin 6 mg/mL AUC, paclitaxel 200 mg/m² and B 15 mg/kg every three weeks for six cycles. Patients were randomized to B 15 mg/kg maintenance therapy or surveillance (only patients without progressive disease after induction therapy were eligible for this arm). Median PFS was significantly higher for B *vs* surveillance at 6.2 mo *vs* 4.5 mo ($P < 0.001$). Median OS was significantly higher for B *vs* surveillance at 12.3 mo *vs* 10.3 mo ($P = 0.003$). No tumor molecular testing was completed for this study.

In 2009, the JMEN study, an international randomized, double-blind, phase III study of maintenance pemetrexed with BSC *vs* placebo plus BSC for NSCLC resulted in pemetrexed being approved by the FDA for use as maintenance therapy in NSCLC^[10]. Patients were treated with one of six induction regimens (gemcitabine-carboplatin, gemcitabine-cisplatin, paclitaxel-carboplatin, paclitaxel-cisplatin, docetaxel-carboplatin or docetaxel-cisplatin) every 3 wk for four cycles. Patients were assigned randomized 2:1 to receive pemetrexed 500 mg/m² or placebo. Median PFS plus induction was 7.7 mo for pemetrexed *vs* 5.9 mo for placebo ($P < 0.0001$, HR = 0.50, 95%CI: 0.42-0.61). Median OS plus induction was 16.5 mo with pemetrexed *vs* 13.9 mo with placebo ($P = 0.012$, HR = 0.79, 95%CI: 0.65-0.95). Overall, switch maintenance therapy with pemetrexed demonstrated improved PFS and OS and was well-tolerated. In this study, no tumor tissue molecular testing was conducted.

In a phase III study of advanced NSCLC patients receiving induction therapy with gemcitabine 1000 mg/m² and carboplatin AUC = 5 every 21 d for four cycles, patients that did not demonstrate disease progression were randomized to immediate docetaxel 75 mg/m² every 21 d for six cycles or were given docetaxel with the same dosage and schedule once they presented with disease progression^[12]. Immediate administration of docetaxel maintenance therapy demonstrated a statistically significant increase in median PFS compared with delayed docetaxel (5.7 mo *vs* 2.7 mo, $P = 0.001$). Median OS was not statistically significant for either arm of the study and no molecular testing on patients' tumors was completed.

The "PointBreak" study randomized advanced NSCLC patients to pemetrexed 500 mg/m², carboplatin AUC = 6, B 15 mg/kg induction every 21 d with four cycles, with maintenance pemetrexed 500 mg/m², B 15 mg/kg [pemetrexed and bevacizumab (PB)] *vs* paclitaxel 200 mg/m², carboplatin AUC = 6, B 15 mg/kg induction every 21 d for four cycles, with maintenance B 15 mg/kg^[17,18]. The maintenance therapy for both arms was given until disease progression. Median PFS was significantly higher for PB *vs* B at 6 mo *vs* 5.6 mo, respectively ($P = 0.012$, HR = 0.83, 95%CI: 0.7-0.96). Median OS was not significantly different for PB *vs* B at 12.6 mo *vs* 13.4 mo, respectively. The primary endpoint of improved median OS was not met. While tumor molecular testing was conducted, the

Table 1 A list with pertinent details of large randomized maintenance therapy trials in non-small cell lung cancer

Maintenance	PFS (mo)	HR	P	95%CI	PFS + induction	HR	P	95%CI	OS (mo)	HR	P	95%CI	OS + induction (mo)	HR	P	95%CI	Genotype	Post-TX
Vinorelbine 25 mg/m ² q weekly <i>vs</i>	5.0				NR				12.3				NR				NC	Etoposide 80 mg/m ² , cisplatin 30 mg/m ²
Observation	3.0	0.77	0.110	0.56-1.07	NR	NR	NR	NR	12.3	1.08	0.65	NR	NR	NR	NR	NR		
Gemcitabine 1250 mg/m ² q21 d <i>vs</i>	3.6 (TTP)				6.6 (TTP + induction)				10.2				13.0				NC	Second line chemotherapy/ radiation
BSC	2.0 (TTP)	0.70	0.001	0.50-0.90	5.0 (TTP + induction)	0.70	0.001	0.50-0.90	8.1	0.79	0.003	0.67-0.92	11.0	NR	0.195	NR		
Bevacizumab 15 mg/kg q3 wk <i>vs</i>	6.2	0.66	0.001	0.57-0.77	NR	NR	NR	NR	12.3	0.79	0.003	0.67-0.92	NR	NR	NR	NR		
Observation	4.5				NR				10.3				NR				NC	None reported
Pemetrexed 500 mg/m ² q21 d	4.3				7.7				13.4				16.5				NC	Pemetrexed, docetaxel, erlotinib, gefitinib, vinorelbine, gemcitabine, carboplatin, cisplatin, paclitaxel
<i>vs</i>																		
Placebo Immediate docetaxel 75 mg/m ² q21 d <i>vs</i>	2.6 5.7	0.50	0.0001	0.42-0.61	5.9 NR	0.50	0.0001	0.42-0.61	10.6 12.3	0.79	0.012	0.65-0.95	13.9 NR	0.79	0.012	0.65-0.95	NC	Best supportive care, observed for PD/ survival
Delayed docetaxel 75 mg/m ² (start at PD) q21 d <i>vs</i>	2.7	NR	0.0001	2.60-2.90 m	NR	NR	NR	NR	9.7	NR	0.853	NR	NR	NR	NR	NR		
Pemetrexed 500 mg/kg + bevacizumab 15 mg/kg q21 d <i>vs</i>	6.0				8.6				12.6				17.7	NR			Collected- no specific results	None reported
Bevacizumab 15 mg/kg q21 d <i>vs</i>	5.6	0.83	0.012	0.70-0.96	6.9	NR	NR	NR	13.4	1.00	0.949	NR	15.7	NR	NR	NR		
Cetuximab 250 mg/m ² weekly <i>vs</i>	4.8	0.94	0.390	0.82-1.07	NR	NR	NR	NR	11.3	0.87	0.044	0.76-0.99	NR	NR	NR	NR	Collected- EGFR (IHC)	None reported
Observation	4.8								10.1									
Bevacizumab 7.5 mg/m ² <i>vs</i>	6.7	0.75	0.003		NR	NR	NR	NR	Not sufficient				NR	NR	NR	NR	NC	No bevacizumab
Bevacizumab 15 mg/m ² <i>vs</i>	6.5	0.82	0.030															

vs Placebo Erlotinib 150 mg/d	6.1 4.1	Until PD/ toxicities/ death	NR	NR	NR	12.0	NR	NR	EGFR/wild type/ resistance mutations	Erlotinib (people in placebo group that were egfr +, taxanes (+ docetaxel), antimetabolites (+ pemetrexed), platinum, antineoplastics
vs Placebo Cetuximab 250 mg/m ² weekly	2.75 4.4	Until PD/ toxicities	NR NR	NR NR	NR NR	11.0 9.6	NR NR	NR NR	Not included in study	None reported
Observation Pemetrexed 500 mg/m ² q3 wk	4.2 4.1	Until PD	6.9			8.3 13.9			NC	Erlotinib, docetaxel, gemcitabine, vinorelbine, cisplatin, bevacizumab, investigational drug
vs Placebo Gemcitabine 1250 mg/m ² q3 wk	2.8 3.0	Until PD/ toxicity/death	5.6 NR	0.59 0.0001	0.47-0.74	11.0 12.1	0.78 0.89	0.020 0.387	EGFR mutations/ expressions (exon 19 deletions, mutations in exon 21 and L858R point mutations)	Pemetrexed 500 mg/m ² q21 d, erlotinib, docetaxel
vs Erlotinib 150 mg/d q3 wk	2.9	Until PD/ toxicity/death	NR			11.4	0.87	0.304	NR	NR
vs Observation Pemetrexed 500 mg/kg + bevacizumab 7.5 mg/kg q3 wk	1.9 7.4	Until PD	NR 10.2	0.50 0.001	0.37-0.69	10.0 NR	0.75	0.219	NC	Taxanes/TKI
vs Bevacizumab 7.5 mg/m ² q3 wk	3.7	Until PD	6.6			12.8				15.0

PFS: Progression-free survival; OS: Overall survival; NS-NSCLC: Nonsquamous non-small cell lung cancer; NR: Not reported; NC: Not collected; PD: Progressive disease; TKI: Tyrosine kinase inhibitors; EGFR: Epidermal growth factor receptor.

types of testing and results have not been reported.

The FLEX study, randomized previously untreated advanced NSCLC patients to cisplatin 80 mg/m² plus vinorelbine 25 mg/m² every 3 wk for six cycles, with or without cetuximab 400 mg/m² day 1 and 250 mg/m² day 8 and all subsequent doses weekly^[19]. Cetuximab maintenance was given until disease progression/toxicities. Median PFS was not statistically significant ($P = 0.39$, HR = 0.94, 95%CI: 0.82-1.07). Median OS for cetuximab *vs* observation was 11.3 mo *vs* 10.1 mo ($P = 0.044$, HR = 0.87, 95%CI: 0.76-0.99). Tumor molecular testing was conducted for EGFR immunohistochemistry and was part of the entry criteria for study eligibility.

The AVAIL study, randomized advanced NSCLC patients to cisplatin 80 mg/m² plus gemcitabine 1250 mg/m² every three weeks for six cycles, with either B (7.5 mg/kg), B (15 mg/kg), or placebo every three weeks until disease progression^[20]. Median PFS for low dose B *vs* placebo was 6.7 mo *vs* 6.1 mo ($P = 0.003$, HR = 0.75, 95%CI: 0.62-0.91). Median PFS for high dose B *vs* placebo was 6.5 mo *vs* 6.1 mo ($P = 0.03$, HR = 0.82, 95%CI: 0.68-0.98). Median OS was not analyzed due to insufficient follow-up duration at the time of data reporting. Overall, B as maintenance therapy does improve PFS. No tumor molecular testing was conducted.

The SATURN study evaluated erlotinib as maintenance therapy in advanced NSCLC patients who received one of seven different platinum based doublet chemotherapy regimens (type of regimens were not specified)^[3]. Induction therapy was given for four cycles followed by erlotinib 150 mg/d *vs* placebo until disease progression, toxicity, or death. No B or pemetrexed were used in the induction chemotherapy regimens. Median PFS for erlotinib *vs* placebo was significantly prolonged at 4.1 mo *vs* 2.75 mo, respectively ($P < 0.0001$, HR = 0.69, 95%CI: 0.58-0.82). Median OS with erlotinib *vs* placebo was also significantly improved at 12 mo *vs* 11 mo ($P = 0.0088$, HR = 0.81, 95%CI: 0.70-0.95). From this trial, molecular testing of EGFR immunohistochemistry was reported.

The BMS-099 study, randomized advanced NSCLC patients to carboplatin AUC = 6 plus either docetaxel 75 mg/m² or paclitaxel 225 mg/m² every three weeks for six cycles or carboplatin AUC = 6 plus either docetaxel 75 mg/m² or paclitaxel 225 mg/m² every three weeks for six cycles with cetuximab 400 mg/m² day 1, 250 mg/m² day 8 and each subsequent dose^[21]. Cetuximab was given weekly until disease progression/toxicities. Median PFS and OS were not statistically significant. Maintenance cetuximab added no clinical benefit to PFS or OS. No tumor molecular testing was included in this study.

The PARAMOUNT study evaluated the use of pemetrexed as continuation maintenance therapy in patients with advanced NSCLC nonsquamous histology^[22,23]. Patients were given pemetrexed 500 mg/m² and cisplatin 75 mg/m² every three weeks for four cycles. Patients were then randomized to pemetrexed 500 mg/m² continuation maintenance every three weeks until disease progression or placebo. Median PFS was significantly higher for pemetrexed maintenance *vs* placebo at 4.1 mo *vs* 2.8 mo,

respectively ($P < 0.0001$, HR = 0.62, 95%CI: 0.49-0.79). Median OS was significantly higher for pemetrexed maintenance *vs* placebo at 13.9 mo *vs* 11 mo, respectively ($P = 0.0195$, HR = 0.78, 95%CI: 0.64-0.96). The use of pemetrexed as continuation maintenance therapy can significantly increase median PFS and OS in patients with advanced nonsquamous NSCLC. No tumor molecular testing was conducted.

The IFCT-GFPC 0502 study evaluated gemcitabine (continuation maintenance) *vs* erlotinib (switch maintenance) *vs* observation as maintenance therapy after induction therapy with cisplatin 80 mg/m² and gemcitabine 1250 mg/m² every three weeks for four cycles in patients with advanced NSCLC^[11]. Patients were then randomized to gemcitabine 1250 mg/m² every three weeks, erlotinib 150 mg/d every three weeks, or observation until disease progression, toxicity, or death. Median PFS for gemcitabine *vs* erlotinib *vs* observation was 3.8 mo ($P < 0.001$, HR = 0.56, 95%CI: 0.44-0.72) *vs* 2.9 mo ($P = 0.003$, HR = 0.69, 95%CI: 0.54-0.88) *vs* 1.9 mo. Median OS was not significantly different for gemcitabine *vs* erlotinib *vs* observation at 12.1 mo *vs* 11.4 mo *vs* 10.8 mo; respectively. Molecular testing was completed for EGFR immunohistochemistry (IHC) ($n = 261$) and EGFR mutation ($n = 188$). Fourteen different EGFR mutations were noted [exon 19 deletion ($n = 10$), exon 21 ($n = 4$)]. EGFR IHC had no significant effect on median PFS for gemcitabine or erlotinib therapy and there were too few cases of EGFR mutations for analysis.

The AVAPREL study evaluated the use of B with or without pemetrexed as maintenance therapy in advanced NSCLC with nonsquamous histology with B 7.5 mg/kg, cisplatin 75 mg/m² and pemetrexed 500 mg/m² every three weeks for four cycles as induction chemotherapy regimen^[24]. Patients were randomized to B 7.5 mg/kg alone or B 7.5 mg/kg plus pemetrexed 500 mg/m² (PB) given every three weeks until disease progression/toxicities. Median PFS for PB *vs* B was 7.4 mo *vs* 3.7 mo ($P < 0.001$, HR = 0.48, 95%CI: 0.35-0.66). Median OS was not significantly different between the two arms. No tumor molecular testing was completed in this study.

MOLECULAR ANALYSIS AND ITS IMPACT ON MAINTENANCE THERAPY

Therapy for advanced NSCLC should be individualized based upon the molecular features of the tumor. Whenever possible, tumor tissue should be assessed for the presence of a somatic driver abnormality (*e.g.*, mutated EGFR, ALK rearrangement) which confers sensitivity to a specific inhibitor^[25]. Unfortunately, many clinical trials do not require collection of tumor tissue for molecular analysis as either entry criteria or for subsequent analysis. There are no randomized trials conducted in patients known to have an EGFR mutation or other driver abnormality prior to the initiation of maintenance chemotherapy. After review of maintenance therapy trials cited here, three of ten had molecular subtypes identified

and two of these three trials had pre-planned analysis for molecular subtype *EGFR* mutations. Furthermore, structuring of clinical trials that identify patients with molecular alterations and evaluating their response to standard maintenance therapy has been minimal^[26]. An improved understanding of the molecular pathways that drive malignancy in NSCLC has led to the development of agents that target specific molecular pathways in malignant cells. These agents have been a significant step forward in the treatment of patients whose tumors contain specific mutations in these pathways. Most patients with advanced NSCLC whose tumors contain a driver mutation are initially treated with the appropriate targeted agent (*e.g.*, erlotinib, gefitinib, or crizotinib). For patients with advanced NSCLC who were initially treated with chemotherapy but in whom a driver mutation has subsequently been identified, continuation of therapy with an appropriate targeted agent after the initial cycles of chemotherapy are complete is recommended^[3].

By taking into consideration patient demographics and obtaining molecular testing target treatment plans can be made. *EGFR* mutations and *ALK* rearrangements are more common in NSCLC tumors of patients that have a history of never to light smoking, compared to Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutations which are often found in tumors of heavy smokers^[27]. Treatment with *EGFR* tyrosine kinase inhibitors (TKIs) (such as erlotinib, gefitinib, or afatinib) as single agents is indicated for the initial management of patients whose tumors contain an activating mutation in *EGFR*. In this setting, first-line treatment with an *EGFR* TKI improves PFS compared to standard platinum-based chemotherapy. The impact on OS is less clear, since *EGFR* TKIs were frequently used as second line therapy after chemotherapy in the clinical trials demonstrating the efficacy of this approach. *EGFR* TKIs generally are not combined with platinum-based doublet chemotherapy as initial therapy, since these combinations have not prolonged survival even when patients were selected for sensitivity to these *EGFR* TKIs based upon clinical criteria. In the absence of significant toxicity, treatment with an *EGFR* TKI is continued until there is evidence of progression. An example of second-line therapy that has been shown to be more effective in a specific patient population is pemetrexed and *ALK* rearranged tumors. *ALK*-positive tumors have a significant response to pemetrexed leading to longer PFS when compared to *KRAS* mutant, *EGFR* mutant, or triple negative tumors in patients treated with pemetrexed^[27]. Information on molecular subtypes should be considered^[26]. Pemetrexed is cost effective for patients with non-squamous cell histology and shows the importance in identifying patients who will benefit from pemetrexed maintenance therapy^[8].

Crizotinib, an inhibitor of the *ALK* tyrosine kinase, is preferred as first-line therapy in patients whose tumor contains the *ALK* fusion oncogene. Phase II studies using crizotinib demonstrated an objective response rate over 50 percent in previously treated patients with *ALK* rearrangements, with a median duration of response

greater than 40 wk in responders. A phase III trial demonstrated a significant increase in PFS compared to standard chemotherapy in patients who had previously received one platinum-containing regimen^[28]. Further development and research can help distinguish if *ALK*-positive tumors are responsive to cytotoxic agents or specifically responsive to pemetrexed alone. Such findings can improve the way NSCLC patients with distinct tumor molecular phenotypes are treated and how these treatments can impact outcomes^[27].

DISCUSSION

The role of maintenance treatment for patients with advanced NSCLC is under active investigation. There are several factors to consider when choosing to start a patient on maintenance therapy. These factors include deciding how and whether to continue therapy including patient's tolerance for these agents, absence or presence of molecular mutations, patient specific factors like comorbidities, toxicity associated with the original treatment, and desire to balance clinical benefit vs toxicity of immediate further treatment. The studies reviewed have shown that maintenance therapy could provide clinical benefit in specific advanced NSCLC patients. However, as alluded to earlier, few features that can help identify those most likely to benefit from maintenance therapy have been identified.

Not all advanced NSCLC lung cancer patients are made equal and continuation maintenance therapy to date may improve OS in first-line therapy responders^[11], whereas switch maintenance therapy, can improve OS in patients with stable disease after first-line therapy^[29]. More research into identifying factors that contribute to response rate of various maintenance therapies would allow for better selection of patients to receive maintenance therapy^[26]. A recent study identified patients who normally were not qualifying candidates based on common clinical trial inclusion guidelines (such as socioeconomically disadvantaged and patients with greater symptom burden requiring pre-chemotherapy palliative radiation therapy), and observed that this subset of patients maintained stable disease after first line chemotherapy without additional therapy and at time of disease progression responded well to second-line chemotherapy. This is an example of how some patients may benefit successfully without the use of maintenance therapy^[30]. Identification of these factors will assist providers to better define patient populations who should receive maintenance chemotherapy and decrease costs and toxicities in patients who may or may not benefit from having maintenance chemotherapy.

Measurement of PFS and OS should not be the only factors determining the success of maintenance therapy. Patient perspectives need to be taken into consideration. PFS is valued if disease symptoms are minimal, but these gains can be offset as disease symptoms progress or toxicity burden from treatment impacts that patient^[30]. Clinical benefit is an important determinant in deciding

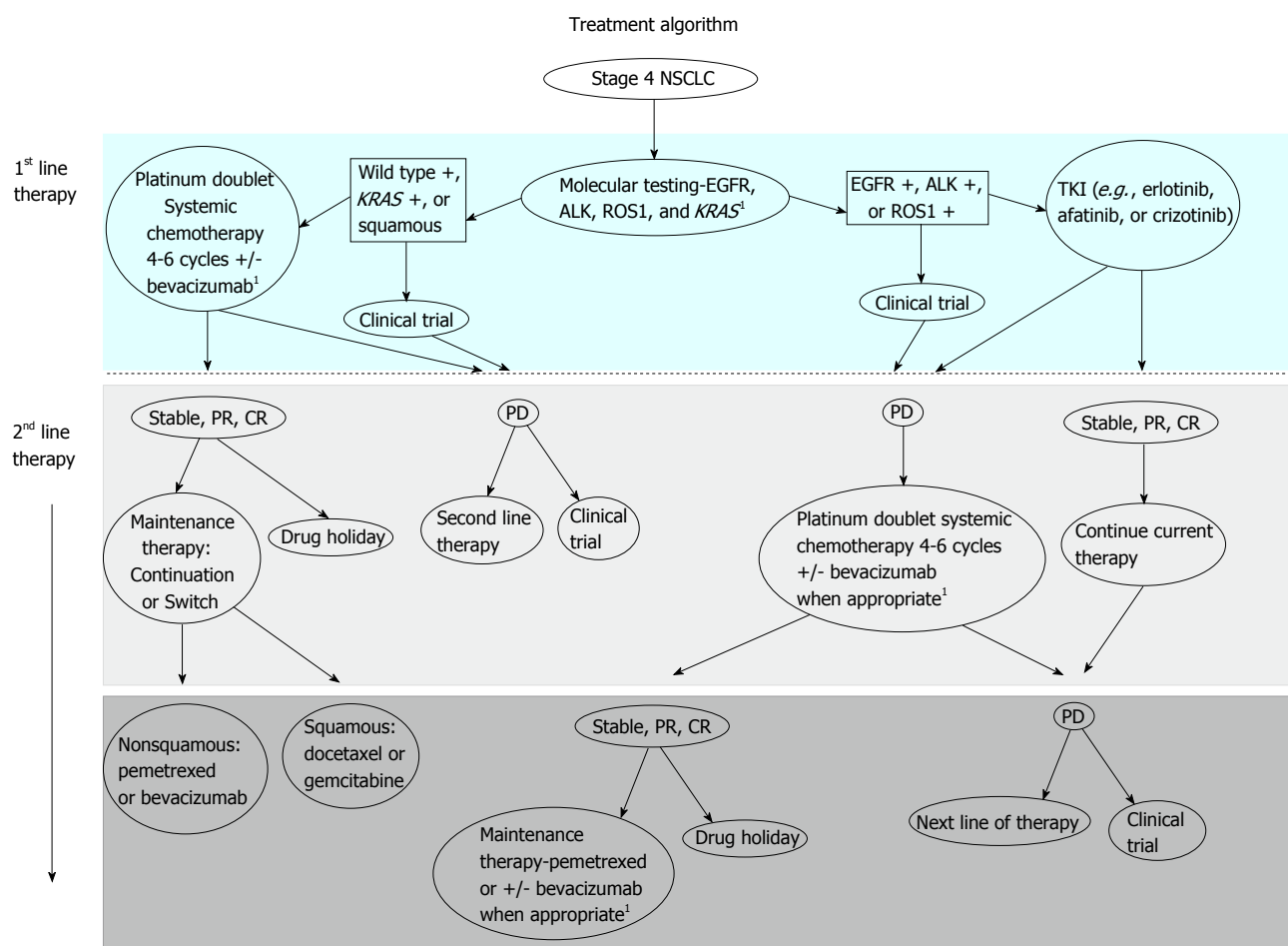


Figure 1 Treatment algorithm. Stage 4 nonsquamous non-small cell lung cancer (NSCLC) should have their tumors analyzed for epidermal growth factor receptor (*EGFR*) mutation or anaplastic lymphoma kinase (*ALK*), Kirsten rat sarcoma viral oncogene homolog (*KRAS*) and ROS1 in a Clinical Laboratory Improvement Amendments-certified laboratory setting. For patients with *EGFR* mutation, we recommend first-line therapy is erlotinib or afatinib or a clinical trial. For patients with *ALK* or ROS1 rearrangements, we recommend first-line crizotinib therapy. We recommend continuation of targeted therapy until disease progression. Upon disease progression, provided the patient is eligible to receive additional therapy, we next recommend a clinical trial or platinum-doublet systemic chemotherapy for 4-6 cycles with or without bevacizumab (drug holiday). Upon disease progression, provided the patient is eligible to receive additional therapy, we next recommend a clinical trial or another National Comprehensive Cancer Network (NCCN) guideline recommended cytotoxic therapy. For patients with squamous, *KRAS* mutation, or wild-type for these 4 molecular phenotypes, we recommend platinum-doublet systemic chemotherapy for 4-6 cycles with or without bevacizumab or a clinical trial. Note: bevacizumab should not be administered to patients with squamous cell carcinoma. Those patients with stable disease or better can proceed on maintenance therapy or have a drug holiday. Those with disease progression on first-line therapy or developing disease progression during maintenance therapy or drug holiday, can be evaluated for a clinical trial or another NCCN guideline recommended cytotoxic therapy, provided the patient is eligible to receive additional therapy. Not for squamous cell lung cancer. TKI: Tyrosine kinase inhibitor; PD: Progressive disease; CR: Complete response.

if patients are candidates for maintenance therapy. By identifying patients' goals and their tolerance of adverse symptoms, determination about the appropriate use of maintenance therapy can be made.

An additional factor when determining the utilization of maintenance therapy is cost effectiveness of maintenance therapy, which can vary depending on location. For example, maintenance pemetrexed is more cost effective compared to other maintenance therapies in the United Kingdom, but is not cost effective in the United States^[30]. Identification of those patients who will gain the greatest benefit from maintenance therapy will help balance efficacy, cost, and patient preferences.

Several of the studies displayed statistically significant results for primary or secondary endpoint PFS. Although PFS was prolonged in many studies, the concern of "statistical significance" in relation to clinical significance

needs attention by the critical eye of a clinician. A result that is statistically significant does not mean that the result is clinically significant and *vice versa*^[31]. Historically and currently, the trend for reporting and interpreting clinical trial results are not based on the prospect of clinical importance^[32]. When interpreting clinical trial results, the *P* value is not the only "value" indicating that the study was statistically significant. The number of subjects in the study contributes largely to reaching a statistically significant number, but not a clinically significant result^[31]. For example, a study with a very large number of subjects commonly will show significant *P* values but overall, the clinical significance and treatment differences are very small^[31].

There have been four maintenance studies to date reporting statistically significant improved PFS and OS. Three out of the four studies, ECOG4599, JMEN, and

PARAMOUNT, did not report tumor molecular analysis. The former study involved B maintenance and the latter two studies involved pemetrexed maintenance. The fourth study, SATURN, did evaluate patient tumors for *EGFR* mutation retrospectively, however, those with *EGFR* mutations had the most dramatic “benefit” of significantly prolonged PFS and OS^[3]. Unfortunately, the majority of maintenance studies reviewed did not conduct molecular testing. To accurately measure the clinical benefit of maintenance therapy in advanced NSCLC patients, their molecular tumor analysis or prospective sample collection should be included as criteria for future clinical trials. As discussed above, the question of clinical *vs* statistical significance is important to point out with all four of these studies. All were very large study populations (at least 663 subjects each) and while the primary results demonstrated statistically significant improvements in median OS, the reality is these are not blockbuster changes for clinically meaningful improvement over standard platinum based doublet therapy in exchange for potential increased treatment-related toxicity and financial-related toxicity.

Other considerations to take into account for maintenance therapy as more oral biologic agents come to the clinic, is patient adherence with their prescribed anticancer therapy. Adherence to treatment is a major factor that can impact outcomes, though the quality of data on this topic and interventions to improve adherence need improvement as well^[33].

Precision-based oncology care allows treatment of advanced NSCLC to be personalized to the patient not the cancer. Just as TKIs have been incorporated into standard of care for treatment of patients with specific tumor molecular mutations^[4], TKIs and metabolic inhibitors have and may continue to demonstrate more significant prolongation of PFS and OS in patients with molecular mutations. As oncologists and advanced practitioners create treatment plans for advanced NSCLC patients, testing for molecular mutations is crucial for selecting the right treatment and stratifying how best to treat patients eligible for systemic therapy. A suggested algorithm for treating stage 4 NSCLC is outlined in Figure 1. By taking histology and molecular subtypes into consideration, more succinct and clear identification of patients that would benefit from one maintenance therapy agent *vs* other alternatives is likely important. Molecular subtypes may behave differently to various standard therapies resulting in the need for developing of targeted therapies for patients with NSCLC^[27]. More advancement is needed in treating NSCLC patients that do not display molecular mutations^[9]. By recognizing these new developments as well as limitations, there is a need for clinicians to be able to identify patients who will have the greatest benefit and effectiveness from maintenance therapy.

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Squamous cell carcinoma of the oral cavity and circulating tumour cells

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Abstract

Due to a lack of substantial improvement in the outcome of patients suffering from oral squamous cell carcinoma (OSCC) during the past decades, current staging methods need to be revised. This disease is associated with poor survival rates despite considerable advances in diagnosis and treatment. The early detection of metastases is an important indicator of survival, prognosis and relapse. Therefore, a better understanding of the mechanisms underlying metastasis is crucial. Exploring alternative measures apart from common procedures is needed to identify new prognostic markers. Similar to previous findings predominantly for other solid tumours, recently published studies demonstrate that circulating tumour cells (CTCs) and disseminated tumour cells (DTCs) might serve as prognostic markers and could supplement routine staging in OSCC. Thus, the detection of CTCs/DTCs is a promising tool to

determine the individual need for therapeutic intervention. Encouraging results and new approaches point to the future use of targeted therapies for OSCC, an exceedingly heterogeneous subgroup of head and neck cancer. This review focuses on summarising technologies currently used to detect CTCs/DTCs. The translational relevance for OSCC is highlighted. The inherent challenges in detecting CTCs/DTCs will be emphasised.

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Key words: Circulating tumour cells; Disseminated tumour cells; Oral squamous cell carcinoma; Head and neck squamous cell carcinoma; Bone marrow; Peripheral blood; Micrometastasis; Minimal residual disease; Epithelial-mesenchymal transition

Core tip: Oral squamous cell carcinoma (OSCC), among head and neck cancer, is related to poor survival rates despite considerable advances in diagnosis and treatment. Therefore, detecting tumour cell dissemination early and understanding the underlying mechanisms are crucial for predicting prognosis, relapse and survival. According to previous findings, circulating tumour cells (CTCs) and disseminated tumour cells (DTCs) might serve as prognostic markers to supplement routine staging and support determining individual therapeutic interventions. This review focuses on summarising the current knowledge about the detection of CTCs/DTCs with special emphasis on patients suffering from OSCC. The translational relevance of CTCs/DTCs and challenges for clinical application are highlighted.

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INTRODUCTION

Because of its biological heterogeneity and complex behaviour, head and neck cancer must be considered a conglomeration of several tumour types. The vast majority of patients are diagnosed with squamous cell carcinoma (SCC). With an estimated yearly incidence worldwide of half a million persons, nearly 50% of patients die due to tumour-related complications^[1,2]. The disease ranks age dependently among the leading types of solid cancer and is responsible for more than 65000 annual deaths in Europe^[3,4]. Early stage carcinomas are only observed in one third of the patients [Union for International Cancer Control stage (UICC) I - II] while two thirds present with advanced disease (UICC-Stage III-IV). More than 50% of patients suffering from head neck squamous cell carcinoma (HNSCC) sustain local relapses while only up to 25% develop distant metastases. However, the prognosis of these patients is still poor^[5-7].

Although the most important prognostic indicator for relapse is lymph node metastasis in the neck, the incidence of distant metastasis has increased dramatically^[8,9]. Despite considerable advances in diagnosis^[10] and therapeutic options^[11], the 5-year survival rate has remained stable at approximately 50% during the past decades among all tumour stages^[12,13]. This finding might also be due to an altered pattern of the clinical disease itself. While fewer patients currently present with a locoregional relapse, more patients develop a distant disease and recurrences due to occult cervical metastasis. These micrometastases are assumed to contribute to increased mortality and morbidity^[14,15]. Thus, improved methods in staging oral squamous cell carcinoma (OSCC) are needed to detect early metastatic spread and any residual tumour, hence improving decisions about individual therapeutic interventions. The establishment of a resilient model of genetic instability, such as a loss of heterozygosity at distinct chromosomes and the consecutive progression from premalignant lesions to invasive tumours in head and neck cancer, led to a better understanding of the disease^[2,16-19]. Due to the paradigm shift of considering solid cancers a systemic disease, the value and promise of circulating tumour cells (CTCs) in peripheral blood (PB) and disseminated tumour cells (DTCs) in bone marrow (BM) for cancer patients regarding diagnosis, prognosis, and response to therapy came into focus. While sampling BM from the easily accessible iliac crest is well established in diagnosing leukemic diseases, the full acceptance of this practice as an additional diagnostic tool for patients with solid tumours is still lacking. For carcinomas, including SCC, BM should operate as a so-called “homing organ” for CTCs becoming DTCs in the nomenclature. The clinical relevance of CTC/DTC detection in carcinoma patients has been part of extensive research, and encouraging results exist for an association between CTC detection and the prognosis in patients with other solid tumours, such as metastatic breast, prostate, and gastrointestinal cancers^[20-28]. For HNSCC, a putative prognostic value was identified as well, revealing a positive correla-

tion between CTCs/DTCs to relapse and metastasis^[29-32]. In this context, it is important to note that the clinical behaviour and outcome of OSCC differ from those of the oropharynx and other HNSCC. Although there is no sharp distinction between OSCC and HNSCC in general, not all aspects and findings are transferrable. Even the main therapeutic options vary between the OSCC and other HNSCC. For OSCC, surgical removal is the primary treatment, whereas there is an ongoing debate about the preferred treatment options, such as primary radiation, (induction-) chemotherapy and supplemental targeted therapy, for laryngeal or nasopharyngeal SCC^[33,34]. However, recently published data suggest that DTCs and CTCs in patients with OSCC are independent prognostic markers of disease-free survival^[35]. Identifying clinical behaviour and understanding the inherent patterns of OSCC dissemination might result in meaningful contributions to the treatment of those patients. The application of new technologies that make the detection of CTCs/DTCs feasible might allow for more accurate staging and could help select patients and provide targeted therapies. To identify patients in need of systemic therapy in addition to local resection and irradiation, it is important to identify patients suffering from non-localised but “circulating” disease to expand the indication for systemic therapy. With new prognostic markers in prospect, the therapeutic options could substantially improve^[36-38].

DETECTION AND CHARACTERISATION OF CTCs

Several techniques have been established to detect the rare CTCs/DTCs. To put the rarity of these cells in perspective, by applying currently available methods, it is estimated that there might be only one tumour cell among millions of other cells, even in metastatic disease. To increase the efficiency of detecting these cells, an initial enrichment step is needed. Basic enrichment approaches rely on either physical properties, such as size, density or dielectrophoretic mobility that might be characteristic at least for subgroups of CTCs/DTCs, or on the expression of specific surface molecules captured by magnetic bead-coated antibodies. Subsequently, one has to decide between cytometric/immunological approaches on one hand and molecular [mostly real-time reverse transcription-polymerase chain reaction (RT-PCR)-based] techniques on the other hand to detect the CTCs/DTCs among the simultaneously enriched non-tumoural cells^[37].

A widely used method is gradient centrifugation using Ficoll-Hypaque, which utilises the density of different cell types. However, during enrichment, a substantial loss of cell-material is described^[39]. The advantages of using immunocytochemical detection of enriched CTCs/DTCs include the possibility to characterise these cells by size and shape as well as by the nucleus-plasma relation. A set of monoclonal antibodies against various epithelial proteins is available, including various keratins of the cytoskeleton, surface adhesion molecules or growth fac-

tor receptors. The pan-keratin-antibody A45-B/B3 is still widely used for detecting DTCs *via* an alkaline phosphatase anti-alkaline phosphatase technique with New Fuchsin as a substrate^[35,40]. Automated screening for potential CTCs/DTCs and visualisation can be performed with the ACISTM-system (Chromavision, San Juan Capistrano, CA, United States)^[41,42].

Thus far, one of the most advanced methods for capturing and enumerating CTCs from PB is represented by the CellSearchTM-system (Veridex, Raritan, NJ, United States), which provides automated enrichment and immunostaining of CTCs. It is the first food and drug administration-cleared device in CTC detection for solid cancers and has been approved for metastasised prostate, breast and colon cancers^[23,43-46]. Gröbe *et al.*^[35] succeeded in detecting CTCs in a small subset of OSCC patients as well. The underlying principle uses immunomagnetic bead separation of EpCAM-positive tumour cells followed by immunofluorescent staining with anti-keratin antibodies and the exclusion of leukocytes using an anti-CD45-antibody. The CellSearch system benefits from selecting for epithelial features, such as the epithelial cell adhesion molecule (EpCAM) exposed at the surface of the vast majority of epithelial but not on normal blood cells.

A semi-automated process *via* fluorescence microscopy scans visualises the results. Subsequently, nucleated cells, with a diameter of at least 4 µm and characterised by keratin-positivity and CD45-negativity, are accepted and designated as epithelial cells as surrogates for tumour cells^[20,44]. Other promising EpCAM-based tools were presented recently, including the microfluidic-based “CTC”-chips consisting of arrays of anti-EpCAM antibody-coated microposts able to capture EpCAM-positive cells under controlled laminar flow of whole blood^[47,48]. However, these techniques still lack tumour cell specificity and an application for head and neck tumours. Moreover, widespread application must be proven in large clinical trials.

Furthermore, other techniques were used for the detection of CTCs in patients with HNSCC. The Surfaced enhanced Raman Spectroscopy with epidermal growth factor receptor (EGFR) as a targeting ligand has been proven for the detection of putative CTCs in 19 patients by Wang *et al.*^[49]. The potential use of EGFR expression and activation in finding CTCs during the course of combined chemo- or bioradiotherapy regimens, accentuating the aspect of monitoring the therapeutic response in HNSCC, was described by Tinhofer *et al.*^[50] using flow cytometry^[49-51]. Alternative approaches should be mentioned, however, these approaches still lack proof for their utilisation in OSCC. For example, the detection of CTCs from large blood volumes after leukapheresis is promising and paves the way for the analysis of higher numbers of CTCs either by fluorescence-activated cell sorting, immunocytochemistry or molecular approaches^[52,53]. Moreover, real time monitoring of CTCs is desirable, such as during the course of therapeutic interventions. In this context, CTC detection *in vivo* is provided by

the GILUPI cell collector device using a wire coated with anti-EpCAM antibodies ready to accumulate CTCs after insertion into a vein for 30 min. The detection of CTCs and the exclusion of leukocytes can be confirmed by immunostaining with anti-keratin and anti-CD45-antibodies, respectively^[54]. To prove reliability and applicability for different tumour types and clinical relevance, several clinical trials are ongoing^[37].

The detection of viable CTCs/DTCs after secretion of specific proteins during a 48 h short term culture is possible using epithelial immunospots (EPISPOT)^[55]. For the EPISPOT approach, leukocytes must be depleted by negative selection of haematopoietic cells, such as using the common leukocyte antigen CD45.

To conclude, of the various introduced techniques, consideration should be given to molecular technologies. These technologies involve either DNA or complementary DNA (mRNA)-based PCR-amplification. These methods rely on gene expression patterns or the detection of known gene mutations, amplifications, other genomic aberrations or methylation patterns in tumour cells with the restriction that currently no universally applicable marker exists due to strong inter- and intra-tumoural heterogeneity. Nevertheless, using epithelium-specific targets, such as keratin 19 encoding transcripts, RT-PCR approaches are promising^[56,57].

Wicha *et al.*^[58] have recently suggested, for breast and other carcinomas, that not all CTCs in these patients can be found with the approaches that are currently available and that not all detected CTCs are aggressive and have the potential to initiate metastases or recurrences, and these suggestions might also be true for OSCC. Therefore, strong efforts must be made to detect CTCs that have lost their epithelium-specific features and instead favour mesenchymal characteristics as the cells may be undergoing epithelial-mesenchymal transition (EMT). Investigations applying ultrasensitive imaging procedures, gene expression analyses and next generation sequencing approaches are ongoing and have already yielded interesting data^[38,59-61].

A DISCUSSION OF THE RESULTS FROM STUDIES ON HEAD AND NECK CANCER

In comparison to other cancer types, the clinical impact of CTCs in OSCC and other HNSCC patients (Table 1) is still unclear. Therefore, investigating a correlation between these cells and clinico-pathological features is crucial. The research on the outcome of patients suffering from OSCC is impaired by comorbidities, such as chronic obstructive pulmonary disease, malnutrition, loss of function due to surgery and therapy, which can influence decisive parameters. Moreover, a sharp distinction between OSCC and HNSCC is hindered by similar but different causative pathogens, such as the contribution of human papillomaviruses (HPV) in the carcinogenesis of a subset of HNSCC, which is found less often in OSCC^[62]. The incidence of the distinctly different disease

Table 1 Detection of circulating and disseminated tumour cells in oral squamous cell carcinoma and head neck squamous cell cancer

Source	Entity	n	Assay system	CTC-positive (%)	DTC-positive (%)	Clinical relevance	Number of analysed OSCC	Ref. yr of release
PB	OSCC	20	RT-PCR: CK19 mRNA	20.0		Detection of CK19-mRNA when tumour is incised	OSCC only	[85], 2000
PB	HNSCC	77	RT-PCR: CK19 mRNA	2.1		No significance for CTCs	Various sites n = 13 OSCC n = 23 oropharyngeal	[79], 2001
PB/ CVB BM	HNSCC	40	ICC: Keratins CK1-8, 10, 14-16 and 19 RT-PCR: E48 mRNA	10.0 (PCR) 20.0 (ICC)	20.0 (PCR) 32.0 (ICC)	E48 mRNA and DMFS/DFS preoperative in BM ($P = 0.001$ / $P = 0.002$) and CVB ($P < 0.001$) E48-mRNA and DMFS/DFS intraoperative in CVB ($P = 0.026$ / $P = 0.001$) ICC and DMFS preoperative in BM ($P = 0.0006$) and CVB ($P < 0.001$) ICC and DFS preoperative in BM ($P = 0.036$) and CVB ($P = 0.042$) DTC on DFS ($P = 0.1460$) DMFS ($P = 0.2912$) N > or = 2 DMFS ($P = 0.0210$) OS and N-involvement ($P = 0.0001$), OS and DTC (0.0066)	OSCC only	[81], 2003
BM	HNSCC	162	RT-PCR: E48 mRNA		40.0		Various sites n = 13 OSCC n = 23 oropharyngeal	[80], 2004
BM	HNSCC	176	ICC: CK19		30.7		Various sites n = 37 OSCC n = 43 oropharyngeal	[29], 2004
PB	HNSCC	21	Immunomagnetic separation: EpCAM	33.3		Occurrence of CTCs more frequent in stage III-IV than in stage I - II ; connection with recurrence presumed; NS	Various sites n = 7 OSCC / oropharyngeal	[83], 2007
PB	OSCC	25	RT-PCR: CK19 mRNA	16		Detection of CK19-mRNA when tumour is incised	OSCC only	[108], 2008
PB	HNSCC	48	Immunomagnetic separation: ICC: CK3-6H5	71		DFS and NO CTC ($P = 0.01$) clinical outcome and CTCs ($P = 0.04$)	Various sites n = 25 OSCC n = 10 oropharyngeal	[30], 2010
PB	HNSCC	42	Flow cytometry: EpCAM, CD45 RT-PCR: EGFR mRNA	43		CTC and N in flow cytometry ($P = 0.014$), CTC and N in RT-PCR	Various sites n = 9 OSCC n = 17 oropharyngeal	[31], 2011
PB	HNSCC	15	CellSearch™	40		CTC and M ($P = 0.04$)	Various sites n = 4 OSCC n = 7 oropharyngeal	[84], 2012
PB	HNSCC	31	Flow cytometry: EpCAM; EGFR, CD45	29 pEGFR in 55% of CTC ⁺ -cases		Increasing number of CTC-positive cases at end of treatment	Various sites n = 5 OSCC n = 18 oropharyngeal	[50], 2012
PB	Under RCT HNSCC	33	Flow cytometry: EpCAM CD45 keratins 7, 8 EGFR	33 pEGFR in 36.4% of CTC ⁺ -cases		None given for comparing two methods	Various sites n = 0 OSCC (not specified) n = 13 oropharyngeal	[51], 2012
PB	HNSCC	73	CellSearch™	15.1		Decreasing CTC counts and clinical response ($P = 0.017$)	Various sites n = 3 OSCC n = 39 oropharyngeal	[82], 2012
BM	HNSCC	105	ICC: keratins 8, 18 and 19		14.5	No significance for DTCs (76/105 tested)	Various sites n = 72 OSCC n = 8 oropharyngeal	[68], 2012
PB BM	OSCC	110	CellSearch™ ICC: keratins 8, 18 and 19	12.5 (Cell search)	20.0 (ICC)	CTCs and T ($P = 0.04$), N and DTCs ($P = 0.02$), M and CTCs ($P = 0.004$), M and DTCs ($P = 0.005$), DTCs and RFS ($P < 0.001$)	OSCC only	[35], 2014

BM: Bone marrow aspirates; CD: Cluster of differentiation; CK: Cytokeratin; CTC: Circulating tumour cell; CVB: Central venous blood; DFS: Disease-free survival; DMFS: Distant metastasis free survival; EGFR: Epidermal growth factor receptor; pEGFR: Phospho-EGFR; HNSCC: Head neck squamous cell cancer; EpCAM: Epithelial cell adhesion molecule; M: Metastatic; NS: Nodal status; OS: Overall survival; OSCC: Oral squamous cell cancer; PB: Peripheral blood; RT-PCR: Reverse transcriptase-polymerase chain reaction; RFS: Relapse-free survival; T: Tumour stage; ICC: Immunocytochemistry; RCT: Randomized control trial; DTC: Disseminated tumour cells.

of HPV-related head and neck cancers from other head and neck carcinomas is increasing^[63]. This finding will influence further studies regarding etiology and outcome and therefore must be included when evaluating these studies, especially those of patients undergoing different therapy-regimens.

With reference to the concept of field cancerisation (FC) introduced by Slaughter *et al.*^[64] in the 1950's, related problems in OSCC should also be considered because locoregional failure can lead to distant metastasis in HNSCC^[65]. FC is thought to be undetectable by routine procedures thereby potentially causing a second primary tumour^[66]. Furthermore, the entire mucous membrane of the respiratory and upper digestive tract is at risk for neoplasia^[67]. Graveland *et al.*^[68] investigated the potential association of minimal residual cancer in deep surgical margins and DTCs in BM aspirates with clinico-histopathological parameters and outcomes and concluded that the presence of vaso-invasive and

infiltrative growth in HNSCC is a significant risk factor for locoregional recurrence and disease-free survival. Furthermore, they concluded that, at present, there seemed to be no role for the molecular analysis of deep surgical margins and BM aspirates in predicting outcomes with the methods used for 105 HNSCC patients enrolled in this study. To improve histopathological staging in previously tumour cell-negative margins, the overexpression of p53 frequently found in primary tumours was revealed to be indicative of an increased risk of local recurrence^[69,70]. Clinically relevant tumour cell spread is rarely found beyond the level of histopathological detection. Thus, a molecular assessment of minimal residual disease is needed to fill the gap between histopathological findings and outcomes in HNSCC, including OSCC^[71]. Several serum tumour markers have been evaluated with regard to clinical value in HNSCC and OSCC, but most of them demonstrated poor sensitivity^[72,73]. This article focuses on the potential haematogenous spread of tumour cells while OSCC most often spreads in lymphatic vessels^[74-76], potentially decreasing the relevance regarding OSCC/HNSCC. However, there are increasing signs that CTCs are clinically relevant in the pathogenesis and recurrence of head and neck tumours. The total number of suspicious cells most likely depends on the method applied. OSCC is characterised by specific expression patterns for different keratins, capable of being detected in CTCs/DTCs^[77], leading to technical limitations that must be considered. The detection methods have been optimised for CTCs or DTCs derived from adenocarcinomas such as breast, prostate, colorectal or hepatocellular carcinomas, as reported by Schulze *et al.*^[78] in the latter case. Therefore, an adaption to the unique biology of OSCC may be required.

Nevertheless, one of the first reports of prognostic value in a homogenous group of OSCC has been recently published by Gröbe *et al.*^[35], which describes a strong and independent prognostic impact of CTCs and DTCs for disease-free survival, which outperformed current prognosticators. This finding might provide evidence that patients who are positive for tumour cells in the BM or PB may suffer from a more aggressive disease. Thus, CTCs/DTCs detected in OSCC patients might serve as prognostic markers, predicting relapse at various disease stages, supplementing the current routine staging procedures. In this study, 110 patients were enrolled. Anti-keratin immunostaining after Ficoll density gradient centrifugation to detect DTCs and applying the CellSearch™-System to analyse PB represent techniques with the current highest degree of international standardisation^[42,44]. Nineteen patients (21%) exhibited DTCs in the BM, while 11 patients (13.6%) were positive for CTCs in the PB. According to other literature data, CTCs/DTCs are traceable in the PB of HNSCC patients^[29,31]. Wollenberg *et al.*^[29], for example, detected DTCs from BM aspirates, depending on tumour stage.

Gröbe *et al.*^[35] found no significant correlation between the detection of CTCs and the presence of DTCs ($P = 0.68$), suggesting that both analyses might provide

supplementary results. Distant haematogenous metastases were less frequently observed compared to locoregional relapses. Consistent with other studies examining more heterogeneous groups of HNSCC patients^[29,79,80], the subsequent development of recurrent disease was observed more frequently when patients were positive for CTCs/DTCs^[35,81]. However, there are several incongruous and moderately significant results demonstrating that the higher the disease stage, the higher the yield of detected CTCs. Although statistically not significant, a trend regarding this conclusion was shown by Buglione *et al.*^[82] last year, comparing T1 to higher tumour stages in HNSCC, but in the OSCC subgroup (13/152 patients), no CTCs were found. Previously, Guney *et al.*^[83] found similar results for HNSCC but in a smaller cohort of patients. A correlation to tumour stage was found, though not significant for DTCs, and the total number of CTCs was higher in T3-4 tumours compared to T1-2 tumours^[29]. These findings suggest that the size of the primary tumour is not always prognostically relevant. This hypothesis is strengthened by significantly higher incidences of CTC positivity in patients with locally advanced disease when considering the N-stage, holding true even in a multivariate analysis^[31]. Moreover, CTC detection was significantly associated with the presence of lung nodules^[84]. The tumour mass limits local resection by potentially decreasing safety margins because of neighbouring structures, such as the sinus at the skull base, which reaffirms the problem of an assumed CF. Nevertheless, a longer disease-free survival of patients without detectable CTCs compared to CTC-positive patients was stated by Jatana *et al.*^[32]. Unfortunately, most of the studies cited were performed on small patient cohorts for other groups of HNSCC^[83,84]. In a distinctly larger group of 176 HNSCC patients, the presence of DTCs in BM showed prognostic relevance for overall survival. In half of the DTC-positive patients, the disease recurred, whereas in the total cohort, recurrences were only observed in 27% of the patients^[29].

The importance of CTC detection in the context of therapy is still controversially discussed. For OSCC patients, there are studies investigating the dissemination of tumour cells after surgery and tumour cell spread due to surgical procedures^[32,85], but among these studies, only a few have investigated the impact of metastases and survival regarding this issue. These facts are crucial in OSCC as surgery is the current gold standard and primary treatment option.

Before, during and following radiotherapy the detection of keratin-19-positive cells coincided with local failure, distant metastasis and moreover, the diagnosis of anaemia^[79]. Regarding the therapeutic response in HNSCC with decreased levels of previously detected CTCs, a positive effect on outcome was presumed by Buglione *et al.*^[82] because partial or complete response could be stated along with these findings.

A fact that might impair the analysis of detected CTCs/DTCs is the subjective evaluation by even experienced independent readers of common immunocytochemical methods, such as the use of the anti-keratin

antibody A45-B/B3. However, false-positive results are seldom found^[86]. The criteria to evaluate the morphology of potential CTCs and immunostaining reactions are crucial in defining comparable results^[39,42]. This knowledge is even important to evaluate results obtained for blood samples analysed with the semi-automated, highly standardised CellSearch system. To overcome these problems, different international ring studies have begun to reduce interobserver variability of the results^[44,87]. The successful CellSearch system has proven its usefulness in OSCC/HNSCC, although not in all advanced stage patients. However, even in metastasised, advanced disease, CTCs were not detectable in all cases^[35]. Nichols *et al.*^[84] detected almost 100% of spiked HNSCC cell line cells (FaDu) using the CellSearch system, however only 6 out of 15 HNSCC patients tested CTC-positive using this approach. The results are in accordance with those of other studies but suggest that, in patients, either the incidence or number of potentially detectable CTCs is very low or that not all present CTCs are detectable with the currently applied approaches. Furthermore, the system is ruling out non-EpCAM and/or non-keratin expressing CTCs. This escape from detection has been previously demonstrated for breast cancer where, in aggressive tumours, EpCAM expression might be down-regulated, and this finding has also been assumed for aggressive forms of solid cancers other than OSCC^[88].

Hence, serious consideration should be given to EMT, which is associated with a loss of epithelial features, enhancing the migratory capability due to altered plasticity^[88,89]. Stem cell-like cells seem to be most notably affected, as dynamic changes in these cells were identified by Mani *et al.*^[90] and Yu *et al.*^[91]. Thus, increasingly false-negative results might correlate to EMT^[92]. The detection of EpCAM-negative cells might be possible by using other surface markers, such as N-cadherin, EGFR or cancer stem cell markers, such as CD44^[89,93,94]. In colorectal carcinomas, *platin3* was identified as a potential target for cells undergoing EMT because its expression is not altered during this process^[95]. Superiority over EpCAM-based methods must be proven, and expression of *platin3* in cancers other than colorectal cancer must be analysed. EGFR, frequently overexpressed in HNSCC, is a meaningful target for CTC enrichment^[50,96]. Due to the biological heterogeneity of CTCs/DTCs, the most aggressive subsets initiating metastasis must still be categorised^[97,98]. In OSCC, Nagata *et al.*^[99] performed molecular analyses, identifying integrins as potential biomarkers for the risk of locoregional and haematogenous dissemination. The role of the expression rates of integrin α -3 for locoregional and ITGB4 for haematogenous dissemination and, therefore, cancer cell motility and anchorage-independent survival, which are vital for OSCC recurrence and metastasis, has previously been shown. Furthermore, the importance of integrin β 1 in HNSCC and stimulated vascular endothelial growth factor secretion by α B-crystallin in HNSCC was specified^[100,101].

In addition to these considerations, there might be a spread of tumour-derived molecules on a subcellular lev-

el, such as micro-RNAs. Their role in the formation of metastases derived from OSCC is currently unclear^[102,103].

According to current hypotheses, most DTCs remain in a dormant state and probably never initiate a relapse or metastasis^[56,104], whereas others return to a proliferating state by still unknown molecular alterations or changes in environmental conditions^[105,106]. Interestingly, also a re-circulation from the dormant site back to the primary location of the cancer has been reported in an mouse breast cancer model^[107], but because of the high rate of locoregional failure, the concept does not seem apparent for OSCC in the reviewers opinion due to the concept of FC^[67]. Therefore, more obvious challenges must be faced when considering of this phenomenon. The spread of tumour cells during surgery remains unclear but has been proven for fine needle aspiration or incision biopsy in OSCC^[85,108]. A total of 71% of 48 patients harboured CTCs detected by Jatana *et al.*^[30] in HNSCC. Here, samples were taken at the time of surgery. Circulating epithelial cells also can be found in non-malignant diseases, such as inflammatory diseases of the colon, with the current assays^[109].

Nevertheless, the appealing potential of detecting CTCs/DTCs as prognostic markers in OSCC from non-invasively accessible blood samples must be emphasised. When occult tumour cells are ubiquitously spread, PB may be a way to detect these cells, facilitating a “liquid biopsy”^[37]. Out of a set of several markers, a decision must be made, and the occurrence of CTCs is a very rare event. Researchers are faced with the problem that often processed samples cannot be used a second time for other methods, and the samples display only a cross section of the entire circulating blood. A reliable, morphological identification, for example, with fixation of the cells and nuclear staining might prevent subsequent molecular characterisation. These analyses provide just a snapshot of tumour cell dissemination in contrast to BM, which may represent a reservoir where DTCs can be collected over a longer period of time^[104]. Many CTCs are assumed to undergo apoptosis quickly^[110], as the lifespan of CTCs in the circulation is estimated to be only between one and two hours^[111]. Alternative approaches, such as the “CTC-Chip”, punctuate an improvement in CTC yield and purity but only give a possible outlook due to the small number of examined cohorts and have not been tested for OSCC yet^[47,112].

To identify potential targets for individualised therapies and to use repeated CTC assessments in individual patients for treatment surveillance is of the utmost importance^[37]. A potential marker in breast cancer is the human epidermal growth factor receptor 2 (HER2), the expression of which can also be monitored on CTCs, thus giving patients with HER2-negative primary tumours but HER2-positive CTCs a chance to be treated by a HER2-targeted therapy^[113,114]. Currently, there is no biomarker available for OSCC yet. BM might be an interesting target organ for therapeutic interventions by considering DTCs as therapeutic targets. The data described here point to the potential future utility of drugs targeting DTCs in the

BM of OSCC patients. The present high expenses will decrease when evidence is found that these methods are suitable for OSCC and can be included in standard protocols for tumour staging.

CONCLUSION

A set of promising techniques for the detection of CTCs/DTCs has been developed in the past decade, proving their usefulness in solid cancers with predominantly haematogenous dissemination. Less invasive diagnostic tools might be appropriate to refine the current staging methods. The non-invasiveness of collecting samples from PB and obtaining BM specimens might provide additional treatment options in patients suffering from OSCC. For OSCC, the current data show that CTCs/DTCs might have a clinical impact on the prognosis and survival, even outperforming current prognosticators. Circulating tumor cells and DTCs must be considered independent markers in OSCC as a subgroup of head and neck tumours, but the ongoing debate remains too controversial to be considered evidence. The findings presented in this review are not convincing for oral cancer in all aspects yet. Nevertheless, the potential is striking, guiding therapy towards individual-centred medicine with the possibility of early intervention in this severe disease. Despite encouraging results, further investigation of the clinical relevance of CTCs and DTCs is needed as validation in large multicentre trials with synchronised measuring methods and defined outcomes and time points is lacking. In particular, the risk of an early locoregional relapse is difficult to assess because of different problems in the field of HNSCC and OSCC. It is important to continue investigations in homogenous cohorts to decrease the still unaltered morbidity in patients suffering from OSCC. Due to being one of the most active areas of translational cancer research, the technologies presented herein offer a number of compelling advantages. The enumeration of CTCs and DTCs as biomarkers to predict tumour relapse may become feasible in the near future, but researchers in this extraordinarily dynamic field are still facing technical and clinical hurdles. In addition to further improvements, there are challenges beyond the mere detection of CTCs/DTCs. In particular, the detection of carcinoma cells undergoing EMT, a down-regulation of epithelial features when entering vessels, the transient circulation of cells, the mechanisms behind leaving the “homing organs” and the states of dormancy need to be investigated. Whether EpCAM- or keratin-positive cells detected by the described techniques are involved in initiating metastasis is also controversially discussed. To address this question further, molecular analysis of CTCs/DTCs is needed.

A profound understanding of OSCC as a systemic disease supported by molecular investigation of CTCs/DTCs gives new perspectives on the disease and provides the chance to identify potential targets for individualised therapies. Repeated CTC assessments might offer treatment surveillance in individual patients prospectively and

might predict local and systemic relapse with a higher sensitivity at various disease stages compared to routine staging procedures in OSCC.

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Systemic treatment strategies for triple-negative breast cancer

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Abstract

Triple-negative breast cancer (TNBC) is defined by the lack of immunohistochemical expression of the estrogen and progesterone receptors and human epidermal growth factor receptor 2 (EGFR2). Most TNBC has a basal-like molecular phenotype by gene expression profiling and shares clinical and pathological features with hereditary *BRCA1* related breast cancers. This review evaluates the activity of available chemotherapy and targeted agents in TNBC. A systematic review of PubMed and conference databases was carried out to identify randomised clinical trials reporting outcomes in women with TNBC treated with chemotherapy and targeted agents. Our review identified TNBC studies of chemotherapy and targeted agents with different mechanisms of action, including induction of synthetic lethality and inhibition of angiogenesis, growth and survival pathways. TNBC is sensitive to taxanes and anthracyclins. Platinum agents are effective in TNBC patients with *BRCA1* mutation, either alone or in combination with poly adenosine diphosphate polymerase 1 inhibitors. Combinations of ixabepilone and capecitabine have added to progression-free survival (PFS) without survival benefit in metastatic TNBC. Antiangiogenic agents, tyrosine kinase inhibitors and EGFR inhibitors

in combination with chemotherapy produced only modest gains in PFS and had little impact on survival. TNBC subgroups respond differentially to specific targeted agents. In future, the treatment needs to be tailored for a specific patient, depending on the molecular characteristics of their malignancy. TNBC being a chemosensitive entity, combination with targeted agents have not produced substantial improvements in outcomes. Appropriate patient selection with rationale combinations of targeted agents is needed for success.

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Key words: Breast cancer; Triple negative; Basal like; *BRCA1*; Poly (ADP-ribose) polymerase 1; Targeted therapy; Chemotherapy

Core tip: Breast cancer is a heterogeneous disease entity with different biological characteristics and clinical behavior. There are no treatment guidelines for triple-negative breast cancer (TNBC). TNBCs are sensitive to taxanes and anthracyclins but there are high rates of local and systemic relapses. Recently there has been great interest in platinum agents, either alone or in combination with poly adenosine diphosphate polymerase 1 inhibitors. Combinations of ixabepilone and capecitabine have shown improved response rates (RRs). Other useful drugs are antiangiogenic agents, tyrosine kinase and epidermal growth factor receptor inhibitors with variable RRs but no survival benefit. In this review, we discuss various systemic treatment strategies available for TNBC and the benefit from each of them.

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INTRODUCTION

Breast cancer is the second most common cancer in the world and the most common cancer among women. However, in the past three decades, the mortality rate has declined as a result of a range of measures, including implementation of screening, improvements in the local management of early breast cancer and most importantly, the introduction of adjuvant systemic treatment and the development of directed therapies for hormone receptor-positive and human epidermal growth factor receptor-2 (HER-2/neu)-positive tumors^[1].

Breast cancer is a complex disease entity with different biological characteristics and clinical behavior. Many clinical and pathological features have been defined to predict treatment response and outcome in breast cancer. Classically these include: age, tumor size, axillary node involvement, angio-lymphatic invasion, histological grade, hormonal receptor status (estrogen and progesterone) and HER-2/neu expression. If the last three features are not expressed in breast cancer cells it is called triple-negative breast cancer (TNBC)^[2]. Chemotherapy is the only systemic therapy for TNBC patients.

Management of TNBC is challenging because of a lack of targeted therapy, aggressive behavior and relatively poor prognosis. There are no specific treatment guidelines for TNBCs and they are managed with standard treatment. Treatment options are limited as most patients have been treated with adjuvant anthracyclins, taxanes and cyclophosphamide. It has been evidenced by various studies that these tumors are highly chemosensitive^[3-7] and in some cases are represented by complete pathological response (pCR), but the results remains unsatisfactory^[8-18]. pCR to the neoadjuvant chemotherapy (NACT) is higher in the TNBC subset of patients but the disease free survival (DFS) and OS are still lower than non-TNBC patients^[3,5]. Sporadic TNBCs show heterogeneity in response to chemotherapy, with pCR rates ranging from 12% for single-agent to 27%-65% in multi-agent NACT trials^[3,5,11,19]. Since the achievement of pCR with primary chemotherapy is of crucial importance in TNBC patients, a maximal effort should be made in selecting the best possible drugs, doses and administration timing. The following are the therapeutic options available in TNBC.

CYTOTOXIC AGENTS

Anthracyclins

Anthracyclins are considered to be among the most active drugs for the treatment of breast cancer. These agents that act by destabilising the DNA through intercalation also prove useful in TNBC due to a degraded DNA repair cascade. Many studies show that TNBC is sensitive to anthracyclin containing regimens^[3-5]. The impact of NACT in patients with TNBC was clearly analyzed by Liedtke *et al*^[3] and in a retrospective analysis they reported a pCR rate of 22% in TNBCs compared to 11% in non-TNBCs with paclitaxel/5-FU, doxorubicin,

Table 1 Pathological complete response to triple-negative breast cancer in triple-negative breast cancer and non-triple-negative breast cancer patients

Ref.	Yr	No.	Therapy	PCR (%)	
				TNBC	Non-TNBC
Liedtke <i>et al</i> ^[3]	2008	255	FAC → P	22	11
Chappuis <i>et al</i> ^[4]	2002	9	FEC 3 wk × 3-4	44	4
Skrypnikova <i>et al</i> ^[10]	2011	15	ACC	29.4	NR
Rouzier <i>et al</i> ^[11]	2005	82	P → FAC	45	6

FAC: 5-Fluorouracil, doxorubicin, cyclophosphamide; ACC: Doxorubicin, cyclophosphamide, capecitabine; P: Paclitaxel; PCR: Pathological complete response; TNBC: Triple-negative breast cancer; FEC: 5-Fluorouracil, epirubicin, cyclophosphamide.

cyclophosphamide/5-Fluorouracil, epirubicin, cyclophosphamide (FEC) (Table 1). The 3-year DFS was similar in both the groups (pCR 94% *vs* 98%, $P = 0.24$), while those who failed to achieve pCR had worse 3-year DFS compared to non-TNBCs (68% *vs* 88%, $P = 0.0001$). This was because the rate of early relapse in patients with residual tumor was dramatically higher in TN patients compared with the others.

In a study by Chappuis *et al*^[4] of TNBC patients treated with FEC regimen, pCR was 44%. Carey *et al*^[5] showed that the clinical response to doxorubicin and cyclophosphamide was markedly higher among patients with TNBCs than non-TNBCs. pCR to NACT was higher in patients with TNBCs but still these patients had a worse DFS and OS compared to non-TNBCs. An intergroup study (C9741) found differences in favor of dose density with adriamycin and paclitaxel in patients with negative ERs, but not in ER-positive patients (32% *vs* 19%). This study highlights the importance of chemotherapy in hormone-independent tumors^[6]. Review of TNBC subgroups in the CALGB 9344 study in node positive patients where they compared the addition of paclitaxel to different anthracyclin doses showed significant benefits for this combination. Although the benefits were independent of HER2 status, ER negative patients derived the greatest benefit in both DFS and OS^[7]. In a large cohort of patients with TNBC treated with anthracyclins and taxanes, Hernandez-Aya *et al*^[9] concluded that, independently of the size of the tumor, once there is evidence of lymph node involvement, the prognosis may not be affected by the number of positive lymph nodes.

Recently, Skrypnikova *et al*^[10] in a prospective pilot trial evaluated the efficacy of a metronomic schedule of doxorubicin, cyclophosphamide and capecitabine in locally advanced and metastatic TNBC. The overall response rate (RR) was 58% with 24% of CR and 34% of PR. Five patients (29.4%) achieved a pCR. In MBC patients, the median progression-free survival (PFS) was 8.3 mo. The most common grade 3 toxicities were hand-foot syndrome (HFS) (28.8%) and mucositis (17.7%), which resulted in discontinuation of doxorubicin in 7 patients. There was 26.4% of grade 3-4 neutropenia. Although the RR was good, this combination is quite toxic.

Table 2 Chemotherapy regimen with their outcomes in triple-negative breast cancer

Ref.	Yr	No.	Therapy	Outcome
Martin <i>et al</i> ^[12]	2010	171	FEC	7 yr DFS 56% <i>vs</i> 74%
			FEC + P	
Byrski <i>et al</i> ^[23]	2009	25	Cisplatin + P + GSF	5 yr DFS-76% DDFS-84%
Koshy <i>et al</i> ^[32]	2010	17	Cisplatin + gemcitabine	PFS-5.3 mo TNBC <i>vs</i> 1.7 mo non-TNBC
Maisano <i>et al</i> ^[31]	2011	31	Carboplatin + gemcitabine	ORR-32% PFS-5.5 mo

Anthracyclin and taxane pretreated TNBC patients. GSF: Granulocyte stimulating factor; ORR: Overall response rate; PFS: Progression free survival; DFS: Disease free survival; DDFS: Distant disease free survival; TNBC: Triple-negative breast cancer; P: Paclitaxel; FEC: 5-Fluorouracil, epirubicin, cyclophosphamide.

Taxanes

Taxanes produce benefits in TNBC by targeting genomic instability. Many studies reveal the benefits produced by paclitaxel when added to other chemotherapeutic agents. In the neoadjuvant setting, Rouzier *et al*^[11] showed that TN and Her2-positive subtypes of breast cancer are more sensitive to paclitaxel and doxorubicin chemotherapy than the luminal and normal-like cancers. pCR was seen in 45% patients with basal like breast cancer (BBC) compared to 6% in luminal subtypes. In a retrospective analysis by Hayes *et al*^[8] where paclitaxel was added to cyclophosphamide and doxorubicin in node positive patients, they observed a 5 years DFS and OS of 27% and 32% respectively. The results were similar in TNBC and HER-2/neu positive patients.

In an adjuvant setting, two meta-analyses have shown benefit with taxanes^[12,13]. Many studies have demonstrated that taxanes are more effective in receptor-negative patients. Jones *et al*^[14] found that docetaxel and cyclophosphamide were equally effective in TNBC and non-TNBC patients. In a study by Jacquemier *et al*^[15], there was greater benefit with the addition of docetaxel to the conventional 6 cycles of FEC in BBC patients. A further study (Table 2) showed maximum benefit in TNBC patients when 4 cycles of FEC were followed by weekly paclitaxel for 8 wk compared to just 6 cycles of FEC^[16]. Loesch *et al*^[17] with the same kind of combination, paclitaxel 3 times weekly *vs* weekly after 4 courses of adriamycin-paclitaxel every 3 wk, showed statistically significant results in 378 TNBC patients treated with weekly paclitaxel. As far as a schedule is concerned, weekly paclitaxel is much more effective than paclitaxel every 3 wk and at least as effective as docetaxel every 3 wk^[18]. On the whole, shortening the administration interval from 3 to 2 wk could substantially improve efficacy, at least in TN patients. The role of anthracyclins alone in TNBC is debatable; however, a definite benefit is seen when used in combination with taxanes.

Platinum agents

It has been postulated that TNBC has phenotypic and

Table 3 Response to neoadjuvant platinum based chemotherapy trials in triple-negative breast cancer

Ref.	Yr	No.	Regimen	PCR
Garber <i>et al</i> ^[20]	2006	28	Cisplatin	21%
Silver <i>et al</i> ^[21]	2010	28	Cisplatin 3 wk × 4	22%
Byrski <i>et al</i> ^[22]		12	Cisplatin	83%
² Byrski <i>et al</i> ^[23]	2009	25	Cisplatin + paclitaxel + GSF	72%
Ryan <i>et al</i> ^[24]	2009	51	Cisplatin + bevacizumab 3 wk × 4	72%
Frasci <i>et al</i> ^[25]	2009	74	Cisplatin + epirubicin + paclitaxel wk × 8	65%
Sirohi <i>et al</i> ^[26]	2008	62	Platinum ¹ + epirubicin + 5-FU (infusion)	88% (cCR)
Sikov <i>et al</i> ^[27]	2007	10	Carboplatin 3 wk × 4 + paclitaxel wk × 16	50%
Leone <i>et al</i> ^[28]	2009	125	Platinum ¹ + docetaxel 3 wk × 4	29%
			Platinum + docetaxel → AC 3 wk × 4	40%

¹Cisplatin or carboplatin; ²In *BRCA1* mutation carriers. AC: Doxorubicin + cyclophosphamide; 5-FU: 5-Fluorouracil; PCR: Pathological complete response; cCR: Clinical complete response; GSF: Granulocyte stimulating factor.

molecular similarity to *BRCA1* related cancers that would confer sensitivity to cytotoxic agents like cisplatin. The platinum agents act by producing intra and inter strand cross links of double stranded DNA, prevent the replication fork formation and produce double strand breaks and replication lesions, and finally due to *BRCA1* mutation, the DNA repair cascade is non functional and produces cell death^[19]. In the last few years, there has been a renewed interest about the role of platinum compounds in the treatment of breast cancer patients. Clinical studies have also suggested that TNBC are more sensitive to DNA damaging agents like cisplatin. In a phase-II study, Garber *et al*^[20] have shown a pCR of 21% with neoadjuvant cisplatin in patients with TNBC. Among 28 patients, two were *BRCA1* carriers, both (100%) of whom achieved pCR; 4 (15%) of the 26 women with sporadic TNBC also achieved pCR to cisplatin. Overall, 50% of the patients had a good response to cisplatin. In a similar study by Silver *et al*^[21] with 4 cycles of single agent cisplatin, a pCR rate of 22% was seen (Table 3). Two patients with *BRCA1* mutation had pCR. They also found a significant association of tumor p53 protein-truncating mutations with cisplatin response. The largest series of *BRCA1* mutation was reported by Byrski *et al*^[22]; out of 6903 patients, 102 patients had *BRCA1* mutation. Out of this, 12 patients were treated with neoadjuvant cisplatin and 10 (83%) had pCR. Anthracyclins and taxane based regimens could only produce a pCR ranging from 7% to 22%. In another study which explored role of platins in TNBC with *BRCA1* mutation, out of 25 patients, 72% had a pCR. Projected 5 year DFS and DDFS were 76% and 84% respectively^[23]. Ryan and co-investigators found that when VEGF-A inhibitor bevacizumab was added along with cisplatin, a pCR rate of 16% was observed^[24]. The results with a single agent or in combination with

bevacizumab are somewhat disappointing as the proportions of CRs are significantly less (16%-22%) than that achieved with multiagent NACT (30%-65% in other studies).

Platin and taxane-based primary chemotherapy has also proven to be highly effective in patients with locally advanced breast cancer (LABC). In a study by Frasci *et al.*^[25] where neoadjuvant cisplatin was used with paclitaxel and epirubicin in a weekly schedule for 8 cycles in LABC patients, a pCR of 65% was achieved. After surgery, patients were treated with 4 or 8 cycles of CMF based on whether lymph nodes were positive at the time of surgery. Patients with pCR had a 5-year DFS of 90% compared to 56% with residual disease. Severe neutropenia and anemia occurred in 23 (31%) and 8 (10.8%) patients, respectively. Thus, lack of achievement of pCR in TNBC is a poor prognostic factor.

Sirohi *et al.*^[26] found that when platins are used in combination with epirubicin and 5-FU, a very high complete clinical response of 88% was achieved. This may be contributed to by epirubicin and 5-FU which was given as a 24 h infusion for 18 wk. Other investigators^[27,28] have observed a pCR from 29% to 50% with platins in combination with taxanes (Table 3). The results are encouraging and merit further validation and testing. At present, platinum agents in the neoadjuvant setting cannot be recommended over established regimens outside of a clinical trial. So, platins should always be used in combination with taxanes or anthracyclins to increase response and survival rates. However, patients with *BRC41* mutation tend to have maximum benefit in the neoadjuvant setting.

In the metastatic setting, cisplatin or carboplatin have shown an ORR of 20%-40%. Cisplatin is very active in first line chemotherapy in MBC with a RR of 50%, whereas carboplatin is moderately active with an ORR of 30%. In a study by Fountzilas *et al.*^[29], carboplatin in combination with paclitaxel demonstrated an ORR of 41% in MBC. PFS was better in the paclitaxel and carboplatin arm compared to paclitaxel and epirubicin. However, there was no difference in ORR and OS between the two arms. Gemcitabine (GC) and platinum agents in combination have synergistic antitumor activity that results in inter strand DNA crosslinks and double strand DNA breaks, both of which are preferentially repaired by homologous recombination. Both agents have demonstrated activity in MBC^[30], with RR ranging from 26% to 50%. Maisano *et al.*^[31], in a phase-II study with combination of carboplatin and GC in pretreated 31 metastatic TNBC patients, reported a RR of 32%. Median PFS was 5.5 months and median OS was of 11 mo. Many patients required dose reductions. Similarly, in a study by Koshy *et al.*^[32] in MBC patients, TNBC had a better PFS (5.3 *vs* 1.7 mo) compared to non-TNBC when treated with a cisplatin and GC combination (Table 2).

Lastly, in a retrospective study Staudacher *et al.*^[33] reported that median OS and median PFS were improved in patients responding to platinum based chemotherapy: 27 *vs* 8 mo ($P < 0.001$) and 10 *vs* 4 mo ($P < 0.001$), respectively. Therefore, combination of platins with taxanes or

GC or vinorelbine are good alternatives for patients in whom anthracyclins may pose as toxic or who are already exposed to these in the adjuvant setting. So, once again platins have generated interest among investigators for its role in TNBC. The heterogeneous outcome with platins may be related to the heterogeneity of TNBC. Cisplatin appears to be more effective than carboplatin.

Newer chemotherapeutic agents, antitubulin agents

Ixabepilone is a potent tubulin polymerizer that has recently been added to the armamentarium of drugs available for the treatment of breast cancer. Similarly to taxanes, ixabepilone stabilizes microtubules and causes cell cycle arrest and apoptosis. It is active in taxane refractory and LABC as well as in TNBC. The clinical activity and toxicity profile of ixabepilone are similar to the taxanes, with neuropathy and myelosuppression as dose-limiting toxicities^[34,35]. It has the advantage of bypassing the resistance mechanisms associated with drug efflux pumps and specific paclitaxel resistance associated with β -tubulin. In the neoadjuvant setting, a pCR rate of 26% in breast tumor and 19% when there was axilla involvement was seen in 42 patients with TNBC. A low expression of ER gene was identified as a predictor of response to ixabepilone^[34].

In patients with anthracyclin and taxane resistant metastatic TNBC, a combination of ixabepilone and capecitabine has an improved RR and PFS compared to capecitabine alone (RR 27% *vs* 9%; PFS 4.1 *vs* 2.1 mo)^[35]. Subsequently, in the pooled results of the 046 study (taxane resistant) and the 048 study (population pretreated with anthracyclins and taxanes), benefits were found for the ixabepilone-capecitabine combination in terms of objective responses (31% *vs* 15%) and PFS (4.2 *vs* 1.7 mo), but not for OS (10.3 *vs* 9.0 mo)^[36]. These outcomes are comparable to cisplatin combination regimens. So, the ixabepilone and capecitabine combination can be used in patients who do not tolerate cisplatin combinations or when renal function is compromised. The magnitude of benefit also appears comparable to other combinations, such as GC plus paclitaxel or capecitabine plus docetaxel. Another novel mitotic inhibitor currently being studied for the treatment of breast cancer is eribulin. Its activity in TNBC is yet to be seen.

TARGETED THERAPY

Currently, a lot of research is going on to further characterize TNBC with different molecular markers and find targets for therapy in order to improve its outcome.

PARP inhibitors

Poly (adenosine diphosphate ribose) polymerase also plays a vital role in DNA repair like *BRC4*. Unlike *BRC4*, it recognises single strand breaks and repairs by the base excision repair pathway. PARP inhibitors are effective in TNBC because damage to one strand of DNA cannot be repaired by homologous recombination due to *BRC4* mutation and PARP inhibition in synergism creating a state of "synthetic lethality". The inhibition of

Table 4 Clinical outcomes with targeted therapy in metastatic triple-negative breast cancer

Ref.	Line of treatment	Regimen	No.	ORR (%)	CBR (%)	PFS (mo)	OS (mo)
O'Shaughnessy <i>et al</i> ^[40]	First line	Gemcitabine +	61	52	56	5.9	12
		Carboplatin ± Inipari ²	62	32	34	3.6	7.7
Isakoff <i>et al</i> ^[42]	First line	Veliparib ² + TMZO	41	37.5	62.5	5.5	NR
Carey <i>et al</i> ^[44]	First line	Cetuxim ^{1,2} ±	71	18	31	2 ²	12
		Carboplatin ¹	54	6	10		
O'Shaughnessy <i>et al</i> ^[45]	First or second line	Irinotecan +	52	49	NR	5.1	15.5
		Carboplatin ± Cetuxim ^{1,2}	51	30		4.7	12.3
Finn <i>et al</i> ^[49]	First line	Dasatin ²	44	4.6	9.2	8.3 wk	NR
Baselga <i>et al</i> ^[46]	First or second line	Cisplatin ±	115	20	NR	3.7 ³	12.9
		Cetuxim ^{1,2}	58	10		1.5	9.4
Gray <i>et al</i> ^[50] (E2100)	First line	Paclitaxel ±	122	48	NR	11.8 ³	NR
		Bevacizum ^{1,2}	111	22		5.9	
Miles <i>et al</i> ^[51] (AVADO)	First line	Docetaxel ±	58	64	NR	10 ³	NR
		Bevacizum ^{1,2}	53	46		8	
Robert <i>et al</i> ^[52] (RIBBON-1)	First line	Tax/Anthr ¹	96	NR	NR	6.5	NR
		± Bevacizum ^{1,2}	46			6.2	
		Cap ±	87	NR	NR	6.1 ³	NR
		Bevacizum ^{1,2}	50			4.2	
Brufsky <i>et al</i> ^[53] (RIBBON-2)	Second line	Cap, tax, gem/ vinorel, ±	112	41 ³	NR	6.0 ³	17.9
		Bevacizum ^{1,2}	47	18		2.7	12.6

¹Cross over to cetuximab + carboplatin arm after progressive disease; ²For entire cohort; ³Significant. TMZO: Temozolamide; ORR: Overall response rate; CBR: Clinical benefit rate; PFS: Progression free survival; NR: Not reported; Tax: Taxanes; Cap: Capecitabine; Gem: gemcitabine.

poly (ADP-ribose) polymerase 1 (PARP1) potentiates the effects of ionizing radiation, DNA methylating agents, topoisomerase I inhibitors and platinum compounds^[19]. Several *PARP1* inhibitors are at different stages of clinical development. In a phase- I study of olaparib in patients with ABC, 9 (15%) patients had an objective response. Of the 3 patients with *BRC42* mutation, CR occurred in one and another one had SD for 7 mo^[37]. In a phase- II study by Tutt *et al*^[38] in 54 patients with known *BRC4* mutations in ABC, 27 received olaparib 400 mg twice a day, of which 11 (41%) experienced a response with a median PFS of 5.7 mo. A second cohort of 27 women received 100 mg of per day and 6 patients (22%) experienced a response with a median PFS of 3.8 mo. The majority of patients in the 400 mg dose had *BRC41* mutation. This agent was fairly well tolerated, with nausea and fatigue being the most common adverse events. A recent phase- I study by Dent *et al*^[39] demonstrated that it was not feasible to administer the 200 mg daily dose of olaparib in combination with weekly paclitaxel due to significant myelosuppression, in spite of prophylaxis with growth factor support.

In a phase- II randomised study, O'Shaughnessy *et al*^[40] found that the addition of iniparib to carboplatin and GC in metastatic TNBC resulted in significant improvements in RR, PFS (Table 4) and OS from 7.7 to 12.3 mo. The addition of iniparib was well tolerated. However, a randomised phase- III study by the same investigators failed to prove significant benefit of iniparib in combination with GC in metastatic TNBC in terms of PFS (4.1 *vs* 5.1 mo) or OS (11.1 *vs* 11.8 mo); although, the addition of iniparib did not significantly add to the toxicity profile of GC alone^[41].

Another drug, veliparib, is a novel oral inhibitor of PARP1 and PARP2. It has shown a synergistic effect with temozolamide in TNBC^[42]. In *BRC41* and *BRC42* mutation carriers, ORR was 37.5% and CBR was 62.5% with a PFS of 5.5 mo. Since both the drugs are given orally, they can be good options for patients in whom there is difficulty in accessing a venous line from the above subgroup.

PARP inhibitors have shown clinical activity in *BRC4* mutation carrier breast cancer and TNBC. These drugs are also being evaluated in the neoadjuvant setting but experience is limited and patient selection, combination with other chemotherapy drugs, route of administration, duration of therapy and toxicity of combination therapy are factors that need to be addressed. However, their role in unselected TNBC patients is uncertain and future trials may address these issues.

Epidermal growth factor receptor inhibitors

Epidermal growth factor receptor (EGFR) is over expressed in TNBC and so it is also one of the targets in its treatment. Cetuximab, a chimeric monoclonal antibody, binds specifically to the extracellular domain of the EGFR and inhibits its activation^[43]. In a phase- II randomised study by Carey *et al*^[44] in metastatic TNBC, patients were treated with cetuximab alone or in combination with carboplatin. Patients in the combination arm had a high RR and CBR (Table 4). In patients with cetuximab monotherapy, carboplatin was added at the time of disease progression. However, patients in both arms had a rapid progression, with a median PFS of only 2 mo. In another randomised phase- II study, pre-treated patients with MBC (78 patients had TNBC) were randomised to receive carboplatin and irinotecan with or without ce-

tuximab^[45]. TNBC patients in the cetuximab arm had a higher RR than the control arm. However, there was no significant improvement in PFS. Patients in the cetuximab arm had more toxicity in the form of neutropenia, thrombocytopenia and diarrhea. The above trials have failed to achieve the expectation of EGFR being a target for treatment in TNBC. In another study by Baselga *et al*^[46] in metastatic TNBC with cetuximab alone or in combination with cisplatin, ORR and PFS was better in the combination arm compared to cetuximab alone (Table 4). Several phase I - II studies with cetuximab in combination with cytotoxic agents or with other targeted therapies, such as trastuzumab, are currently ongoing in metastatic TNBC.

Tyrosine kinase inhibitors

Tyrosine kinase (TK) is also over-expressed in breast cancer and is associated with metastatic disease progression. There are many agents that target the phosphorylation of the receptor by acting at TK, such as imatinib, erlotinib, gefitinib and lapatinib, used for the treatment of many solid tumors. Lapatinib is more effective in HER-2/neu positive breast cancer patients^[47]. Cristofanilli *et al*^[48] presented data from a small open-label phase-II study of 23 patients with newly diagnosed inflammatory breast cancer treated with neoadjuvant lapatinib 1500 mg once daily and paclitaxel 80 mg/m² weekly for 12 wk. RR was 95% (20/21) in HER-2-positive and 100% (2/2) in HER-1 positive/HER-2 negative patients. Dasatinib is an oral inhibitor of multiple TKs, including the Src and Abl family, c-kit and platelet derived growth factor receptor (PDGFR)- β . Finn *et al*^[49] in a phase II trial showed a CBR of 9% in metastatic TNBC, but discontinuation of therapy and dose reductions weakened the results (Table 4). Presently, several studies are evaluating dasatinib as monotherapy or in combination regimens in this setting.

Antiangiogenic drugs

VEGF expression is higher in TNBC than non-TNBC. Targeted therapy against angiogenesis can cause tumor suppression. Bevacizumab is a recombinant humanised monoclonal antibody targeted against VEGF.

The efficacy of first-line bevacizumab-containing therapy for MBC has been proven in three randomized trials^[50,52]. The E2100 trial showed that adding bevacizumab to paclitaxel as first-line treatment in TNBC patients doubled RR (48% *vs* 22%) and PFS (11.8 *vs* 5.2 mo)^[50]. In the AVADO trial where docetaxel was paired with two different doses of bevacizumab (7.5 and 15 mg/kg) given every 3 wk in 167 patients with TNBC (22%), the addition of bevacizumab at 15 mg/kg led to an improvement in PFS from 6.0 to 8.1 mo^[51]. RIBBON-1 offered investigators the choice of capecitabine, once every 3 wk taxane (docetaxel or nab-paclitaxel), or anthracyclin-cyclophosphamide combinations, each given with or without bevacizumab^[52]. A subset analysis of patients with TNBC demonstrated an improvement in PFS when bevacizumab was used both with capecitabine (6.1 *vs* 4.2 mo) and taxane/anthracyclin cohort (8.2-14.5 mo). In all three trials, there

was statistically significant improvement in PFS and RR with the addition of bevacizumab to chemotherapy but no OS benefit (Table 4). There was also a greater risk of hypertension with any bevacizumab regimen and adverse effects such as headache and nasal congestion, although rarely scored as grade 3 or 4, were also more frequent with bevacizumab. When bevacizumab is paired with taxanes taken once every 3 wk, there is a greater chance of neutropenia. Altogether, between 34% and 57% of patients receiving bevacizumab-based treatment in RIBBON-1 experienced toxicity \geq grade 3, suggesting that there may be limitations for adding extra therapy to these combinations. For instance, attempts to add sunitinib, the multitargeted TK inhibitor, to chemotherapy with bevacizumab have proven unsuccessful as a result of extensive toxicity in patients with breast cancer. In further analysis of RIBBON-2 for the role of bevacizumab as second line therapy in metastatic TNBC, there was significant RR and PFS benefit but again no OS advantage^[53]. Its cost is also a limiting factor and its toxicity further adds to the overall cost. Currently, its accelerated approval in breast cancer has been withdrawn due to the only modest risk-benefit ratio.

In a recent study by Gerber *et al*^[54] where neoadjuvant bevacizumab and anthracyclin-taxane-based chemotherapy was given in 686 TNBC patients, the effect of bevacizumab on pCR was more in patients with TNBC (40.1% *vs* 32.3%). The long term results of this trial will show if this pCR benefit translates to DFS and OS.

Other agents that target VEGF

Sunitinib is a TK inhibitor and inactivates VEGF and PDGFR. Two phase-III trials have shown that combining sunitinib with docetaxel or capecitabine does not offer any benefit in prolonging PFS compared to the cytotoxic regimen alone in patients with ABC when used as a first line therapy or in pre-treated patients^[55,56]. Sunitinib is currently being evaluated in addition to carboplatin and paclitaxel as adjuvant treatment for TNBC. It should always be used in combination as monotherapy is not recommended.

In the clinical trials so far, sorafenib has not shown any absolute benefit when used as the only therapeutic strategy in breast cancer^[57,58]. Median PFS was extended by 2 mo in patients treated with the combination of sorafenib-capecitabine in comparison with the combination sorafenib-placebo, but at the cost of high toxicity (grade III HFS 45% *vs* 13%)^[57]. The second trial evaluated sorafenib in combination with paclitaxel or placebo as first-line therapy in patients with locally recurrent or MBC. Forty percent of patients had TN disease. The hazard ratio for PFS was 0.78 ($P = 0.08$), a trend favoring the sorafenib-paclitaxel group^[58]. The incidence of grade III HFS was 30% *vs* 3% in the placebo group. Such a high incidence of grade III HFS is unacceptable and therefore careful monitoring of patients for HFS and timely dose-reduction should be done. The other agents like vandetanib and montesanib are still in the trial stages.

Currently, a lot of research is going on in TNBC. Recently, Melhem-Bertrandt *et al.*^[59] investigated 1413 patients treated with NACT who used β -blockers (BB), comparing those without BB exposure for pCR, DFS and OS. In 377 TNBC patients, there were significant effects for BB use on both DFS ($P = 0.03$) and OS ($P = 0.05$). Many agents and treatment approaches are under investigation for the treatment of TNBC. Many targets such as α V β 6, cyclin E, c-kit, E-cadherin, O⁶MGMT, FOXp3 and mitogen-activated protein kinase pathway need further exploration to dissect TNBC and may possibly identify new targets for therapy.

Future phase-III breast cancer treatment trials should endeavour to collect prospective data on relevant medication exposures, weight and weight gain, comorbid conditions, and behaviors that have the potential to influence the microenvironment of the tumor as these may be potent mediators of prognosis and survival and may or may not be effectively accounted for in randomization. Key areas of research should include appropriate patient sub-classification for new and existing treatment options in their rationale combination.

CONCLUSION

TNBC is a heterogeneous disease entity. There are no specific treatment guidelines for TNBC and it is managed with standard treatment. Targeted agents have not produced substantial improvements in outcomes. The result of targeted therapy depends on the existence and level of expression of the target protein. The treatment of TNBC will continue to evolve as we learn more about the heterogeneity of this disease and this will underscore the need for treatments to be tailored for a specific patient, depending on the molecular characteristics of their malignancy. The tumor microenvironment may be a critical target for future cancer treatment and prevention of recurrence.

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Long non-coding RNAs in stem cells and cancer

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Abstract

An overwhelming majority of the transcribed genome encodes for non-coding RNA (ncRNA) sequences. Deep sequencing of the transcriptome has uncovered tens of thousands of long ncRNA (lncRNA) sequences. However, little is known regarding the possible functions for a vast majority of these sequences. Among those lncRNAs whose function has been experimentally validated, most serve as regulators of gene expression. lncRNAs have been found to be critical to development and homeostasis and they have been implicated in several pathologies including cancer. Here, we examine the functions and underlying mechanisms of lncRNAs in stem cells and in cancer biology, areas linked by the actions of lncRNAs.

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Key words: Long non-coding RNA; Stem cell; Cancer stem cell

Core tip: We discuss long non-coding RNA (lncRNA) in

stem cells, where they are shown to be critical regulators of pluripotency and self-renewal. Next, we examine lncRNAs role in regulating the epigenome. Finally we summarize the suspected involvement of lncRNAs in tumorigenesis.

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INTRODUCTION

A surprising revelation from the human genome project was that less than 2% of the human genome was composed of protein-coding genes^[1]. Although originally thought of as junk DNA, the ENCODE project revealed that 76% of the genome is actively transcribed^[2]. Although transcription alone is not a demonstration that these are indeed functional molecules, through careful examination we now know that a great deal of this non-coding RNA (ncRNA) is functionally relevant to physiology and disease. One important class of ncRNA is long ncRNA (lncRNA). ncRNA species greater than 200 nucleotides in length are all grouped together as lncRNAs. This arbitrary limit to their size likely ignores the diversity present among lncRNAs.

The GENCODE consortium (version 18) has annotated 13562 lncRNAs^[3]. Around 2/3 of lncRNAs are intergenic [long intergenic ncRNAs (lincRNAs)], the rest are overlapping, antisense, or intronic to protein coding genes. Although some lncRNAs have been found to be transcribed by RNA Pol III, a majority of lncRNA is thought to be transcribed by RNA pol II, to be polyadenylated, spliced and 5'-capped^[4]. lncRNAs show lower evolutionary conservation than protein coding genes and show less conserved than other ncRNAs, *i.e.*, microRNAs (miRNAs). This may be because lncRNAs have rapidly

evolved or because selection pressure maintains only short critical sequences or secondary structures of the lncRNAs.

lncRNAs are functionally very diverse, interacting with ncRNAs, mRNAs, proteins and genomic DNA and acting as tethers, guides, decoys, and scaffolds^[5]. Most lncRNAs have been found to be critical regulators of gene expression. lncRNAs have been found to act at nearly every level of gene regulation: epigenetic, transcriptional, posttranscriptional, and translational.

Here, we will discuss lncRNAs in stem cells, where they are shown to be critical regulators of pluripotency and self-renewal. Next, we will examine lncRNAs role in regulating the epigenome. Finally we will summarize the suspected involvement of lncRNAs in tumorigenesis.

LNCRNAS IN STEM CELLS

One area of biology where lncRNAs are emerging as major players is in stem cell biology. Several studies have found that lncRNAs can regulate pluripotency and differentiation in embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Furthermore, lncRNAs are also emerging as important regulators of adult stem cells.

LncRNAs in Embryonic Stem Cells

Guttman *et al.*^[6] examined lincRNA expression and function in ESCs. They first profiled lincRNA expression in ESCs and then performed knockdown of 147 lincRNAs that were expressed in ESCs (out of 226 lincRNAs they could detect in ESCs). They found that a majority of these lincRNAs (93%) were able to influence gene expression patterns in ESCs. Furthermore, they found that 26 lincRNAs were able to significantly impact expression levels of the critical pluripotency factor Nanog. This suggests that lincRNAs are heavily involved in maintaining pluripotency and preventing the differentiation of ESCs. Next, they examined differentiation of ESCs and identified 13 lincRNAs associated with endoderm differentiation, 7 lincRNAs associated with ectoderm differentiation, 5 with neuroectoderm, 7 with mesoderm, and 2 with trophectoderm differentiation. This demonstrates that lincRNAs are critical regulators of lineage-specific differentiation of ESCs. Next, they examined whether lincRNAs in ESCs were regulated by pluripotency-associated transcription factors (Oct4, Sox2, Nanog, cMyc, nMyc, KLF4, ZFX, Smad and TCF3). At least one pluripotency factors was associated with the promoter region of 75% of the 226 lincRNAs expressed in ESCs suggesting that these lincRNAs serve as direct gene targets of pluripotency transcription factors. Finally, they found that 74 (30%) of these lincRNAs were associated with chromatin-modifying proteins known to be important in ESCs. Sheik Mohamed *et al.*^[7] also examined the role of lncRNAs in regulating pluripotency of ESCs. They examined high-confidence binding sites of OCT4 and Nanog as determined by paired end-tag sequencing

of ChIP-PET DNA. Ten percent of the OCT4 binding sites (105/1083) and 11% of the Nanog binding sites (335/3006) occupied proximal regions of lncRNA genes. This strongly suggests that regulation of lncRNA is a critical aspect of pluripotency.

Ng *et al.*^[8] examined pluripotency associated lncRNAs by examining expression following differentiation of ESCs to Neural Progenitor Cells (NPCs). Using custom lncRNA microarray technology they revealed over 934 lncRNAs that were differentially expressed. They identified 36 lncRNAs that were downregulated greater than five-fold in NPCs. Next they chose 3 candidate lncRNAs for functional characterization, lncRNA ES1, ES2, ES3. They found that knockdown of any of these 3 lncRNAs resulted in ESC differentiation. They performed RNA Immunoprecipitation experiments and found that ES1 and ES2 were associated with SUZ12 [Polycomb Group (PcG)] and pluripotency factor SOX2 in the nucleus.

These studies reveal that maintenance of pluripotency and lineage-specific differentiation are carefully regulated by lncRNA networks, many of which are direct targets of master pluripotency transcription factors. Additional functional studies of the lncRNAs should shed more light on the mechanism and interacting partners of these lncRNAs in ESCs.

LncRNAs in iPSCs

Loewer *et al.*^[9] examined lincRNA expression in iPSCs. They discovered that the lincRNA-ST8SIA3 [or Regulator of Reprogramming (ROR)] was upregulated in iPSCs. They found that pluripotency transcription factors (OCT4, SOX2 and NANOG) directly regulated expression of ROR. Furthermore they found that ROR knockdown could inhibit reprogramming and iPSC colony formation. Furthermore, overexpression of ROR enhanced iPSC colony formation. Wang *et al.*^[10] examined ROR function in ESCs and found that ROR was able to regulate self-renewal and differentiation of ESCs. Furthermore, they identified a possible mechanism through which ROR may act, serving as a competitive endogenous RNA (sponge) for miR-145, which is known to target OCT4, SOX2, KLF4 and regulate ESC differentiation^[11].

LncRNAs in adult stem cells

There are several reports of lncRNAs regulating adult stem cell self-renewal and differentiation. Zuo *et al.*^[12] examined osteoblast differentiation of Mesenchymal stem cells and found that 116 lncRNAs were differentially expressed upon differentiation. Next, Kretz *et al.*^[13] found that the lncRNA ANCR was down-regulated during differentiation of epidermal cells and found that ANCR regulates gene expression to suppress differentiation of epidermal progenitors. Ramos *et al.*^[14] found that the lncRNAs Six3os and Dix1as were regulators of glial-neuronal lineage specification from adult neural stem cells. Finally, Yildirim *et al.*^[15] found that the lncRNA Xist was essential for long term survival of hematopoietic stem cells.

Adipocytes and adipogenesis

Much of the information involving lncRNA regulation of adipogenesis came from the recent discoveries by Sun *et al.*^[16] who identified 20 candidate lncRNAs up-regulated during adipogenesis using RNA-seq to analyze gene expression of *in vitro* cultured mouse preadipocytes and brown/white adipocytes, alongside primary isolated mature adipocytes. Using RNAi based loss of function screening and unique score metrics, they found that loss of many of these lncRNAs resulted in either partial or near complete reversion of mature adipocyte to precursor phenotype. In another study, Pang *et al.*^[17] found that an antisense lncRNA for the *PU.1* gene promotes adipogenesis by an forming mRNA/lncRNA duplex with *PU.1* mRNA and blocking translation.

EPIGENETIC REGULATION

lncRNA is emerging as a major player in chromatin remodeling and epigenetic regulation of gene expression. Evidence indicates that lncRNAs play important roles in the critical physiological processes of X-chromosome inactivation (XCI) and genomic imprinting. Furthermore, dysregulation of lncRNAs has been indicated in epigenetic reprogramming in human cancer.

XCI

One of the X chromosomes in female mammalian cells is epigenetically inactivated during early development to ensure balanced expression of X-chromosomal genes known as dosage compensation. The lncRNA, X-inactive specific transcript (Xist), was discovered due to its surprisingly only being expressed by inactive X-chromosomes. Curiously, Xist was not expressed in male cells and was only shown to be expressed in cells with at least two X-chromosomes. It was revealed that Xist plays a central role directing XCI by coating one X-chromosome leading to its epigenetic silencing^[18].

XCI initiation begins with one of the X-chromosomes randomly activating Xist expression in early development. Xist then recruits Polycomb repressor complex 2 (PRC2) to the Xist promoter region in the future inactive chromosome. The Xist promoter is then methylated in cis resulting in further activation of Xist^[19]. The transcribed Xist RNA transfers to specific loci in cis across the X-chromosome using a targeting mechanism based on the three-dimensional conformation of the X-chromosome^[20]. The attachment of Xist to the future inactive X-chromosome (Xi) is mediated by a transcription factor, YY1, which can bind simultaneously both DNA and RNA through different motifs^[21]. Xist finally spreads and coats the Xi and PRC2 is recruited by interacting with the Repeat A region of Xist^[19]. At the end, Xi chromatin is extensively ubiquitinated at histone H2A, resulting in the formation of heterochromatin. This process is suppressed at the active X-chromosomes through the action of the transcribed antisense of Xist, the lncRNA Tsix, which through its complementary sequence can inhibit

Xist expression.

Genomic imprinting

Genomic imprinting is an epigenetic process by which certain autosomal genes express only the maternal or paternal allele. Imprinted genes are usually clustered on chromosomes and these imprinted gene clusters contain both protein-coding genes and lncRNA genes. The *Igf2r* locus contains three imprinted, maternally expressed protein-coding genes, *Igf2r*, *Slc22a2*, *Slc22a3*, and an imprinted, paternally expressed lncRNA gene, *Airn* (antisense *Igf2r* RNA non-coding), which is antisense to *Igf2r* RNA and required for the silencing of *Igf2r*, *Slc22a2* and *Slc22a3* on the paternal chromosome^[22].

The gene product of *Airn*, *Air* lncRNA, represses genes from the *Igf2r* locus *via* multiple different silencing mechanisms. In mouse placenta, the *Air* RNA recruits the H3K9 histone methyltransferase G9a to the *Slc22a3* promoter chromatin, resulting in H3K9 methylation and *Slc22a3* transcriptional silencing^[23]. In contrast, *Air* silences *Igf2r* gene through a transcriptional interference mechanism, in which *Airn* transcriptional overlap of the weaker *Igf2r* promoter results in the silencing of *Igf2r* gene^[24]. The silenced *Igf2r* promoter is then subject to DNA methylation that along with *Airn* expression, maintains *Igf2r* silencing^[25].

lncRNA may also function as scaffolds allowing multiple chromatin modifying complexes to regulate target genes. For example, lncRNA HOTAIR in the mammalian *HOXC* locus can bind both PRC2 and LSD1/CoREST/REST complexes mediating histone H3K27 trimethylation and H3K4 demethylation of target genes^[26]. This complex has been shown to target and silence the *HOXD* gene cluster^[27].

LNCRNA IN CANCER

Profiling of normal and tumor tissues has revealed that lncRNAs are dysregulated in many human cancers including prostate^[28], colorectal^[29], breast^[30], bladder^[31], liver^[32], and brain cancer^[33]. They have been found to function as oncogenes and tumor suppressors and regulate many of the hallmarks of cancer.

Tumor Suppressor lncRNAs

The lncRNA Xist was found to be a potent tumor suppressor of hematologic malignancies *in vivo*. Yildirim *et al.*^[15] found that knockdown of Xist in hematopoietic cells in mice resulted in aggressive myeloproliferative neoplasm and myelodysplastic syndrome. They also demonstrated that Xist was critical for hematopoietic stem cell survival. The authors hypothesized that X reactivation *via* Xist silencing could lead to genomic instability and cancer.

Huarte *et al.*^[34] examined lncRNAs regulated by the tumor suppressor p53. They identified lncRNA-p21 as a direct p53 target. Furthermore, they found that lncRNA-p21 is critical in regulating many of the genes that are repressed in response to p53 activity and they

found that lincRNA-p21 associates with hnRNP-K. The lincRNA-p21/hnRNP-K interaction was found to be necessary for hnRNP-K genomic localization at sites of gene repression.

Oncogenic lncRNAs

Gupta *et al.*^[30] found that lncRNA HOTAIR overexpression was a strong predictor of breast tumor metastasis. Furthermore, they found that HOTAIR overexpression could promote breast tumor cell invasion and knockdown of HOTAIR could inhibit invasiveness. Prensner *et al.*^[35] examined lincRNA expression prostate tumors and found that the lincRNA PCAT-1 was overexpressed in high-grade and metastatic tumors. They found that PCAT-1 expression promoted proliferation of prostate cancer cells.

Epigenetic reprogramming in cancer

Since lncRNAs play such a large role in epigenetic regulation and chromatin remodeling in normal physiology it is possible that they may play a role in the epigenetic reprogramming that is a hallmark of human cancer. One hundred and seventy ncRNAs including HOTAIR are differentially expressed among normal human breast tissue, primary breast tumors, and metastatic breast tumors^[30]. Forced expression of HOTAIR in epithelial cancer cells altered the localization of PRC2 on chromatin. Genome-wide studies revealed PRC2 localization more resembling occupancy in embryonic fibroblasts. This correlated with increased tumor cell invasiveness. Conversely, knockdown of HOTAIR was shown to inhibit cancer cell invasiveness in cells with high levels of PRC2 expression. Similar findings were reported in colorectal cancer^[36].

It has also been shown that the tumor suppressor gene p15 is silenced by its natural antisense RNA, a lncRNA ANRIL^[37,38]. ANRIL directs PRC2 to the p15 locus inhibiting p15 expression^[38]. Similarly, the expression the tumor suppressor gene p21 was shown to be epigenetically repressed by its antisense RNA^[39]. These reports suggest that tumor suppressor gene silencing may be a result of an imbalance in bidirectional transcription.

Prensner *et al.*^[28] found that the lncRNA SCHLAP1 was overexpressed in prostate tumors and where it is critical for tumor cell metastasis. They found that SCHLAP1 antagonized localization of the SWI/SNF chromatin-remodeling complex and inhibited tumor suppressive action of SWI/SNF.

Microvascular invasion and angiogenesis

Angiogenesis is an important hallmark of human cancer. New vasculature is required for providing nutrients and oxygen for tumor growth and proliferation as well as proving an avenue for metastatic spread. Although a variety of genes which can stimulate angiogenesis have been discovered, recently there has been a focus on investigating the roles of ncRNA in the angiogenic switch.

Yuan *et al.*^[40] discovered that the lncRNA MVIH (long noncoding RNA associated with microvascular invasion

in hepatocellular carcinoma) was overexpressed in hepatocellular carcinoma. MVIH overexpression was associated with frequent microvascular invasion and a higher tumor node metastasis stage as well as decrease in recurrence-free survival. Further data showed MVIH could promote tumor inducing angiogenesis through inhibiting the secretion of phosphoglycerate kinase phosphoglycerate kinase 1. This is clear evidence that dysregulation of lncRNA promotes tumor growth through angiogenesis.

Angiogenic signaling can also be activated in response to hypoxia. Normal hypoxia can induce gene expression in response to oxygen sensing accompanied by adaptive responses to the hypoxic environment. However, during tumor growth, hypoxia can alter gene expression to promote angiogenesis, cell proliferation and even metastasis. Ferdin *et al.*^[41] found the tight link between long non-coding transcripts from ultraconserved regions, termed transcribed-ultraconserved regions (T-UCRs) and oxygen deprivation. Several T-UCR were upregulated during hypoxia (several were induced directly by hypoxia-inducible factor), these hypoxia-induced noncoding ultra-conserved transcripts (HINCUTs) were also found overexpressed in colon cancer patients. The author also hypothesized that protein modification through addition of O-linked N-acetyl glucosamine was involved in the sensing oxygen tension as one specific HINCUT lncRNA (HINCUT-1) was part of retained intron of the host protein-coding gene O-linked N-acetyl glucosamine transferase.

Another potential angiogenic lncRNA, Maternally expressed gene 3 (MEG3) was found to be silenced in pituitary adenomas. MEG3 knockout mice showed enhanced angiogenic signaling and increased microvascular density in embryonic brains^[42]. Since MEG3 stimulated p53 pathways and regulated p53 target genes, it is highly desirable to examine MEG3 expression in other cancers.

Metabolism imbalance

Metabolic imbalance is a key hallmark of human cancer. One mechanism lncRNA may contribute to tumorigenesis is through regulating tumor cell metabolism. However, very little is known regarding the role of lncRNAs in metabolism. One report demonstrated that a particular lncRNA on paternal chromosome 15q11-q13 was critical to maintaining energy balance^[43]. The loss of noncoding RNAs on this site is followed by genetic disease called Prader-Willi syndrome (PWS). These imprinted PWS locus cover a long noncoding RNA transcript which are processed into SNORD116 small nucleolar RNA and spliced exons of the host gene 116HG. The author found 116HG are concentrated subnuclearly with transcriptional activator RBBP5 at active genes involved in metabolism. 116HG deficient mice showed dramatic increases in energy expenditures. Although the inculcated tissues are mainly in the brain and the syndrome is characteristic of disorders of neurodevelopment and obesity in children, whether it is also a genetic risk factor for certain cancers is unknown and needs to be examined.

Another well-known lncRNA, ANRIL, is implicated

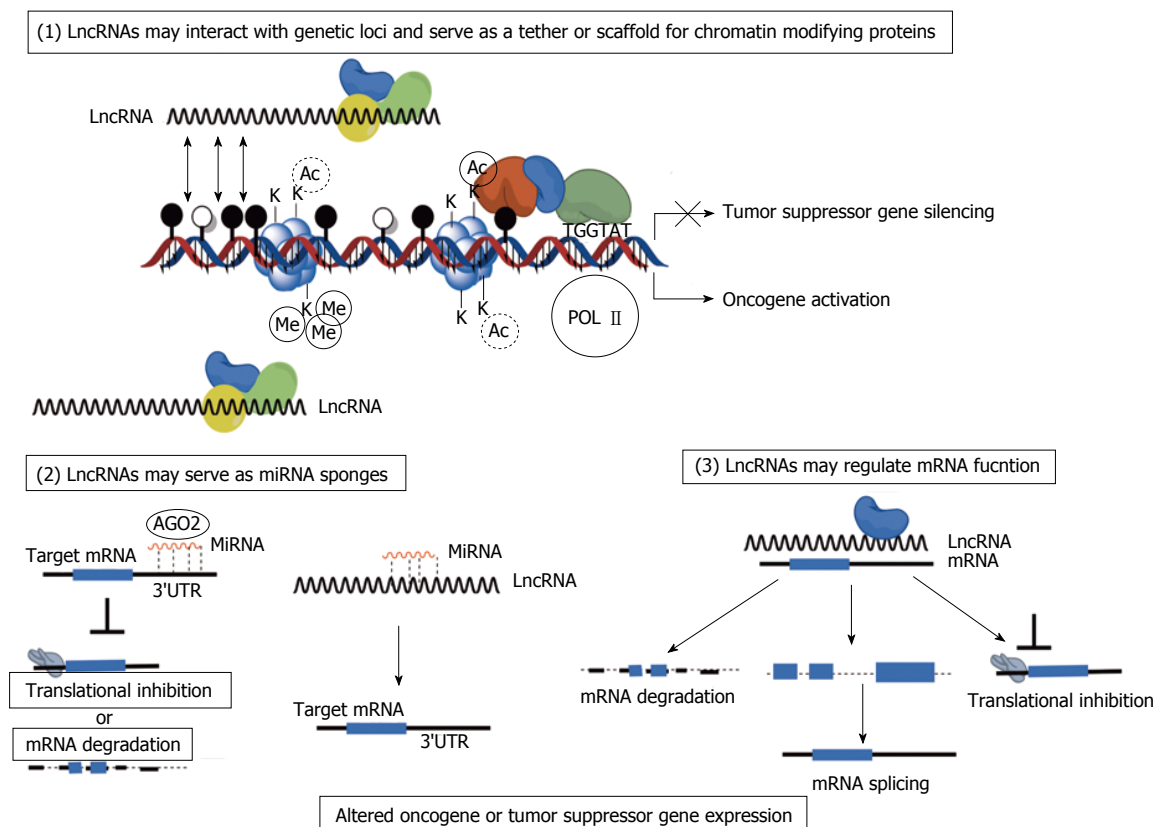


Figure 1 Diverse functions of long non-coding RNA in human cancer cells. Long non-coding RNA (lncRNA) are known to be active regulators of gene expression through transcriptional, post-transcriptional, and translational mechanisms. LncRNA dysregulation in cancer may provide oncogenic signaling or loss of tumor suppressive function. LncRNAs are known to serve as tethers and scaffolds that direct chromatin modifying enzymes such as Polycomb group proteins to regulatory regions of DNA. This may result in upregulated transcription of oncogenic protein coding genes or loss of transcription of tumor suppressor protein coding genes. LncRNAs also may serve as competitive endogenous RNA for microRNAs (miRNAs) (sponges) that then inhibit miRNA function thereby preventing miRNA from interacting with target mRNAs. Finally, lncRNAs can interact directly or indirectly with mRNAs in the nucleus and cytoplasm regulating mRNA splicing, mRNA stability, and mRNA translation.

as a risk factor for breast cancer and is overexpressed in prostate cancer^[44]. Recently, knockdown of ANRIL resulted in downregulation of 3 genes, *ADIPOR1*, *VAMP3* and *C11ORF10*, all of which have a well-established role during the metabolism of fatty acid and glucose and in inflammation^[45].

Extracellular matrix

Extracellular matrix (ECM) is an important component of both normal and malignant tissues. In addition to structural support the ECM supports the communication between nearby cells in order to coordinate signaling and tissue function. The role of lncRNA in the ECM during malignancy has only recently been investigated. In one study, Zhuang *et al.*^[46] investigated the expression of lncRNA during type I collagen stimulation in a 3-D culture. Type I collagen has been shown enriched in tumor microenvironment and can promote tumor growth. In this study, the lncRNA HOTAIR was upregulated in lung adenocarcinoma cells grown in 3-D cell culture supplemented with collagen. Furthermore, the authors observed loss of acini, a sign of hyperproliferation and poor differentiation. This might be the first attempt to determine the function of lncRNA at the level of organ-

otypic culture and it is a very useful strategy for further characterization of lncRNA molecules functional in the tumor microenvironment.

Tumor microenvironment

It is known that the tumor microenvironment plays a role in tumor formation, invasive progression and metastatic dissemination^[47]. Tumor microenvironment is composed of mesenchymal stromal cells, adipocytes, fibroblasts, endothelial and immune cells. The tumor microenvironment is abundant in proinflammatory cytokines that are derived from both cancer cells and nearby endothelial and adipocyte like cells; these secreted cytokines not only stimulate inflammation but also recruit mesenchymal stromal cells and preadipocytes to the tumor. Furthermore, tumor microenvironment can influence tumor cell metabolism and metabolic changes can also affect inflammatory signaling and this feedback can further alter the tumor microenvironment and promote tumor invasion. LncRNAs have been found to be critical regulators of mesenchymal stem cells^[12], endothelial cells^[48], adipocytes^[16] and immune cells^[49,50]. It remains untested what role lncRNAs may have in promoting tumorigenesis through signaling within the tumor microenvironment.

Cancer stem cells

As was discussed earlier, lncRNAs play critical roles in regulating pluripotency in ESCs. The two defining characteristics of stem cells, self-renewal and differentiation capacity, are hijacked by some neoplastic cells in what are often referred to as cancer stem cells. Many of the signaling pathways that are critical in ESCs (OCT4, SOX2, KLF4 and PcG^[51-54]) have been found activated in cancer stem cells. Since the regulation of OCT4, SOX2, KLF4 involves feedback loops with lncRNAs^[10], and as lncRNAs are known to be dysregulated in cancer, it seems likely that lncRNAs may be involved in regulating stem cell signaling in cancer cells. Examining the functions of lncRNAs in cancer stem cells may reveal new therapeutic targets and mechanisms for overcoming chemoresistance.

Many lncRNAs are shown to interact with chromatin modifying complexes. In particular the PcG is known to regulate pluripotency and self-renewal of ESCs. As polycomb signaling has been implicated in some types of cancer^[54], it is likely that lncRNAs may serve to target polycomb complexes to sites in chromatin to silence differentiation programs in cancer stem cells

It is already known that ncRNAs, specifically miRNAs, play critical roles regulating cancer stem cells, e.g., the miR-200 family^[55], let-7^[56], miR-140^[57]. One potential function of lncRNAs is to competitively inhibit miRNAs as a molecular sponge (also referred to as competing endogenous RNAs) (Figure 1). Kallen *et al.*^[58] recently discovered that lncRNA H19 can serve as a sponge for let-7 family of miRNAs in muscle tissues. There is potential for this or other lncRNAs to regulate miRNA signaling in cancer stem cells and future studies will provide further evidence of the importance of ncRNA, both lncRNA and miRNA, in regulating cancer stem cell signaling.

DISCUSSION

It is clear that lncRNAs are critical regulators of gene expression in stem cell biology and in tumorigenesis. Further study of lncRNA will increase our understanding of the complex networks involved in regulating stem cell circuitry and may uncover the mechanisms by which differentiated cancer cells can hijack embryonic or developmental programs to promote self-renewal of cancer cells. Future studies of lncRNAs in cancer stem cells including profiling of sorted cancer cell populations as well as genetic approaches for manipulating lncRNA expression should provide information as to how these molecules regulate cancer stem cells. Ongoing *in vivo* work will provide the strongest evidence for the importance of lncRNAs in physiology and disease. Furthermore, additional tumor profiling should identify which lncRNAs might serve as clinical biomarkers and which are the best candidates for future therapeutic strategies.

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Clinical outcomes following salvage Gamma Knife radiosurgery for recurrent glioblastoma

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GKRS as salvage therapy for malignant gliomas, nine articles from 2005 to July 2013 were identified which evaluated rGBM treatment. In this review, we compare overall survival following diagnosis, overall survival following salvage treatment, progression-free survival, time to recurrence, local tumor control, and adverse radiation effects. This report discusses results for rGBM patient populations alone, not for mixed populations with other tumor histology grades. All nine studies reported median overall survival rates (from diagnosis, range: 16.7-33.2 mo; from salvage, range: 9-17.9 mo). Three studies identified median progression-free survival (range: 4.6-14.9 mo). Two showed median time to recurrence of GBM. Two discussed local tumor control. Six studies reported adverse radiation effects (range: 0%-46% of patients). The greatest survival advantages were seen in patients who received GKRS salvage along with other treatments, like resection or bevacizumab, suggesting that appropriately tailored multimodal therapy should be considered with each rGBM patient. However, there needs to be a randomized clinical trial to test GKRS for rGBM before the possibility of selection bias can be dismissed.

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Key words: Gamma Knife radiosurgery; Malignant glioma; Glioblastoma; Salvage therapy; Stereotactic radiosurgery; Multimodal treatment

Core tip: Glioblastoma is the most common malignant primary neoplasm of the brain. Despite aggressive, upfront therapy, most patients will experience a recurrence of their tumor six months after treatment. This review article analyzes the outcomes of clinical trials that utilized Gamma Knife radiosurgery as salvage therapy for recurrent glioblastoma. Other modalities of radiosurgery were excluded from this study as there is variability in the targeting precision and radiation dos-

Abstract

Glioblastoma multiforme (GBM) is the most common malignant primary brain tumor with a survival prognosis of 14-16 mo for the highest functioning patients. Despite aggressive, multimodal upfront therapies, the majority of GBMs will recur in approximately six months. Salvage therapy options for recurrent GBM (rGBM) are an area of intense research. This study compares recent survival and quality of life outcomes following Gamma Knife radiosurgery (GKRS) salvage therapy. Following a PubMed search for studies using

age fall off. Gamma Knife can be used to target tumors that are adjacent to eloquent brain tissue, thus allowing it to treat a wider population of patients, improving overall survival.

Larson EW, Peterson HE, Lamoreaux WT, MacKay AR, Fairbanks RK, Call JA, Carlson JD, Ling BC, Demakas JJ, Cooke BS, Lee CM. Clinical outcomes following salvage Gamma Knife radiosurgery for recurrent glioblastoma. *World J Clin Oncol* 2014; 5(2): 142-148 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i2/142.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i2.142>

INTRODUCTION

Glioblastoma multiforme (GBM) remains the most common malignant primary brain tumor in adults and is uniformly fatal^[1]. Without treatment, most patients die within a few weeks after presentation of symptoms^[2]. For newly diagnosed GBM, median survival can be extended to 14-16 mo using a standard treatment protocol that includes maximal surgical resection followed by temozolomide chemotherapy and 60 Gy of external beam radiation therapy (EBRT)^[3,4].

Strong predictors of prognosis with these treatments for patients with GBM have been outlined by Radiation Therapy Oncology Group recursive partitioning analysis (RTOG-RPA). These include tumor histopathology, age, Karnofsky Performance Score (KPS), neurologic function, and initial treatments^[5,6]. Patients with GBM histology are categorized in one of four groups, RPA Classes III, IV, V, and VI, with Class VI having the worst prognosis. In 2011, Li *et al*^[7] analyzed the RTOG-RPA with a renewed focus on GBM alone. They suggest that there is not a significant difference between Classes V and VI, and that these groups could be combined. They report median overall survival times for the updated classification as 17.1, 11.2, and 7.5 mo for patient populations in Classes III, IV, and V + VI, respectively^[7]. Additional prognostic factors that may improve survival include small tumor volume, unifocal lesions, use of additional salvage therapies, and O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation^[8-11]. MGMT status, however, was either not known, or not reported for any of the studies reviewed herein.

Despite aggressive adjuvant therapy for GBM, tumors typically recur within 6 mo of treatment. This may be due in part to microscopic infiltrative growth up to 4 cm from visible tumor location along white matter tracts in normal brain tissue^[12] as well as the resistance of the tumor to radiation and chemotherapy treatments. Salvage therapy for recurrent GBM (rGBM) is an area of current interest in order to extend overall survival beyond the aforementioned median survival of 17.1 mo for the most functional patients. Some patients may not be eligible for repeated surgery because of the location of the tumor in critical structures or its diffuse nature. Others may find

that recovery from surgery is too burdensome during the terminal stage of illness. Salvage chemotherapy may be an option for patients who can tolerate the side effects. Repeated conformal radiation therapy comes with uncertain benefit and increased risk of adverse radiation effects including radionecrosis or radiation-induced edema. Stereotactic radiosurgery has shown not to be superior to standard EBRT for newly diagnosed GBM^[13]. However, its role in rGBM is still under investigation.

There are multiple modalities of stereotactic radiosurgery; one of which often utilized for this recurrent disease is Gamma Knife radiosurgery (GKRS). GKRS is capable of delivering a high dose of radiation to a tumor while sparing healthy surrounding tissue. Its advantages in rGBM include the convenience of a single outpatient treatment, minimization of potential radiation necrosis to adjacent brain, and a cost benefit over open resection^[14]. Its disadvantage includes the lack of treatment to regions of tumor not well visualized on magnetic resonance imaging. Therefore, its effect on both local tumor control and overall survival is unclear. Previous review articles on stereotactic radiosurgery for malignant gliomas have taken a broad approach, including gliomas of multiple grades or a variety of radiosurgery treatment modalities. These other articles have excluded some of the smaller studies of radiosurgery for rGBM. The present article reviews the outcomes of clinical studies which used GKRS alone or as part of multimodal treatment regimens as salvage therapy for rGBM. While other modalities, such as CyberKnife® or linear accelerator based systems may have similar outcomes, these radiosurgery modalities are not included in this review due to the differences in cost, conformity, homogeneity, maximum dose, treatment times, dose fall off, invasiveness, treatment planning, and beam properties^[15-20]. Different radiosurgical delivery systems may be more appropriate for other tumor types and locations which are not discussed here^[18,20].

RESEARCH

A PubMed search was performed to identify clinical studies that used GKRS salvage for treatment of rGBM between 2005 and 2013. Key words for this search included "Stereotactic Radiosurgery", "Gamma Knife", "Glioblastoma", "recurrent GBM", "Glioma", and "Glioma salvage therapy". To expand the search strategy, references from articles found in this search were also analyzed for inclusion in this review. Studies which treated World Health Organization Grade I - III Gliomas without treating Grade IV (GBM) were also excluded. Finally, studies which used Gamma Knife as upfront therapy only for newly diagnosed GBM were excluded.

The median overall survival outcomes as compared to RTOG-RPA prognostic indicators^[7] were extracted and collated from these research studies. Other results were also analyzed including median survival post-GKRS salvage, progression-free survival, and time to repeat tumor recurrence. This article will discuss additional factors that may contribute to improved outcomes based on

Table 1 Characteristics of patients with newly diagnosed glioblastoma multiforme

Ref.	rGBM patients	Median age (range)	Median KPS (range)	Median tumor volume (mL) (range)
Skeie <i>et al</i> ^[9]	32	51	73	12.4
	19	50	81	13.9
Park <i>et al</i> ^[10]	11	62 (46-72)	90 (80-100)	13.6 (1.2-45.1)
	44	64 (41-77)	90 (70-100)	9.5 (1.5-48.9)
Koga <i>et al</i> ^[28]	9	43 (17-64)	90 (80-90)	NR
	9	53 (27-79)	90 (80-90)	NR
Elliott <i>et al</i> ^[11]	16	60 (35-69)	90 (80-100)	1.35 (0.37-6.2)
Pouratian <i>et al</i> ^[25]	26	60.7 (12.9-76.9)	80 (40-100)	21.3 (0.3-110.0)
Kida <i>et al</i> ^[21]	54	[Mean 52.4 (11-80)]	NR	(Mean: 29.0 mm) (7-48.3 mm)
¹ Kong <i>et al</i> ^[23]	65	NR	NR	NR
Kohshi <i>et al</i> ^[22]	11	NR	NR	NR
Hsieh <i>et al</i> ^[24]	26	(Mean 58.2)	(Mean: 70)	(Mean: 21.6)

¹Kong *et al*^[23] (2008) includes data from 0-5 cases treated with LINAC. rGBM: Recurrent glioblastoma multiforme; KPS: Karnofsky Performance Status; NR: Data not reported or not segregated for rGBM patients.

Table 2 Upfront treatment modalities for newly diagnosed glioblastoma multiforme

Ref.	rGBM patients	Initial surgery	Adjuvant rad	Adjuvant chemotherapy	Time to 1 st recurrence (mo)
Skeie <i>et al</i> ^[9]	32	All GTR	93.5% of patients had EBRT (39-60 Gy)	37% Temo, 19.9% PCV	11.0 (mean)
	19				10.4 (mean)
Park <i>et al</i> ^[10]	11	4 Bx, 7 GTR	All EBRT (54-60 Gy)	All Temo	NR
	44	NR	NR	NR	NR
Koga <i>et al</i> ^[28]	9	All resection	8 EBRT (48-80 Gy)	6 ACNU, 2 Temo, 1 CE	14.5 (median) (range, 1-51)
	9	2 Bx, 7 resection	All EBRT (60-80 Gy)	All Temo	12 (median) (range, 6-39)
Elliott <i>et al</i> ^[11]	16	NR	All EBRT (60 Gy)	All Temo	7.9 (median) (range, 2-84)
Pouratian <i>et al</i> ^[25]	26	2 Bx, 18 STR, 6 GTR	25 EBRT (dose NR)	16 had chemotherapy (type NR)	NR
Kida <i>et al</i> ^[21]	54	NR	NR	NR	NR
¹ Kong <i>et al</i> ^[23]	65	NR	All EBRT (54-70 Gy)	NR	4.3 (range, 1.5-27.0)
Kohshi <i>et al</i> ^[22]	11	All debulking	All EBRT (50-72 Gy)	All had chemotherapy (type NR)	NR
Hsieh <i>et al</i> ^[24]	26	NR	All EBRT (60 Gy)	NR	NR

¹Kong *et al*^[23] (2008) includes data from 0-5 cases treated with LINAC. rGBM: Recurrent glioblastoma multiforme; GTR: Gross total resection; STR: Subtotal resection; Bx: Biopsy; EBRT: External beam radiation therapy; Temo: Temozolomide; PCV: Procarbazine, lomustine, and vincristine; NR: Data not reported or not segregated for rGBM patients.

these combined results. Additionally, barriers to effective GKRS salvage treatment are considered. Some studies did not report segregated data for GBM versus Grade I - III gliomas^[11,21-23] or salvage GKRS versus upfront GKRS^[24,25] in all categories; however, we considered data as available.

RESULTS

Table 1 outlines the characteristics of patients from the various studies who were newly diagnosed with GBM. Table 2 shows which upfront treatment modalities were used for newly diagnosed GBM and the time until the first recurrence. In Table 3 we show the available details for salvage treatment modalities. Finally, Table 4 describes the outcomes of these salvage therapies.

Median overall survival from initial diagnosis compared to RPA prognosis

Primarily, salvage therapies for rGBM, such as GKRS, are designed to prolong the life of the patient. Taken as a whole, these studies showed an improved survival compared to classical results from the RTOG-RPA study.

Considering the KPS scores and ages of patients in each study, it is expected that the majority of patients would be classified as RTOG-RPA Classes III or IV with an expected overall median survival of 17.1 or 11.2 mo, respectively^[7]. The longest median survival for these salvage studies was reported in the cohort of eleven patients studied by Park *et al*^[10] in 2012 who were treated with both GKRS and bevacizumab as salvage: 33.2 mo (95%CI: 23.7-42.7 mo) survival after initial GBM diagnosis. These patients were matched to a group of 44 patients who were treated with GKRS salvage without bevacizumab resulting in median overall survival of 26.7 mo (95%CI: 21.8-31.6 mo). While the authors attributed this longer survival to selection bias (KPS scores ≥ 70), it is worth mentioning that four of the eleven patients in the bevacizumab treatment group would be classified in RPA Class V + VI as they were older than 50 and received biopsy only when initially diagnosed, and thus their survival would have classically been very limited.

A 2012 study by Skeie *et al*^[9] demonstrated a survival advantage in patients who received salvage GKRS with salvage gross total resection (median 21 mo) or without salvage gross total resection (median 18 mo). Improved

Table 3 Salvage treatment modalities and details for recurrent glioblastoma multiforme

Ref.	rGBM patients	Margin GK dose (range) Gy	Salvage resection	Salvage radiotherapy	Salvage chemotherapy
Skeie <i>et al</i> ^[9]	32	12.2 (8-20)	None	19 EBRT	3 Temo, 15 PCV
	19		All GTR	12 EBRT	1 Temo, 6 PCV
Park <i>et al</i> ^[10]	11	16 (13-18)	2 debulking	None	All BZ (9 w/irinotecan, 1 w/Temo)
	44	15 (10-20)	7 resection	1 EBRT	19 patients (no BZ)
Koga <i>et al</i> ^[28]	9	20	NR	NR	NR
	9	Extended field, 20	NR	NR	NR
Elliott <i>et al</i> ^[11]	16	15 (12-18)	14 GTR, 2 NTR	NR	NR
Pouratian <i>et al</i> ^[25]	26	6.0 (3.0-15.0)	NR	NR	NR
Kida <i>et al</i> ^[21]	54	14.7 (8-25)	NR	NR	NR
¹ Kong <i>et al</i> ^[23]	65	16 (12-50)	NR	NR	13 Temo or PCV
Kohshi <i>et al</i> ^[22]	11	22 (18-27) in 8 fractions followed by HBO therapy	2 patients	NR	NR
Hsieh <i>et al</i> ^[24]	26	12	NR	NR	NR

¹Kong *et al*^[23] (2008) includes data from 0-5 cases treated with LINAC. rGBM: Recurrent glioblastoma multiforme; GK: Gamma knife; HBO: Hyperbaric oxygen; GTR: Gross total resection; NTR: Near-total resection; EBRT: External beam radiation therapy; Temo: Temozolomide; PCV: Procarbazine, lomustine, and vincristine; BZ: Bevacizumab; NR: Data not reported or not segregated for rGBM patients.

Table 4 Outcomes following Gamma Knife salvage therapy for recurrent glioblastoma multiforme

Ref.	rGBM patients	Med. survival from diagnosis (mo)	Progression-free survival	Median survival after GK salvage (mo)	Time to recurrence, post-GK (mo)	Local tumor control	ARE
Skeie <i>et al</i> ^[9]	32	18.0 (95%CI: 16.7-30.8)	NR	9 (95%CI: 8.7-14.9)	6	18.8%	9.80%
	19	21.0 (95%CI: 21.2-55.9)		15 (95%CI: 12.8-40.4)			
Park <i>et al</i> ^[10]	(salvage GTR)						
	11	33.2 (95%CI: 23.7-42.7)	14.9 (95%CI: 6.5-23.3)	17.9 (95%CI: 10.1-25.7)	All 6 tumors > 10 mL: 5.9 (95%CI: 1.8-10.0) 1 of 5 tumors < 10 mL: 9.5	NR	9.00%
	(salvage BZ)						
	44	26.7 (95%CI: 21.8-31.6)	6.7 (95%CI: 5.6-7.8)	12.2 (95%CI: 8.1-16.3)	19 of 21 tumors > 10 mL: 5.1 (95%CI: 4.0-6.2) 21 of 23 tumors < 10 mL: 8.3 (95%CI: 4.2-12.4)	NR	46%
Koga <i>et al</i> ^[28]	9	24.0 (range, 14-57)	NR	10.5 (range, 3-29)	NR	47.0%	5.90%
	9 (EF GK)	21.0 (range, 15-51)		9 (range, 6-27)		93.0%	28.6%
Elliott <i>et al</i> ^[11]	16	26.1	NR	13 (range, 2.5-37.2)	NR	NR	18.8%
Pouratian <i>et al</i> ^[25]	26	17.4 (95%CI: 14.4-20.4)	7.1 (95%CI: 2.9-11.3)	9.4 (95%CI: 7.9-10.9)	NR	NR	0.0%
Kida <i>et al</i> ^[21]	54	27.0	NR	14.0	NR	NR	NR
¹ Kong <i>et al</i> ^[23]	65	23.0 (95%CI: 16.2-29.3)	4.6 (95%CI: 4.0-5.2)	13 (95%CI: 10.6-16.0)	NR	NR	NR
Kohshi <i>et al</i> ^[22]	11	21.0 (95%CI: 16-26)	NR	11 (95%CI: 4-12)	NR	NR	18.0%
Hsieh <i>et al</i> ^[24]	26	16.7	NR	10.0	NR	NR	NR

¹Kong *et al*^[23] (2008) includes data from 0-5 cases treated with LINAC. rGBM: Recurrent glioblastoma multiforme; GK: Gamma Knife; ARE: Adverse radiation effects; GTR: Gross total resection; BZ: Bevacizumab; NR: Data not reported or not segregated for rGBM patients.

survival was shown for salvage GKRS patients in all RPA Classes, the majority of whom were in Class IV. In 2011, Elliott *et al*^[11] showed that salvage GKRS with repeat resection for rGBM patients resulted in 26.1 mo survival. This longer than average survival could be due, in part, to the relatively small volume tumors which were selected: median 1.35 mL (range, 0.37-6.2 mL).

There were two other studies which compared median overall survival outcomes to the RPA Classes of their patients: Hsieh *et al*^[24] and Pouratian *et al*^[25] reported 16.7 and 17.4 mo survival, respectively. Pouratian *et al*^[25] showed that their patients who had GKRS as salvage had significantly longer survival than those who received upfront GKRS therapy and the group which received the greatest benefit were patients in RPA Class III. Contrarily, the study conducted by Hsieh *et al*^[24] found that patients in Class VI received the greatest survival benefit. It is

worth mentioning that both of these studies used a relatively low median marginal dose for the GKRS therapy: 12 Gy in the Hsieh study and 6.0 Gy in Pouratian.

There have not been large studies assessing the number or frequency of repeated salvage GKRS for recurrent GBM. However there have been some reports that suggest that eligible patients may be offered as many repeated therapies as can be tolerated or are feasible with some initial promising results^[26,27].

Other outcomes

Without treatment, the natural history of a recurrent GBM is grim^[2]. It is expected that the same prognostic indicators mentioned previously are the best predictors of survival following recurrence. It is therefore useful when evaluating the efficacy of GKRS salvage therapy to consider the length of median overall survival after treat-

ment, length of progression-free survival, and time to repeat tumor recurrence post-GKRS salvage.

Median overall survival post-GKRS salvage ranged from 9-17.9 mo, and was reported for all nine of these studies ($n = 322$ patients)^[9-11,21-25,28]. Longer survival was associated with a combination salvage treatment of bevacizumab^[10], or repeated surgical treatment^[9]. Those patient cohorts with a shorter post-salvage survival did not use these combined therapies, used a lower radiation dose for GKRS salvage, or did not report any information for other modes of rGBM salvage therapy^[9,22,24,25,28].

Three of these studies reported progression-free survival data following salvage GKRS for their rGBM patients, which might be useful in counseling a patient on their treatment options^[10,23,25]. Park *et al.*^[10] described 6.7 mo progression-free survival in the 44 patient cohort receiving GKRS salvage compared to 14.9 mo progression-free survival in the 11 patients who received GKRS + bevacizumab salvage ($P = 0.035$). Kong *et al.*^[23] reported only 4.6 mo median progression-free survival following GKRS salvage while their patients demonstrated promising median overall survival of 23 mo. Unfortunately, many of the other patient characteristics, treatment histories, and prognostic factors that they reported were grouped together with Grade III gliomas. This makes it difficult to independently assess their rGBM patient population. Lastly, the smaller dosage GKRS study by Pouratian *et al.*^[25] showed median progression-free survival of 7.1 mo.

Only two of the reviewed studies reported the time to tumor recurrence following GKRS salvage^[9,10]. Predicting the timeline of a second recurrence could be helpful in planning later salvage options. Skeie *et al.*^[9] indicated that the second recurrence was at a median of 6 mo after GKRS compared to 2 mo following repeat resection without GKRS ($P = 0.009$). The study by Park *et al.*^[10] found that the pattern of recurrence was dependent upon initial tumor volume. The tumors which were larger than 10 mL tend to recur in approximately 6 mo while smaller tumors did not recur until around 9 mo independent of the addition of bevacizumab treatment^[10].

Factors contributing to outcomes

Due to the infiltrative nature of GBM, most tumors will recur locally, within 4 cm from the original resection bed^[12]. In the hope of stopping or delaying this progression, it is important to develop a treatment strategy that will achieve local control. Skeie *et al.*^[9] assessed 98 of their treated patients and found that 18.8% of those treated with salvage GKRS achieved local control compared to only 2.0% of those receiving repeat resections only ($P = 0.032$). Koga *et al.*^[28] demonstrated 93% local control in their patients who received 20 Gy to a 0.5-1.0 cm extended field GKRS compared to 47% of patients who were treated with 20 Gy to the gadolinium-enhancing margin only.

Other strategies for delaying progression of GBM include radiosensitization methods such as bevacizumab^[10], chloroquine^[29], or hyperbaric oxygen therapy^[22]. If the tu-

mor tissue can be sensitized to radiation, it may be possible to prescribe a smaller dose of radiation while achieving the same therapeutic effect and reducing adverse side effects. This could open up GKRS salvage to a wider patient population: those with larger volume tumors and/or tumors in close proximity to eloquent brain areas (*e.g.*, basal ganglia, optic chiasm, and brainstem). Kohshi *et al.*^[22] used hyperbaric oxygen therapy combined with fractionated GKRS as salvage in an effort to sensitize the tumor while reducing adverse effects of radiation, resulting in a median survival of 21 mo in their rGBM patients. There has not yet been a randomized clinical trial to test these results so the benefits remain theoretical.

Barriers to effective GKRS salvage

Radionecrosis or radiation induced edema present a challenge for repeat conformal radiation treatment as well as aggressive GKRS salvage. In the studies reviewed, the highest rates of symptomatic radiation side effects were associated with the highest prescribed GK dose to the tumor margin. Koga *et al.*^[28] reported adverse radiation effects in 28.6% of patients who received 20 Gy to the extended margin, this suggests that achieving improved local tumor control may come with an increased risk of side effects. Park *et al.*^[10] demonstrated that bevacizumab not only prolonged survival but also reduced detectable adverse radiation effects from 46% to 9% ($P = 0.037$). Two studies reported 0% radiation side effects in their patients: Pouratian *et al.*^[25] used a median margin dose of 6.0 Gy while Kohshi *et al.*^[22] delivered 22 Gy in 8 fractions (2-3 Gy per fraction).

CONCLUSION

The benefits of salvage GKRS for rGBM appear to be promising in a selected group of patients. However selection bias is uncontrolled in these studies, and poses a significant limitation in these retrospective studies. Without a randomized clinical trial, it is unclear whether these salvage therapies truly prolong survival. A future randomized trial is necessary to demonstrate that radiosurgery is beneficial for rGBM. Future studies should evaluate which RPA Classes benefit most from salvage GKRS.

In our center, Gamma Knife of Spokane, each case of rGBM undergoes a multi-disciplinary review followed by individualized treatment. Temozolomide and EBRT are used as upfront therapy and bevacizumab upon recurrence of GBM. When identifying patients for salvage GKRS *via* surveillance magnetic resonance imaging, treatment is typically offered to those with a KPS of 60 or better, at any age. In patients who are candidates for GKRS, we typically treat rGBM to a marginal dose of 16 Gy. The median tumor volume treated has historically been 16.2 cc (range 0.6-52.2 cc).

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Bone metastases: When and how lung cancer interacts with bone

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Abstract

Bone metastasis is a common and debilitating consequence of lung cancer: 30%-40% of patients with non-small cell lung cancer develop bone metastases during the course of their disease. Lung cancer cells find a favorable soil in the bone microenvironment due to factors released by the bone matrix, the immune system cells, and the same cancer cells. Many aspects of the cross-talk among lung tumor cells, the immune system, and bone cells are not clear, but this review aims to summarize the recent findings in this field, with particular attention to studies conducted to identify biomarkers for early detection of lung cancer bone metastases.

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Key words: Lung cancer; Bone metastases; Osteoclast; T cell; Bone microenvironment

Core tip: This review reports current knowledge on the cross-talk among lung tumor cells, the bone microenvironment, and the immune system, that lead to bone metastasis.

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INTRODUCTION

Lung cancer is the most common tumor worldwide, and in Europe alone, it is responsible for the 20.6% of cancer mortality^[1]. It has poor overall survival rates: the majority of advanced stage lung cancer patients die within 18 mo from diagnosis^[2]. The predominant form of lung cancer is non-small cell lung cancer (NSCLC), and is responsible for 80%-85% of all lung tumors^[3].

Lung cancer frequently metastasizes to bone, with 36% of patients presenting with bone lesions at autopsy^[4] and 22%-60% showing bone marrow micro-metastases^[5]. The early diagnosis of NSCLC is difficult, and 30%-40% of patients with NSCLC develop bone metastases during the course of their disease^[6,7]. The prognosis of lung cancer patients with bone metastases is poor, with a median survival time from detection of lesions measured in months^[4]. Even though NSCLC patients have a relatively short survival time, a large percentage develop skeletal-related events (SREs), thus clinicians show particular attention to the management of bone metastases to prevent debilitating skeletal complications. Indeed, bone metastases are often not diagnosed in NSCLC patients until they cause pain and SREs^[8], but once a patient has a first SRE, he is likely to experience subsequent events, leading to debilitating bone lesions and a reduction in the quality of life. Lung cancer bone metastases normally appear as areas of radiolucency and are osteolytic, with poor margination, no matrix and cortical destruction^[9]. They commonly affect the spine, ribs, pelvis and proximal long bones. At an early stage, bone metastases may occur easily at an axial bone through the vertebral vein system, then at appendicular bone in more advanced

stages of the disease^[10]. A unique feature of this tumor is the ability to spread to the bones of the hands and feet. This is probably due to the ability of lung cancer to shed malignant cells directly into the arterial blood flow, from where they can be seeded far and wide.

In this review, I summarize the current knowledge on the cross-talk among lung tumor cells, the bone micro-environment, and the immune system, that lead to bone metastasis formation.

BONE MICROENVIRONMENT IS A FERTILE SOIL FOR DORMANT AND PROLIFERATING TUMOR CELLS

Tumor dissemination is an early event and is not related to the size of the tumor mass, supporting the idea of concomitant progression of the primary tumor and metastasis^[11]. Several studies have shown that disseminated cells, shed from a primary tumor, may lie dormant in distant tissues for long periods before they can be activated to form metastases. The skeleton has a large surface area and a microenvironment conducive and protective to tumor cell growth. Indeed, disseminated tumor cells (DTCs) can persist in a quiescent state in the bone marrow of cancer patients for years, particularly if they lie in the hematopoietic stem cell (HSC) niche^[12], which expresses adhesion molecules and secretes factors contributing to tumor cell dormancy. At present, it is not clear how dormancy is induced or what leads to activation of the dormant cells. One hypothesis is that molecules that induce HSC dormancy are likely to induce dormancy of metastatic tumor cells^[13]. For instance, osteoblasts (OBs) which are a crucial component of the stem cell niche, express stromal derived factor-1 (SDF-1) and annexin-II (Anxa2), that attract both HSCs and cancer cells expressing their receptors, CXCR4 and Anxa2r, respectively^[14]. In this way, the niche attracts, protects and induces dormancy of DTCs, which may be released from quiescence and grow as a consequence of a stimulatory microenvironment, like bone. DTCs detected in bone marrow of breast cancer patients exhibited features of mesenchymal and cancer initiating cells (CICs)^[15]. These latter cells are responsible for both primary tumor generation, maintenance, recurrence and drug resistance^[16,17]. In lung cancer, a subset of cells expressing CD133 and CXCR4 has been shown to have CIC features and to be essential for tumor metastasis formation^[18]. Moreover, chemotherapy may select resistant CICs^[19], as also demonstrated by Bertolini *et al*^[20] who showed that lung CICs developed resistance to cisplatin. The monitoring of DTCs is a potential method to follow the dissemination of tumors, but a recent work reported that in NSCLC patients, DTC detection is not particularly useful to determine the presence of the disseminated disease in its early stages^[21].

Bone has physical properties such as low pH, hypoxia and high levels of extracellular calcium^[22], which sustain the proliferating tumor cells that secrete high quantities of lactic acid, creating a local area of bone with a low

pH, which stimulates osteoclasts (OCs) activity. On the other hand, tumor cells grow better with a low pH and release proteolytic enzymes, that maintain the low pH, thus tumor expansion is perpetuated^[23]. The active bone resorption causes an increase in extracellular calcium level that stimulates the calcium-sensing receptors on surrounding cells and tumor cells, leading to an increased secretion of parathyroid hormone-related peptide (PTHrP), a potent stimulator of OCs^[24,25]. Bone is a hypoxic tissue, thus it promotes the ability of cancer cells to grow under hypoxic conditions, stimulating the expression of hypoxia inducible factor-1 (HIF-1). HIF-1 activates the transcription of target genes which encode proteins that play important roles in many critical aspects of cancer biology^[26]. For instance, HIF-1 is involved in many steps of breast cancer metastasis, including metastatic niche formation, recruitment of bone marrow-derived cells to the metastatic niche, OC formation and OB inhibition^[27-29].

FACTORS PRODUCED BY BONE AND TUMOR CELLS MODULATE OC ACTIVITY

The interaction of tumor and bone cells induces the formation of a vicious cycle leading to bone metastases^[30]. Indeed, tumor cells disrupt normal bone remodeling, a perfectly balanced activity of bone resorption by OCs and bone formation by OBs. Bone resorption induced by cancer cells creates a physical space for tumor expansion, and it induces the release of growth factors and cytokines supporting tumor metastasis^[31].

Bone marrow produces factors, such as SDF-1, which attracts cancer cells expressing the chemokine receptors, CXCR4 and CXCR7, thus it represents a favorable soil for secondary tumor localization^[32,33]. Moreover, activated OCs resorb bone and release growth factors enmeshed in the bone matrix, such as bone morphogenetic proteins, transforming growth factor- β (TGF- β), insulin-like growth factor (IGF), fibroblast growth factor and others that stimulate the growth of metastatic tumor cells in bone, and the production and release of bone resorbing factors from tumor cells^[34,35]. In particular, TGF- β stimulates HIF-1 signaling within bone, contributing to the vicious cycle driving the development of metastatic osteolysis^[36]. Malignant cells produce cytokines, such as interleukin-6 (IL-6), IL-8 and IL-11, which activate OCs^[37]. IL-6 can stimulate tumor cell proliferation, migration and invasion of lung and other tumors^[38,39], and it may induce bone resorption or formation according to the interactions with other factors like PTHrP, IL-1 and receptor activator of nuclear factor-kB ligand (RANKL). IL-8 stimulates OC maturation through binding with its receptor CXCR1 expressed on OC precursors^[40]. IL-11 stimulates osteoclastogenesis and it has been reported to be a predictive factor for development of osteolytic bone metastasis^[41]. Cancer cells also secrete molecules such as parathyroid hormone, PTHrP, prostaglandins, activated vitamin D and TNF, which stimulate RANKL expression on OBs and bone marrow stromal cells^[42].

RANK, the RANKL receptor, is expressed by solid tumors, with high concordance between bone metastasis and corresponding primary tumors^[43]. The activation of the RANK/RANKL axis leads to an increase in OC number, survival and activity, and it may induce migration and homing of tumor cells to the RANKL-rich bone microenvironment^[44]. In clinical practice the use of target therapy to reduce bone metastases gave encouraging results: the fully human monoclonal antibody directed against RANKL reduces bone metastases in breast and lung tumors^[45-47]. Nuclear factor-kappa B is the downstream target of RANKL and it is also activated in lung cancer cells by epidermal growth factor receptor (EGFR), implicating this pathway as pivotal in lung cancer bone metastases^[46]. EGFR-tyrosine kinase inhibitors (TKIs), such as erlotinib, show dramatic anti-tumor activity in a subset of NSCLC patients with an active mutation in the *EGFR* gene. Some lung cancer patients with wild type EGFR respond to EGFR-TKIs, thus EGFR-TKIs have an effect on host cells as well as tumor cells, preventing bone metastases by affecting the host microenvironment irrespective of its direct effect on tumor cells^[48].

PTHrP promotes OC bone resorption^[49-51], and it stimulates OBs and stromal cells to express RANKL, which induces OC maturation^[52,53]. Among the inhibitors of PTHrP, recent data report that the microRNA miR-33a is downregulated in lung cancer cells, and it directly targets PTHrP leading to decreased osteolytic bone metastasis^[54]. Indeed, the downregulation of PTHrP, induced by miR-33a, causes a decreased secretion of IL-8, with consequent reduction in OC differentiation and bone resorption^[54]. Novel data derived from a pre-clinical model of NSCLC reports that miR335 inhibits the expression of RANKL and IGF-1 receptor by lung cancer cells, thus skeletal metastasis is reduced^[55].

In the bone marrow, a direct activation of osteolysis by cancer cells has been shown through the interaction between Notch and Jagged. Jagged1, a downstream mediator of the pro-metastatic TGF- β , promotes tumor growth through stimulation of IL-6 production from OBs, and directly activates OC differentiation^[56]. Moreover, Jagged is overexpressed by bone metastatic tumor cells^[57], whereas its receptor Notch is frequently expressed by progenitors and mature cells in the bone marrow^[58]. In breast cancer, Notch-Jagged interactions activate biological responses in OCs and OBs, which promote both tumor invasion of bone and tumor cell growth in bone^[56]. In NSCLC, the expression of Notch-3 receptor correlates with a poor prognosis and the stage of the disease^[59].

ROLE OF IMMUNE SYSTEM CELLS IN REGULATION OF OCS AND TUMOR GROWTH IN BONE

Oxidative stress is implicated in the initiation and progression of lung cancer^[60]. In particular, myeloid-derived suppressor cells (MDSC), which infiltrate different tumors,

generate reactive oxygen species (ROS) and cytokines, that suppress host T cell responses, promoting tumor progression and metastasis^[61]. Both the production of ROS and nitric oxide are involved in osteoclastogenesis, and bone marrow-derived MDSCs have been showed to be able to differentiate into OCs; thus, all these factors contribute to enhanced bone destruction in tumor osteolysis^[62,63].

Direct involvement of T cells in regulating OC activity has been demonstrated in patients affected by multiple myeloma, lung cancer and other solid tumors with bone metastasis^[64-67]. These bone metastatic patients showed an increase in circulating OC precursors compared with both healthy controls and cancer patients without bone metastases^[66]. OC precursors differentiate into mature, multinucleated and bone resorbing OCs *in vitro*, without adding exogenous pro-osteoclastogenic factors, such as TNF- α and RANKL, which instead are released by T cells. Cell cultures of PBMCs derived from cancer patients without bone metastasis, and depleted of T cells, do not differentiate into OCs without adding macrophage colony stimulating factor and RANKL^[65,66], confirming that T cells regulate osteoclastogenesis and play an important role in the cancer cycle of bone destruction.

Recently, it has been demonstrated that T cells are additional regulators of bone tumor growth. In particular, their activation diminishes bone metastases, whereas their depletion enhances them, even in the presence of zoledronic acid^[68]. Indeed, some patients treated with anti-resorptive therapies develop further skeletal metastases, suggesting that T cells modulate bone tumor growth. Zoledronic acid can activate cytotoxic γ/δ -T cells and inhibit populations of myeloid-derived cells with T-cell-suppressor capabilities^[69]. The anti-bone metastatic therapy based on the blockade of TGF- β at metastatic sites may locally activate an anti-tumor T cell response, because normally TGF- β , released in bone marrow by OC activity, inhibits T cell proliferation^[70]. Tumor cell-derived IL-6, IL-1 and TGF- β can drive T-cell differentiation towards a Th17 secretory helper-cell phenotype able to induce RANKL production by OB and OC activation through IL-17 production^[71]. All these data demonstrate the fundamental role of immune system cells in the control of bone metastatic disease.

SERUM MARKERS FOR EARLY DETECTION OF LUNG CANCER BONE METASTASES

The delayed demonstration of skeletal involvement may seriously affect survival, thus an early diagnosis of bone metastases is necessary. The early detection of asymptomatic bone disease due to lung cancer can be obtained through positron-emission tomography scans^[72], but European guidelines recommend a bone scan only in the presence of bone pain^[3], thus other systems for an early diagnosis are required. The sensitivity of common serum tumor markers is low, and they are used mainly for monitoring the efficacy of therapy and detection of

recurrence. The use of serological markers is desirable, but unfortunately, the identification of potentially useful and specific biomarkers is difficult. According to data in the literature, serum markers of bone turnover may be able to determine the time to tumor progression, the metastatic potential, and the overall survival of NSCLC patients. Furthermore, they may contribute to a more accurate follow-up and tailored treatment options^[73,74]. In particular, there is a statistically significant relationship between levels of biochemical markers of bone metabolism and clinical outcome: both N-telopeptide and bone-specific alkaline phosphatase levels were highly predictive of SRE recurrence, the time to a first SRE, and the occurrence of disease progression and death^[7,75]. Also, the carboxy-terminal telopeptide of type I collagen and amino-terminal propeptide of type I collagen measurement can be useful in monitoring patients with NSCLC during follow-up, with the aim of detecting bone metastases early^[76].

Among the molecules potentially involved in the pathogenesis of bone metastases from lung cancer, there is IL-7, which has been studied as serum marker for monitoring bone metastasis development in NSCLC patients. Indeed, IL-7 is an important regulator of the interaction between bone and immune system, and it has a role in bone homeostasis, in particular in bone loss in estrogen-deficient conditions^[77,78], psoriatic arthritis^[79] and periodontitis^[80]. Other studies support an active role of IL-7 in promoting bone lesions from solid tumors^[81,82] and multiple myeloma^[83]. In culture of PBMCs derived from patients with bone metastases due to lung cancer and other solid tumors, IL-7 was mainly released by B cells, and it directly sensitized T cells to produce pro-osteoclastogenic factors, such as tumor necrosis factor- α and RANKL, which enhanced osteoclastogenesis^[82,83]. Moreover, in lung cancer patients with bone metastases, IL-7 serum levels were found to be significantly higher than in non-bone metastatic patients and in healthy controls^[84]. This increase in serum IL-7 directly depends on tumor production, and indeed strong IL-7 expression was detected in human tumor masses in a mouse model of bone metastases and in human bone metastatic biopsies^[85]. Further studies on a large cohort of patients needs to be performed, but IL-7 could be very useful to monitor the progression and early development of lung cancer bone disease.

CONCLUSION

The current literature reports the existence of an important interaction among lung tumor cells, the bone micro-environment and immune system cells. Many factors are involved in this cross-talk, which may lead to the discovery of new biomarkers useful for early detection of lung cancer bone metastases.

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mRNA expression of DOK1-6 in human breast cancer

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Abstract

AIM: To examine the expression of downstream of tyrosine kinase (DOK)1-6 genes in normal and breast cancer tissue and correlated this with several clinico-pathological and prognostic factors.

METHODS: DOK1-6 mRNA extraction and reverse transcription were performed on fresh frozen breast cancer tissue samples ($n = 112$) and normal background breast tissue ($n = 31$). Tissues were collected between 1991 and 1996 at two centres and all patients underwent mastectomy and ipsilateral axillary node dissection. All tissues were randomly numbered and the details were only made known after all analyses were completed. Transcript levels of expression were determined using real-time polymerase chain reaction and analyzed against TNM stage, tumour grade and clinical outcome over a 10-year follow-up period.

RESULTS: DOK-2 and DOK-6 expression decreased with increasing TNM stage. DOK-6 expression decreased with increasing Nottingham Prognostic Index (NPI) [NPI-1 vs NPI-3 (mean copy number 15.4 vs 0.22, 95%CI: 2.7-27.6, $P = 0.018$) and NPI-2 vs NPI-3 (mean copy number 7.6 vs 0.22, 95%CI: 0.1-14.6, $P = 0.048$)]. After a median follow up period of 10 years, higher

levels of DOK-2 expression were found among patients who remained disease-free compared to those who developed local or distant recurrence (mean copy number 3.94 vs 0.0000096, 95%CI: 1.0-6.85, $P = 0.0091$), and distant recurrence (mean copy number 3.94 vs 0.0025, 95%CI: 1.0-6.84, $P = 0.0092$). Patients who remained disease-free had higher levels of DOK-6 expression compared to those who died from breast cancer.

CONCLUSION: Decreasing expression levels of DOK-2 and DOK-6 with increased breast tumour progression supports the notion that DOK-2 and DOK-6 behave as tumour suppressors in human breast cancer.

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Key words: Adaptor protein; Breast cancer; Downstream of tyrosine kinase-2; Downstream of tyrosine kinase-6; Mitogen-activated protein kinase; Tyrosine kinase, Tumour suppressor

Core tip: Several members of the downstream of tyrosine kinase (DOK) protein family are identified as modulators of cell proliferation/growth pathways. In addition deregulation of specific DOK members has been associated with specific cancers. This study identifies DOK-2 and DOK-6 as potential tumor suppressors in breast cancer.

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INTRODUCTION

Downstream of tyrosine kinase (DOK) family of adaptor proteins consists of 7 members (DOK1-7) that share a structural topology characterized by an NH2-terminal

pleckstrin homology domain, a central phosphotyrosine-binding domain, followed by SH₂ target motifs in the carboxyl-terminal moiety^[1-9]. They act as common substrates for multiple protein-tyrosine kinases (PTK's), including receptor tyrosine kinases (RTK's) and non-RTK's from which they modulate signalling pathways involved in various natural cellular processes including proliferation, apoptosis, growth and migration^[3,5-8,10-17]. Additionally, PTK signalling has been implicated in the development and progression of various cancers, including breast cancer^[18-22].

Principally based on patterns of expression, two sub-groups exist within the DOK family. DOK-1, DOK-2 and DOK-3 comprise one sub-group primarily expressed in haematopoietic tissues^[7,9,11,23-28], whereas, DOK-4, DOK-5 and DOK-6 are expressed in non-haematopoietic tissues, predominantly within the nervous system^[2-4,29-33]. Separately, expression of DOK-7 is concentrated to skeletal muscle and the heart^[34,35]. DOK-1(p62) was initially identified as a substrate for the Bcr-Abl tyrosine kinase oncoprotein in chronic myelogenous leukaemia (CML)^[7,9]. Consequently, DOK-1 knockdown in murine bone marrow-derived mast cells resulted in increased cell proliferation following mitogenic stimulus^[23]. It was discovered that DOK-1 opposes leukemogenesis by Bcr-Abl through inhibition of the mitogen-activated protein kinase (MAPK) pathway^[7,9,10,12,23].

DOK-1, DOK-2 and DOK-3 have all been found to negatively regulate immunoreceptor signalling^[6,7]. It was initially posited, DOK-1 and DOK-2 both inhibit the Ras/ERK pathway through interaction with RasGAP^[6,7]. Conversely, DOK-3 is understood to inhibit c-Jun N-terminal kinase activation by interacting with SHIP-1 and Grb2^[5,36]. However, evidence exists that DOK-1 and DOK-2 can inhibit the MAPK pathway independent of RasGAP *via* epidermal growth factor receptor (EGFR) signalling^[18,37,38]. DOK-2 can facilitate the successive recruitment of c-Src and its inhibitor Csk to EGFR, inhibiting MAPK^[38]. Individual and concomitant up-regulation of EGFR and c-Src is associated with breast cancer progression. Tyrosine-kinase inhibitors targeted against EGFR are currently in use for breast cancer treatment whilst several against c-Src are being trialled^[20,39-41]. Since DOK-1 association with CML, down-regulation of the DOK-1, DOK-2 and DOK-3 sub-group has been linked with several other haematopoietic cancers, including histiocytic sarcoma and Burkitt's lymphoma^[6,7,9,24-26]. Investigation into non-haematopoietic tissues identified lung adenocarcinoma development in DOK-1, DOK-2 and DOK-3 knockout mice and down regulation of DOK-2 expression in human lung cancer^[1]. Additional non-haematopoietic cancers have since been associated with the DOK-1, DOK-2 and DOK-3 sub-group^[42,43]. In particular, DOK-2 has recently been proposed as a potential marker of poor prognosis in patients with gastric cancer after curative resection^[43].

The other sub-group of the DOK family, consisting of DOK-4, DOK-5 and DOK-6, are positive regulators of the MAPK pathway and promote neurite out-

growth^[3,4,29,32,44]. DOK-4 and DOK-6 are phosphorylated by the tyrosine kinase encoded by the proto-oncogene RET (rearranged by transfection)^[3,31,32,44]. Over expression of RET is primarily observed in oestrogen-receptor-positive (ER+) breast cancers where, in models, it is seen to cause increased cell migration and proliferation. In addition, studies have observed RET expression to correlate with poor prognosis of metastasis-free and overall survival in breast cancer patients^[18,21,45,46]. Conflictingly, DOK-4 has also been found to inhibit Ret-mediated activation of Elk-1, a member of the ETS oncogene family. Elk-1 is a transcription activator associated with cell survival in breast cancer and has previously been implicated as a potential therapeutic target^[18,21,45,46]. Aberrant expression of DOK-4 has been witnessed in non-small cell lung cancer and clear cell renal cell carcinoma^[29,30]. Increased expression of DOK-5 is observed in malignant pheochromocytoma and has been suggested to promote cell survival *via* tropomyosin-receptor-kinase (Trk)C receptor signalling, of which DOK-6 is also a substrate^[33,47]. Jin *et al.*^[48] identified TrkC as a critical regulator of breast cancer cell growth and metastasis. It has been established that DOK-7 plays a relatively unrelated role to the rest of the DOK-family in which it promotes acetylcholine receptor clustering on post-synaptic membranes at neuromuscular junctions *via* activating muscle-specific kinase^[34,35].

In view of DOK proteins adaptor role in tyrosine kinase signalling pathways regulating cell growth and proliferation and their altered levels of expression in other cancers, we have examined the expression profile of DOK1-6 in a cohort of archival normal and breast cancer specimens. Transcript levels were evaluated against established pathological and prognostic parameters in addition to clinical outcome.

MATERIALS AND METHODS

Patients and samples

Institutional guidelines, including ethical approval and informed consent were followed. Primary breast cancer tissues ($n = 112$) and adjacent non-cancerous mammary tissue ($n = 31$) were collected immediately after surgical excision and stored at -80°C until use. Tissues were collected between 1991 and 1996 at two centres (St Georges Hospital in London and University Department of Surgery at Cardiff University School of Medicine). All patients underwent mastectomy and ipsilateral axillary node dissection. All tissues were randomly numbered and the details were only made known after all analyses were completed. Follow-up data were recorded in a custom database. Patients were routinely followed up after surgery (June 2004 was the final comprehensive follow up for the cohort). An independent specialist pathologist examined haematoxylin and eosin stained frozen sections to verify the presence of tumour cells in the collected samples. Where normal non-neoplastic tissues were used, no tumour cells were found in the sections.

All patients were treated according to local algorithms

Table 1 Clinical and pathological data

Parameter number	Category	n
Node status	Positive	54
	Negative	73
Tumour grade	1	24
	2	43
	3	58
Tumour type	Ductal	98
	Lobular	14
	Medullary	2
	Tubular	2
	Mucinous	4
TNM staging	Other	7
	1	70
	2	40
	3	7
	4	4
NPI	1	68
	2	38
	3	16
Clinical outcome	Disease-free	90
	With local recurrence	5
	Alive with metastasis	7
	Died of breast cancer	16

Missing values reflect discarded/un-interpretable values. NPI: Nottingham Prognostic Index.

of management following a multidisciplinary discussion. Patients treated with breast-conserving surgery received adjuvant radiotherapy. Those with hormone-sensitive malignancy received tamoxifen. Fit patients with node-positive breast cancer or hormone-insensitive large and/or high-grade cancer were offered adjuvant chemotherapy. Medical notes and histology reports were used to extract clinico-pathological data (Table 1)^[49].

Materials

RNA extraction kits and reverse transcription kits were obtained from Sigma-Aldrich Ltd (Poole, Dorset, England, United Kingdom). The polymerase chain reaction (PCR) primers were designed using Beacon Designer (Palo Alto, CA, United States) and synthesized by Sigma-Aldrich. Custom made hot-start Master mix for quantitative PCR was obtained from Abgene (Surrey, England, United Kingdom)^[49-51].

Tissue processing, RNA extraction and cDNA synthesis

Frozen sections of tissue were cut at a thickness of 5-10 µm and kept for routine histological analysis. Additional 15-20 sections were mixed and homogenized using a hand-held homogenizer in ice-cold RNA extraction solution. The concentration of RNA was determined using UV spectrophotometry. Reverse transcription was carried out using a reverse transcription kit with an anchored oligo (dT) primer supplied by Abgene, using 1 µg of total RNA in a 96-well plate. The quality of cDNA was verified using Cytokeratin 19 (CK19) primers (Table 2)^[49].

Quantitative analysis

The level of DOK1-6 transcripts from the above pre-

Table 2 DOKs and CK19 primers

Gene	Forward	Reverse
DOK-1	TGGCCCTACACTCTGTG	ACTGAACCTGACCGTACAG GAAGGTGAAGGTTCCAG
DOK-2	ACTGGCCCTACAGGTTTC	ACTGAACCTGACCGTACAC TCAAAGTTGCCCTCTCC
DOK-3	AGAAGGGGAAGTGTGAGG	ACTGAACCTGACCGTACAT CCTTGATAGGGGTCTCC
DOK-4	GCCTCAACGACATCAGTC	ACTGAACCTGACCGTACAC CATACACGTCCAGGTTG
DOK-5	CGTGGTTCACTTTGTAGG	ACTGAACCTGACCGTACAG CAGCAGAGTGGACTTTC
DOK-6	AGAACACGCTTGGTGAAA	ACTGAACCTGACCGTACAA GCTGGGAAATGTCTGTG
CK19	CAGGTCCGAGGTTACTGAC	ACTGAACCTGACCGTACAC ACTTCTGCCAGTGTGCTTC

DOK: Downstream of tyrosine kinase; CK19: Cytokeratin 19.

pared DNA were determined using real-time quantitative PCR based on the Amplifluor technology, modified from a method reported previously^[49,52]. The PCR primers were designed using Beacon Designer software, but to the reverse primer an additional sequence, known as the Z sequence (5'-ACTGAACCTGACCGTACA-3') which is complementary to the universal Z probe (Intergen Inc., Oxford, United Kingdom) was added. The product expands one intron. The primers used are detailed in Table 2. The reaction was carried out using Hotstart Q-master mix (Abgene), 10 pmol of specific forward primer, 1 pmol reverse primer which had the Z sequence, 10 pmol of FAM (fluorogenic reporter dye, carboxyfluorescein) tagged probe (Intergen Inc.), and cDNA from 50 ng of RNA. The reaction was carried out using the IcylerIQ (Bio-Rad Ltd, Hemel Hempstead, England, United Kingdom), which is equipped with an optic unit that allows real-time detection of 96 reactions, under the following conditions: 94 °C for 12 min and 50 cycles of 94 °C for 15 s, 55 °C for 40 s, and 72 °C for 20 s. The levels of the transcript were generated from a standard that was simultaneously amplified with the samples. The levels of gene expression were then normalized against the reference gene *CK19*, which was already quantified in these specimens, to correct for varying amounts of epithelial tissue between samples^[53]. The primers used for CK19 are detailed in Table 2. With every PCR run, a negative control without a template and a known cDNA reference sample as a positive control, were included.

Statistical analysis

The two-sample *t* test was used for statistical analysis of absolute and normalised gene copy number. The transcript levels within the breast cancer specimens were compared to normal background tissues and analyzed against conventional pathological parameters and clinical outcome over a 10 year follow-up period. The statistical analysis was carried out using Minitab version 14.1 (Minitab Ltd. Coventry, England, United Kingdom) using a custom written macro (Stat 2005.mtw).

Table 3 Downstream of tyrosine kinases (1-3) mRNA expression levels (mean \pm SD)

Patient and tumour characteristics	DOK-1	P	DOK-2	P	DOK-3	P
NPI						
1 vs 2	2250 \pm 4652 vs 3988 \pm 7824	0.48	4.37 \pm 10.32 vs 4.14 \pm 12.26	0.950	6802 \pm 18036 vs 37024 \pm 127523	0.150
1 vs 3	2250 \pm 4652 vs 942 \pm 1395	0.19	4.37 \pm 10.32 vs 1.57 \pm 4.55	0.270	6802 \pm 18036 vs 9808 \pm 19782	0.600
2 vs 3	3988 \pm 7824 vs 942 \pm 1395	0.21	4.14 \pm 12.26 vs 1.57 \pm 4.55	0.420	37024 \pm 127523 vs 9808 \pm 19782	0.210
Tumour grade						
1 vs 2	1346 \pm 2587 vs 3886 \pm 6155	0.18	0.651 \pm 1.837 vs 4.63 \pm 12.49	0.190	1259 \pm 2797 vs 37803 \pm 126737	0.084
1 vs 3	1346 \pm 2587 vs 2100 \pm 5520	0.58	0.651 \pm 1.837 vs 4.23 \pm 10.15	0.092	1259 \pm 2797 vs 8954 \pm 23276	0.022
2 vs 3	3886 \pm 6155 vs 2100 \pm 5520	0.37	4.63 \pm 12.49 vs 4.23 \pm 10.15	0.910	37803 \pm 126737 vs 8954 \pm 23276	0.170
TNM						
1 vs 2	2507 \pm 4800 vs 3553 \pm 7084	0.62	6.62 \pm 13.46 vs 1.195 \pm 3.782	0.052	26175 \pm 101693 vs 4508 \pm 9938	0.100
1 vs 3	2507 \pm 4800 vs 0.782 \pm 1.38	0.01	6.62 \pm 13.46 vs 0.00562 \pm 0.00942	0.015	26175 \pm 101693 vs 16879 \pm 39061	0.640
1 vs 4	2507 \pm 4800 vs 626 \pm 1252	0.11	6.62 \pm 13.46 vs 0.00001 \pm 0.00001	0.015	26175 \pm 101693 vs 83.2 \pm 80.9	0.050
2 vs 3	3553 \pm 7084 vs 0.782 \pm 1.38	0.07	1.195 \pm 3.782 vs 0.00562 \pm 0.00942	0.190	4508 \pm 9938 vs 16879 \pm 39061	0.440
2 vs 4	3553 \pm 7084 vs 626 \pm 1252	0.15	1.195 \pm 3.782 vs 0.00001 \pm 0.00001	0.190	4508 \pm 9938 vs 83.2 \pm 80.9	0.014
3 vs 4	0.782 \pm 1.38 vs 626 \pm 1252	0.39	0.00562 \pm 0.00942 vs 0.00001 \pm 0.00001	0.320	16879 \pm 39061 vs 83.2 \pm 80.9	0.300
Survival						
DF vs LR	1839 \pm 4089 vs 1957 \pm 2149	0.95	3.94 \pm 8.96 vs 0.00001 \pm 0.00001	0.009	18203 \pm 86910 vs 16027 \pm 40813	0.910
DF vs DR	1839 \pm 4089 vs 12051 \pm 17043	0.55	3.94 \pm 8.96 vs 0.00246 \pm 0.00349	0.009	18203 \pm 86910 vs 11083 \pm 22940	0.620
DF vs DR	1839 \pm 4089 vs 3023 \pm 6758	0.72	3.94 \pm 8.96 vs 5.93 \pm 17.43	0.750	18203 \pm 86910 vs 21264 \pm 60618	0.880

DOK: Downstream of tyrosine kinase; NPI: Nottingham Prognostic Index; DF: Disease Free; LR: Local Recurrence; DR: Distant Recurrence.

RESULTS

DOKs1 - 6 mRNA expression by quantitative PCR

The DOKs1-6 expression profiles were determined both in absolute terms and normalised against CK19.

DOK-1: DOK-1 (Table 3) was found to be expressed in both normal/benign breast tissue and breast cancer specimens. No significant difference was found between DOK-1 expression in breast cancer specimens and its expression in normal background tissue. The expression of DOK-1 mRNA did not significantly differ with increasing Nottingham Prognostic Index (NPI) or between normal background breast tissue and tumour tissues of patients with different NPI levels. No significant difference in the transcript levels observed with different tumour grades or TNM classes. After a median follow up of 10 years, we found DOK-1 mRNA expression levels did not differ among women who remained disease free compared to those who developed recurrence (local or distant) and compared to those who died from breast cancer.

DOK-2: DOK-2 (Table 3) was found to be expressed in both normal/benign breast tissue and breast cancer specimens. No significant difference was found between DOK-2 expression in breast cancer specimens and its expression in normal background tissue. No significant difference in the transcript levels observed with different tumour grades and the decrease in DOK-2 with increasing NPI was not significant. The expression of DOK-2 mRNA was demonstrated to significantly decrease with increasing TNM stage; TNM-1 vs TNM-3 (mean copy number 6.6 vs 0.0056, 95%CI: 1.4-11.8, $P = 0.015$), and TNM-1 vs TNM-4 (mean copy number 6.6 vs 0.0000072, 95%CI: 1.4-11.8, $P = 0.015$). After a median follow up

of 10 years DOK-2 expression was significantly higher among those who remained disease free compared to those who developed local (mean copy number 3.94 vs 0.0000096, 95%CI: 1.0-6.85, $P = 0.0091$), and distant recurrence (mean copy number 3.94 vs 0.0025, 95%CI: 1.0-6.84, $P = 0.0092$).

DOK-3: There was neither significant difference in DOK-3 (Table 3) mRNA expression levels between cancer tissue and normal background tissue nor there was a significant difference in the transcript levels with different tumour grades or NPI.

DOK-3 mRNA expression was found to significantly decrease with increasing tumour TNM stage: TNM-1 vs TNM-4 (mean copy number 27175 vs 83.2, 95%CI: 41-52143, $P = 0.05$); and TNM-2 vs TNM-4 (mean copy number 4508 vs 83.2, 95%CI: 956-7894, $P = 0.014$).

DOK-4: DOK-4 (Table 4) was found to be expressed in both normal/benign breast tissue and breast cancer specimens. No significant difference was found between DOK-4 expression in normal background tissue and its expression in breast cancer tissue. The expression of DOK-4 mRNA did not significantly differ with increasing NPI or between normal background breast tissue, tumour tissues of patients with different NPI levels and tumour grade. After a median follow up of 10 years, we found DOK-4 mRNA expression levels to be higher among women who remained disease free compared to those who developed local recurrence (mean copy number 328 vs 2.22, 95%CI: 19-631.4, $P = 0.038$). No significant difference in expression levels were found between patients who remained disease free and those that developed distant recurrence or died from breast cancer.

DOK-5: There was neither significant difference in

Table 4 Downstream of tyrosine kinases (4-6) mRNA expression levels (mean \pm SD)

Patient and tumour characteristics	DOK-4	P	DOK-5	P	DOK-6	P
NPI						
1 <i>vs</i> 2	415 \pm 1444 <i>vs</i> 94.7 \pm 275	0.170	31504 \pm 163256 <i>vs</i> 2466 \pm 7194	0.23	15.4 \pm 39.98 <i>vs</i> 7.55 \pm 18.37	0.270
1 <i>vs</i> 3	415 \pm 1444 <i>vs</i> 103.4 \pm 182.6	0.180	31504 \pm 163256 <i>vs</i> 88648 \pm 287508	0.52	15.4 \pm 39.98 <i>vs</i> 0.218 \pm 0.468	0.018
2 <i>vs</i> 3	94.7 \pm 275 <i>vs</i> 103.4 \pm 182.6	0.910	2466 \pm 7194 <i>vs</i> 88648 \pm 287508	0.32	7.55 \pm 18.37 <i>vs</i> 0.218 \pm 0.468	0.048
Tumour grade						
1 <i>vs</i> 2	589 \pm 1860 <i>vs</i> 188 \pm 819	0.410	68655 \pm 261421 <i>vs</i> 5756 \pm 14725	0.32	12.96 \pm 28.62 <i>vs</i> 12.41 \pm 39.55	0.960
1 <i>vs</i> 3	589 \pm 1860 <i>vs</i> 147.5 \pm 623.8	0.350	68655 \pm 261421 <i>vs</i> 28639 \pm 155311	0.55	12.96 \pm 28.62 <i>vs</i> 6.22 \pm 21.56	0.430
2 <i>vs</i> 3	188 \pm 819 <i>vs</i> 147.5 \pm 623.8	0.830	5756 \pm 14725 <i>vs</i> 28639 \pm 155311	0.35	12.41 \pm 39.55 <i>vs</i> 6.22 \pm 21.56	0.440
TNM						
1 <i>vs</i> 2	229 \pm 829 <i>vs</i> 403 \pm 1568	0.610	27103 \pm 152075 <i>vs</i> 5096 \pm 19916	0.30	10.26 \pm 33.54 <i>vs</i> 13.68 \pm 32.53	0.680
1 <i>vs</i> 3	229 \pm 829 <i>vs</i> 83.9 \pm 222	0.330	27103 \pm 152075 <i>vs</i> 200882 \pm 446722	0.44	10.26 \pm 33.54 <i>vs</i> 2.07 \pm 4.62	0.130
1 <i>vs</i> 4	229 \pm 829 <i>vs</i> 49.4 \pm 62.1	0.160	27103 \pm 152075 <i>vs</i> 0.198 \pm 0.184	0.20	10.26 \pm 33.54 <i>vs</i> 0.0734 \pm 0.1464	0.048
2 <i>vs</i> 3	403 \pm 1568 <i>vs</i> 83.9 \pm 222	0.340	5096 \pm 19916 <i>vs</i> 200882 \pm 446722	0.38	13.68 \pm 32.53 <i>vs</i> 2.07 \pm 4.62	0.098
2 <i>vs</i> 4	403 \pm 1568 <i>vs</i> 49.4 \pm 62.1	0.280	5096 \pm 19916 <i>vs</i> 0.198 \pm 0.184	0.11	13.68 \pm 32.53 <i>vs</i> 0.0734 \pm 0.1464	0.047
3 <i>vs</i> 4	83.9 \pm 222 <i>vs</i> 49.4 \pm 62.1	0.720	200882 \pm 446722 <i>vs</i> 0.198 \pm 0.184	0.37	2.07 \pm 4.62 <i>vs</i> 0.0734 \pm 0.1464	0.340
Survival						
DF <i>vs</i> LR	328 \pm 1205 <i>vs</i> 2.22 \pm 2.5	0.038	23013 \pm 139049 <i>vs</i> 10477 \pm 22630	0.53	11.38 \pm 33.96 <i>vs</i> 9.24 \pm 18.47	0.840
DF <i>vs</i> DR	328 \pm 1205 <i>vs</i> 40.5 \pm 77.9	0.074	23013 \pm 139049 <i>vs</i> 1022 \pm 2240	0.21	11.38 \pm 33.96 <i>vs</i> 20.7 \pm 35.8	0.700
DF <i>vs</i> DR	328 \pm 1205 <i>vs</i> 84.1 \pm 185	0.140	23013 \pm 139049 <i>vs</i> 81855 \pm 276356	0.47	11.38 \pm 33.96 <i>vs</i> 0.209 \pm 0.45	0.013

DOK: Downstream of tyrosine kinase; NPI: Nottingham Prognostic Index; DF: Disease Free; LR: Local Recurrence; DR: Distant Recurrence.

DOK-5 (Table 4) mRNA expression levels between cancer tissue and normal background tissue nor there was a significant difference in the transcript levels with different tumour grades or TNM classes. The expression of DOK-5 mRNA did not significantly differ with increasing NPI and after a median follow up of 10 years, no significant difference in expression was observed between survival statuses.

DOK-6: DOK-6 (Table 4) was found to be expressed in both normal/benign breast tissue and breast cancer specimens. No significant difference was found between DOK-6 expression in normal background tissue and its expression in breast cancer tissue. The expression of DOK-6 mRNA was found to decrease with increasing NPI; NPI-1 *vs* NPI-3 (mean copy number 15.4 *vs* 0.22, 95%CI: 2.7-27.6, $P = 0.018$) and NPI-2 *vs* NPI-3 (mean copy number 7.6 *vs* 0.22, 95%CI: 0.1-14.6, $P = 0.048$), but there was no significant decrease with higher tumour grade. The expression of DOK-6 mRNA was demonstrated to significantly decrease with increasing TNM stage; TNM-1 *vs* TNM-4 (mean copy number 10.3 *vs* 0.073, 95%CI: 0.1-20.3, $P = 0.048$), and TNM-2 *vs* TNM-4 (mean copy number 13.7 *vs* 0.073, 95%CI: 0.2-27.04, $P = 0.047$). After a median follow up of 10 years, patients who died from breast cancer had significantly lower DOK-6 expression compared those whom remained disease free (mean copy number 11.4 *vs* 0.21, 95%CI: 2.5-19.9, $P = 0.013$).

DISCUSSION

Here we analyze the mRNA expression profiles of the DOK1-6 family members in breast cancer specimens and identify decreased expression of individual members with

increasing clinical and pathological stage.

The results show a decreased DOK-2 expression with increasing TNM stage. Down regulation of DOK-2 has previously been documented in several human epithelial cancers including lung, colorectal and gastric cancer^[1,24,42,43]. Additionally, we have found higher DOK-2 levels to correlate a significantly lower chance of both local and distant recurrence within the 10-year period following surgical resection. Similarly, a recent study also found higher DOK-2 expression to be a good indicator of non-recurrence in gastric cancer patients following resection^[43]. The prediction of recurrence and metastasis after curative resection can determine the need for intensive follow-up and adjuvant therapy.

In addition to this the *DOK2* gene is localized to chromosome 8p21.3, one of the most frequently deleted regions in human lung cancer and a region hypothesized to contain multiple tumour suppressor genes^[54,55]. DOK-2 has previously been thought to associate with cancer progression *via* its regulatory role of the MAPK pathway. Initially, DOK-2 was found to inhibit the MAPK pathway through interaction with the Ras inhibitor RasGAP^[12,23]. Further studies identified a RasGAP-independent route of MAPK inhibition with DOK-2 shuttling c-Src to the EGFR where, after transient activation of c-Src, a phosphorylated DOK-2 recruits the negative regulator of c-Src, Csk. Both EGFR and c-Src are up-regulated in a large percentage of human breast tumours supporting a potential tumour suppressor role of DOK-2 in breast cancer^[38].

Furthermore, our results display decreased DOK-6 expression with increased TNM stage as well as NPI, suggesting DOK-6 could hold prognostic value. Interestingly, significantly higher levels of DOK-6 expression were found in specimens of patients that remained dis-

ease free compared to those that died of breast cancer within the following 10-year period. Together these results highlight DOK-6 as potentially possessing tumour suppressor function. In comparison to other family members, less research has been undertaken into DOK-6 function and to our knowledge it has not previously been directly associated with any human cancer, *in vivo*^[3,32]. DOK-6 has been suggested to play a role in neurite outgrowth *via* both Ret and TrkC signalling^[3,32]. However, it has been indicated that DOK-6 is involved in Ret signalling with less influence when compared with DOK-1 and DOK-4^[32]. The exact function of DOK-6 in TrkC signalling has of yet to be established, although knockdown of DOK-6 has been observed to decrease neurite outgrowth in cortical neurons upon NT-3 stimulation^[33]. TrkC has recently been proposed to act as a critical regulator of breast cancer cell growth and metastasis. Interaction between TrkC and c-Src was found to activate MAPK cascade *in vivo* in human breast cancer tissues^[21,38,48,56,57]. It is worth noting that despite DOK-5 also regulating MAPK activity *via* TrkC interaction, unlike DOK-6, we observe no difference in its expression^[47].

DOK-2 and DOK-6 are members of different subgroups within the DOK adaptor protein family and although each is a substrate of multiple tyrosine kinases, current literature places their natural functions and interactions relatively distinct from one another. Our results, however, indicate that they both function within breast tumour cells and loss of their expression is associated with tumour progression. The two DOK proteins may regulate independent pathways or could potentially function together in an as yet undefined pathway.

Goel *et al.*^[58] have recently published qualitative data of a lucid differential pattern of DOK-1 expression in 8 breast cancer cell lines compared to a non-tumourigenic breast epithelial-derived cell line. We believe our study to be the first that has quantitatively measured DOK-1 expression in human breast cancer specimens. No significant difference was observed between normal/background tissue and breast cancer tissue. Using histologically normal appearing samples as the sole control tissue is probably less appropriate. The use of donor tissues from reduction mammoplasty specimens (ideally obtained under similar conditions as the tumour tissue) will serve as a better control. Donor control, in addition to normal adjacent to tumours, precancerous lesions, and tumour samples will provide the best sample set for resolution of genetic alterations that are relevant to the disease process by minimizing the potential implications of field cancerisation. As there was no normal donor tissue used in this study, it was not clear whether the absence of a difference in DOK family expression between tumour and matched normal background tissue in our cohort was due to the effect of field cancerisation.

To our knowledge, this study is the first to analyze the mRNA expression of the DOK family members 1-6 in breast cancer specimens. Our data support the notion that DOK-2 behave as a tumour suppressor. For

the first time we also provide novel data predicting a role of DOK-6 as a potential tumour suppressor in breast cancer. Nevertheless, our understanding of the molecular mechanisms involved, across all DOK members, in their adaptor function to multiple tyrosine kinases requires further study. Our data could be used in further validation studies in order to define clear mechanisms attributing to the development and progression of breast cancer and develop new prognostic markers and novel therapeutic strategies.

COMMENTS

Background

Identification of molecular biomarkers has shown great promise in the identification of metastatic risk and survival rate in breast cancer as well as requirement for adjuvant therapies. The downstream of tyrosine kinase (DOK) family of adaptor proteins have previously been associated with the progression of other cancers including other human epithelial cancers.

Research frontiers

Several members of the DOK protein family are identified as modulators of cell proliferation/growth pathways. In addition deregulation of specific DOK members have been associated with specific cancers. This study identifies DOK-2 and DOK-6 as a potential tumor suppression in breast cancer.

Innovations and breakthroughs

The identification of molecular biomarkers and their subsequent use to determine risk of recurrence and prognosis in breast cancer has begun to make real clinical impact. This is the first study correlate expression of several DOK family members with clinical and pathological parameters of breast cancer and identify DOK-2 and DOK-6 as having potential tumour suppressor function as well as hold prognostic value.

Applications

Further understanding and identification of members of pathways through which breast cancer can progress is essential in the development of effective therapeutic strategies. In addition, the potential prognostic value of DOK-2 and DOK-6 may determine need for post-curative therapies.

Peer review

This work tackles a novel and interesting issue: the expression profile of DOK proteins in breast cancer. The importance of this study lies in that by first time it is suggested a role of DOK proteins as tumour suppressors in breast cancer.

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Evolution of costs of cancer drugs in a Portuguese hospital

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RESULTS: The overall costs of cancer drugs increased gradually between 2004 and 2008 (from €1911947 to €3666284), with an increase in the number of patients treated during this period. The expenditure decreased in 2009 (€3438155) and increased again in 2010 (€3673116), but the costs increment was not the same as in previous years. Chemotherapy and targeted therapy were responsible for most of the expenditure. Drugs placed on the national market before 1999 accounted for more than 50% of the expenditure up to 2007. From 2008, these drugs represented less than 50% of the total expenditure. Cancer drugs placed between 1999 and 2002 accounted for 25%-35% of the costs in all the years studied, while drugs placed between 2003 and 2005 accounted for less than 30%. Drugs placed between 2006 and 2010 were responsible for less than 10% of the expenditure.

CONCLUSION: In this study, older drugs were responsible for most of the expenditure up to 2007, which is in agreement with the Karolinska study.

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Key words: Cancer; Costs; Drugs; Economy; Treatment

Abstract

AIM: To analyze the costs of cancer drugs administered in a Portuguese Hospital compared with the Karolinska Institute study.

METHODS: To evaluate spending on cancer drugs, we retrospectively analyzed data on the overall costs of cancer drugs, obtained at the Department of Medical Oncology of the Centro Hospitalar de Entre Douro e Vouga, between 2004 and 2010. In this comparative study we selected only drugs belonging to the following groups: chemotherapy, targeted therapy, immunotherapy and endocrine therapy. The selected drugs were further grouped according to their market placement year: ≤ 1998, 1999 to 2002, 2003 to 2005, and 2006 to 2010. Drugs used as supportive therapy and bisphosphonates were excluded.

Core tip: In the last decade costs related to cancer drugs have increased significantly. This growth seems to be explained by the increase in cancer incidence, new indications for treatment with previously approved cancer drugs and to placement of new drugs on the market, which are frequently more expensive than those already on sale. The results of the Karolinska Institute study demonstrated a substantial increase in available cancer drugs and costs between 1998 and 2007. The cost increment was not only related to the introduction of new drugs, but 68% of the costs in 2007 were due to drugs approved before 1999.

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E, Magano A, Loureiro M, Araújo A. Evolution of costs of cancer drugs in a Portuguese hospital. *World J Clin Oncol* 2014; 5(2): 164-169 Available from: URL: <http://www.wjg-net.com/2218-4333/full/v5/i2/164.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i2.164>

INTRODUCTION

In Portugal, the number of inhabitants and the average life expectancy at birth have increased in the last century^[1,2]. The most recent data revealed that the average life expectancy in Portugal is 80.8 years, similar to the European average^[3]. This increase reflects improvements in the population's socioeconomic conditions and in the resources dedicated to health care^[1,4,5].

In the aging population, among other aspects, an increase in the incidence in chronic and incurable diseases has been observed^[4,6,7]. Of these diseases, the incidence of cancer dominates^[4,6,7]. Cancer incidence has increased over the last decades and in 2008 there was an estimated 12.7 million new cancer cases worldwide^[4,6,7]. In Portugal, the cancer incidence rate standardized by age is 428:100000 in men and 289:100000 in women^[1]. Globally, cancer is the second most common cause of death after cardiovascular diseases^[1,8]. In recent years, there has been a slight decrease in the mortality rate related to cancer^[1,4,6-8]. However, this rate is still high^[1,3-5]. In 2008, 7.6 million cancer deaths occurred worldwide^[7].

The burden of cancer to society can be measured by direct and indirect costs^[4,6,9,10]. Direct costs are related to prevention and treatment, while indirect costs include loss of production due to inability to work caused by disease, disability and death^[4,6,9,10]. Drugs are one of the most investigated components of Oncology, consuming most of its economic resources^[4,8,11]. In the past few years, direct costs related to cancer treatment have increased significantly^[4,12-15]. This increment in costs can be explained by the increase in cancer incidence, new indications for treatment with previously approved cancer drugs and to placement of new drugs on the market, which are frequently more expensive those already on sale^[4,12,16-19]. Despite the continuous growth in expenditure due to cancer drugs, this growth is not expected to be the same as in the last decade^[4].

According to the Karolinska Institute study, in 2007 the cost increment was not only related to the introduction of new drugs, but 68% of the costs were due to drugs approved before 1999 in Europe^[4]. The increased cost of these drugs, from €4.3 to €26.3 per capita, was the major cause of the rise in costs related to cancer drugs^[4]. In this study, cancer drugs (chemotherapy, targeted therapy, endocrine therapy and immunotherapy) were grouped according to their market placement year: ≤ 1998; 1999 to 2002; 2003 to 2005; 2006 to 2007. Supportive drug treatments were excluded^[4].

In Portugal, the growth in public spending on cancer drugs has been the subject of great debate^[1,9]. However, data related to cancer treatment, particularly the direct

costs of drug treatments are scarce^[9]. For this reason, we conducted the current study to better understand the costs involved in cancer drug therapies in Portugal.

The aims of the current study were the analysis the cost evolution of cancer drugs from data collected from 2004 to 2010 at the Department of Medical Oncology of the Centro Hospitalar de Entre Douro e Vouga. An analysis of costs according to the type of drug and the date of its placement on the national market was also performed and compared with the results obtained in the Karolinska Institute study.

MATERIALS AND METHODS

Study design

After obtaining the necessary authorization from our Administration Board, we conducted a retrospective observational study to analyze the evolution of costs of cancer drugs from 2004 to 2010 in the Department of Medical Oncology of the Centro Hospitalar de Entre Douro e Vouga. The first year studied was 2004 because there was difficulty in obtaining data relating to previous years, and the last year studied was 2010 as data collection was conducted in 2011.

The Centro Hospitalar de Entre Douro e Vouga, Portugal, is a medium-sized hospital, established in 1999, with 409 beds and is responsible for the health care of 350000 inhabitants. Until 2010, the Department of Medical Oncology treated solid (unless sarcomas, melanomas and tumors of the central nervous system) and hematologic malignancies, mostly in outpatient settings. Thereafter, the Department only treats solid malignancies.

Patients and drug selection

The patients selected were treated in the Department of Medical Oncology from 2004 to 2010. Due to incomplete records on the number of patients treated by the various types of treatment in the first four years analyzed, it was not possible to calculate the average cost per patient and the cost per type of treatment.

According to data provided by the Pharmacy Department, we selected all the cancer drugs used during the study period in both inpatients and outpatients, and divided this by the type of drug: chemotherapy (cytostatics), targeted therapy (monoclonal antibodies, tyrosine kinase inhibitors, mammalian target of rapamycin inhibitors), immunotherapy and endocrine therapy. To analyze the costs, we considered the absolute global cost (purchasing cost to the hospital) for each drug, which was provided by the Pharmacy Department.

To compare our results with the Karolinska Institute study, drugs used in the Department of Medical Oncology were grouped according to their market placement year: ≤ 1998; 1999 to 2002; 2003 to 2005; 2006 to 2010. To access dates of placement on the national market, we consulted the Portuguese National Authority for Medication and Healthcare Products database. As supportive drugs were not analyzed in the Karolinska study, these drugs were excluded in the present study. Other drug

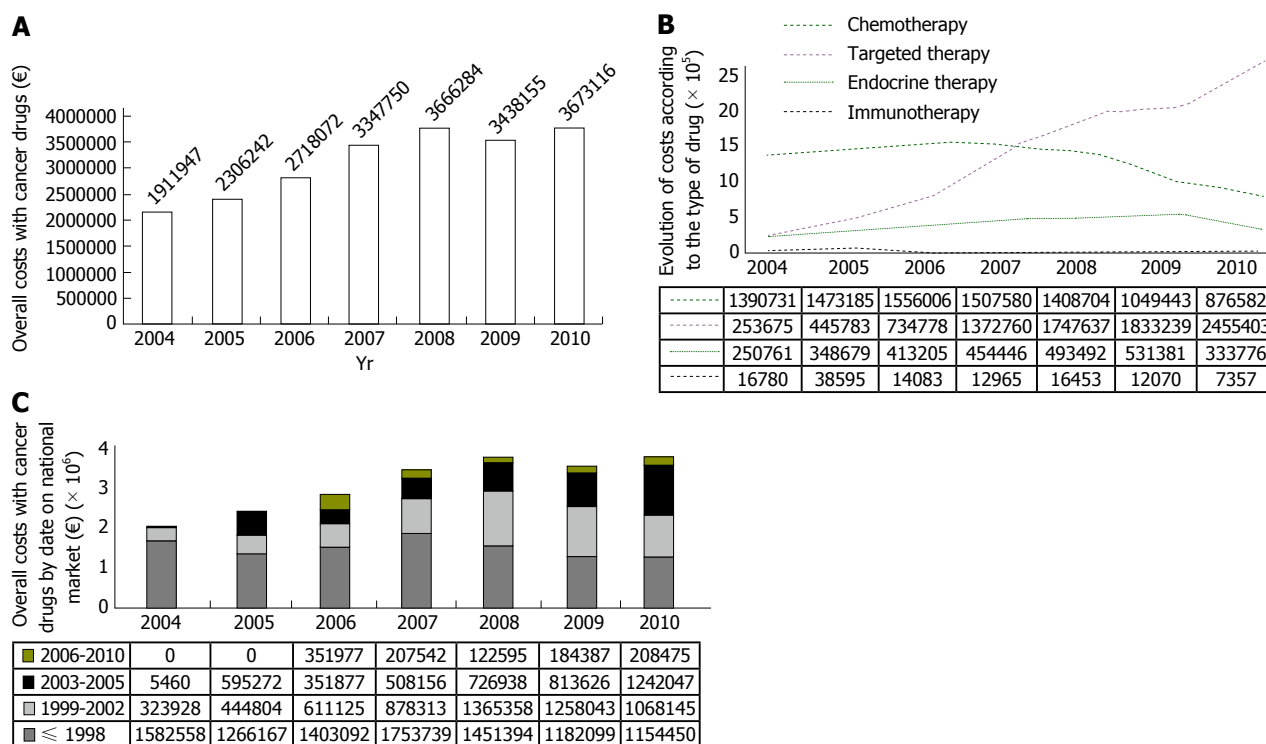


Figure 1 Evolution of global costs of cancer drugs. A: Evolution of global costs of cancer drugs used at the Department of Medical Oncology of Centro Hospitalar de Entre Douro e Vouga; B: Evolution of costs by type of drug administered at the Department of Medical Oncology of Centro Hospitalar de Entre Douro e Vouga; C: Evolution of costs of cancer drugs used at the Department of Medical Oncology of Centro Hospitalar de Entre Douro e Vouga, according to their market placement year.

costs not used in cancer treatment were also excluded.

RESULTS

From 2004 and 2010 there was a gradual increase in the number of patients treated in the Department of Medical Oncology (924 patients in year 2004, 1111 in year 2005, 1222 in 2006, 1376 in 2007, 1550 in 2008, 1589 in 2009 and 1560 patients in year 2010). According to data provided by the Pharmacy Department and specifically for the period under analysis, this increase was followed by an increment in the overall costs of cancer drugs up to 2008 (Figure 1A). In 2009, the global expenditure decreased, and in 2010 there was an increase in expenditure to values similar to those in 2008 (Figure 1A).

The evolution of costs, according to the type of drug, is presented in Figure 1B. Chemotherapy and targeted therapy accounted for most of the expenditure, followed by endocrine therapy. The drugs studied according to the year of placement on the national market are described in Table 1.

The global distribution of the drugs used by the Department, according to placement on the national market, is shown in Figure 1C.

Drugs placed on the national market before 1999 accounted for more than 50% of the expenditure on drugs up to 2007 (in 2004 these accounted for 83%; in 2005 for 55%; in 2006 and 2007 for 52% of the expenditure, respectively). After 2008, these drugs represented less than 50% of the total expenditure, and in the last two years

of the study expenditure overlapped in drugs placed between 2003 and 2005 (in 2008 these represented 40%; in 2009 34% and in 2010 31% of the costs). Drugs placed between 1999 and 2002 accounted for 25%-35% of the expenditure on drugs in each year analyzed. When the drugs placed between 2003 and 2005 were analyzed, there was a progressive increase in the costs (in 2004 these accounted for 0.3% of the expenditure; in 2005 for 26%; in 2006 for 13%; in 2007 for 15%; in 2008 for 20%; in 2009 for 24% and in 2010 for 34%). The most recent drugs (from 2006 to 2010) accounted for less than 10% of the expenditure, with higher spending in 2006 and increased expenditure again after 2008.

Expenditure on chemotherapy, targeted therapy and endocrine therapy according to the date of placement on the national market is shown in Figure 2, respectively. The expenditure on immunotherapy is not represented here as it was found to have the lowest cost.

With regard to the expenditure on chemotherapy (Figures 1B and 2A), the costs of cytostatics decreased after 2006 and these drugs were no longer responsible for the main costs related to cancer treatment after 2007. The drugs placed on the market before 1999 accounted for the largest expenditure in all the years analyzed, while drugs placed between 1999 and 2002 accounted for less than 25% of the expenditure. The drugs placed between 2003 and 2005 accounted for about 20% of costs, showing a progressive decrease. More recent cytostatics accounted for less than 10% of the expenditure.

The expenditure for targeted therapy increased over

Table 1 Drugs selected in the study and their date of introduction to the Portuguese market

Drugs on the national market before 1999	Market placement yr	Drugs on the national market before 1999	Market placement yr	Drugs on the national market between 1999 and 2010	Market placement yr
Aldesleukin	1992	Ifosfamide	1979	Bleomycin	2001
Amifostine	1995	Interferon Alfa-2A	1998	Capecitabine	2001
Anastrozole	1996	Intravenous vinorelbine	1993	Exemestane	1999
Bicalutamide	1998	Irinotecan	1997	Hydroxyurea	2001
Bleomycin	1998	Letrozole	1997	Imatinib	2001
Carboplatin	1989	Megestrol	1987	Ketoconazole	2002
Carmustine	1983	Melphalan	1966	Leuporelin	1999
Chlorambucil	1966	Mercaptopurine	1997	Liposomal Doxorubicin	2000
Cisplatin	1980	Methotrexate	1993	Oral vinorelbine	2001
Cyclophosphamide	1960	Mitomycin	1984	Raltitrexed	2001
Cyclosporine	1990	Mitoxantrone	1998	Temozolomide	1999
Cyproterone	1994	Octreotide	1989	Trastuzumab	2000
Cytarabine	1996	Oxaliplatin	1993	Anagrelide	2004
Dacarbazine	2000	Paclitaxel	1997	Bevacizumab	2005
Dactinomycin	1980	Pegylated liposomal doxorubicin	1996	Bortezomib	2004
Docetaxel	1995	Procarbazine	1998	Cetuximab	2004
Doxorubicin	1998	Rituximab	1998	Erlotinib	2005
Epirubicin	1992	Tamoxifen	1984	Fulvestrant	2004
Etoposide	1998	Tegafur	1985	Interleukin 2	2005
Estramustine	1982	Thalidomide	1961	Pemetrexed	2004
Fludarabine	1995	Topotecan	1996	Azacitidine	2008
Fluorouracil	1997	Vaccine, Bacillus Calmette-Guerin	1992	Lapatinib	2008
Flutamide	1998	Vinblastine	1991	Sorafenib	2006
Gemcitabine	1996	Vincristine	1993	Sunitinib	2006
Goserelin	1998			Temsirolimus	2007
Idarubicin	1995			Trabectedin	2008

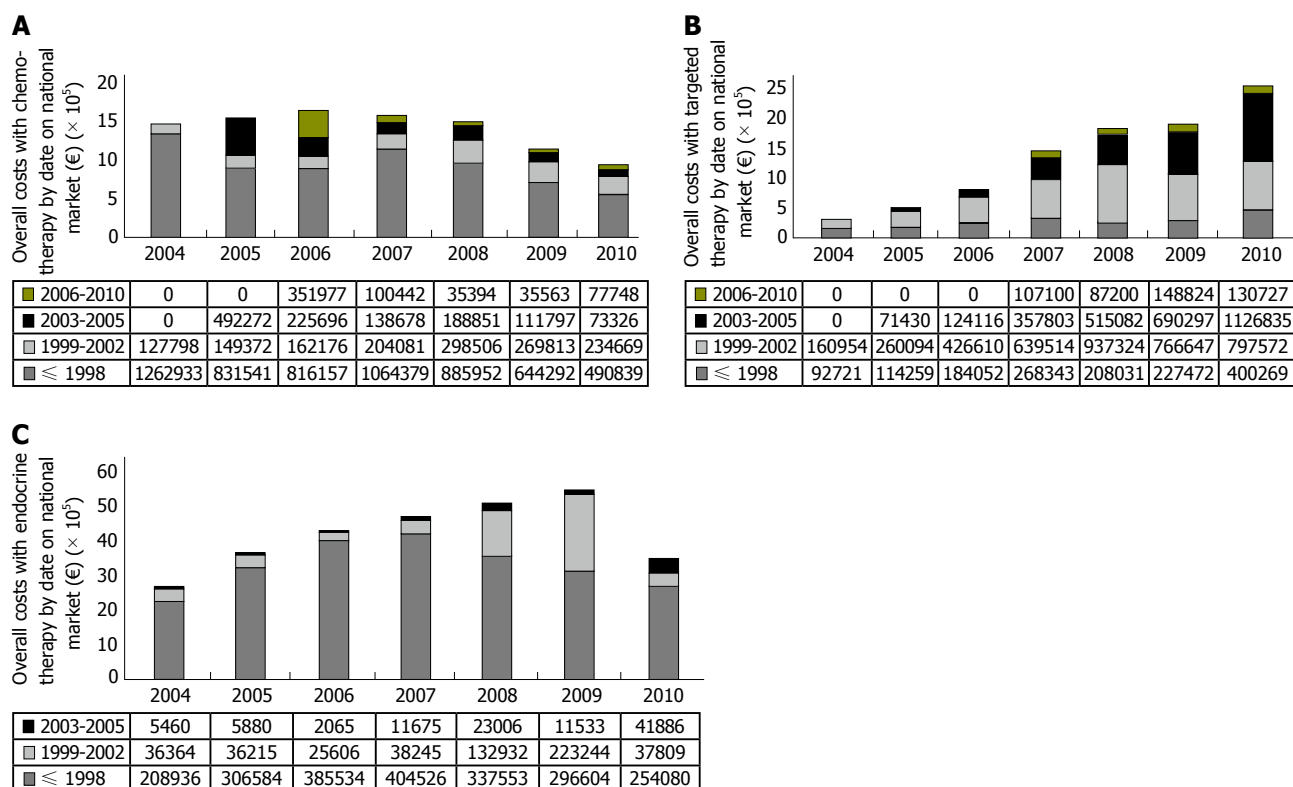


Figure 2 Evolution of costs associated with therapy. A: Evolution of costs associated with chemotherapy treatment at the Department of Medical Oncology of Centro Hospitalar de Entre Douro e Vouga according to the market placement year; B: Evolution of costs associated with targeted therapy at the Department of Medical Oncology of Centro Hospitalar de Entre Douro e Vouga according to the market placement year; C: Evolution of costs associated with endocrine therapy at the Department of Medical Oncology of Centro Hospitalar de Entre Douro e Vouga according to the market placement year.

the years, and was responsible for most costs after 2007 (Figures 1B and 2B). Drugs placed on the market be-

tween 1999 and 2002 cost most in each year studied, except in 2010 where most of the expenditure was on

more recent drugs (placed between 2003 and 2005). When analyzing the drugs placed before 1999, there was an increase in expenditure due to these drugs up to 2006, after which a decrease in costs was observed, with a new increment in the last year studied. The cost of drugs placed between 2003 and 2005 increased gradually during the study period. The most recent drugs (placed between 2006 and 2010) used in cancer treatment cost least and this cost remained stable.

When analyzing the expenditure on drugs for endocrine therapy (Figure 2C), it was observed that drugs placed before 1999 represented the biggest share of the expenditure. Drugs placed between 1999 and 2002 accounted for 25% to 33% of the expenditure in each year and drugs placed between 2003 and 2005 showed a progressive increase in costs, and was more prominent in 2010.

DISCUSSION

Oncology has registered important progress in available treatments, especially cancer drugs, resulting in a significant improvement in healthcare in recent years, both in terms of overall survival and quality of care^[20-24].

In Europe, between 1998 and 2007, there was an important increase in direct costs related to cancer drugs^[4,16,25]. The primary reasons for this were new indications for already approved drugs and the introduction of new drugs which cost significantly more than most of the older cancer drugs^[4,6,7]. According to the Karolinska study, the increase in costs was mainly due to the growth in sales of drugs already on the market^[4]. In 2007, drugs placed on the market before 1999 accounted for 68% of the total costs of drugs for cancer; drugs placed between 1999 and 2002 accounted for 17%; drugs placed between 2003 and 2005 accounted for 11%; and drugs placed between 2006 and 2007 accounted for 3%^[4].

In our study, chemotherapy was mainly responsible for the costs of cancer drugs up to 2007, after which targeted therapy was responsible for most of the expenditure. In this work, we observed an increase in global costs between 2004 and 2008. Explanations for this increment may be related to the increased number of patients treated, to more drug administration cycles per patient (data not shown) in part due to better overall survival, and to the placement of new drugs on the market, which are frequently more expensive than those already on sale. The expenditure decreased in 2009 and increased again in 2010, but the costs increment was not the same as in previous years. Possible reasons for the cost stability over the last 3 years of the study may be at the local level. The Department of Medical Oncology has adopted certain strategies which may explain this cost stability, such as the acquisition of drugs in smaller doses, a study of cytostatics stability to determine the time allowed to administer a drug after its reconstitution, implementation of treatment guidelines at the Department for the most frequent cancer pathologies, and specific scheduling during weekdays to administer certain drugs such as monoclonal antibodies,

and reducing waste. Other reasons for this observed stability in expenditure may be more general, but of great importance, and related to patent expiration, greater use of generics, increased competition and optimization on the negotiation of prices between the Healthcare Centers Board and Pharmaceutical Industries.

The overall results of our study were in accordance with those published by the Karolinska Institute^[4]. Up to 2007, drugs placed on the national market before 1999 were responsible for most of the expenditure. Reasons for this may include new treatment indications for these drugs and the loss of some drug patents leading to a reduction in their price. Drugs available between 1999 and 2002 were the second leading cause of expenditure, followed by drugs placed on the market between 2003 and 2005. Recently placed drugs (from 2006) accounted for a smaller percentage of the costs. However, after 2008 there was a reduction in costs for previously available drugs followed by a gradual increase in the expenditure for new drugs.

Nevertheless, these results may be affected by several confounding factors which influence the price of pharmaceuticals, such as reimbursement and pricing policy, and the Portuguese financial system. Other limitations in our study are related to the retrospective analysis and the period studied that may have resulted in selection bias with many confounding factors.

In summary, in this study older drugs were responsible for most of the expenditure in cancer treatments up to 2007, after which we observed an increase in expenditure related to new drugs. Despite this increase in expenditure on new drugs in the last 3 years analyzed, the increase in costs for cancer drugs was not the same as in the previous years. However, more studies must be undertaken to fully understand this situation in Portugal.

COMMENTS

Background

Drugs are one of the most investigated components of Oncology, consuming most of its economic resources. Over the past few years, direct costs related to cancer treatment have increased significantly. This growth seems to be explained by the increase in cancer incidence, new indications for treatment with previously approved cancer drugs and to the placement of new drugs on the market, which are frequently more expensive than those already on sale. However, data related to cancer treatment cost is scarce in Portugal.

Research frontiers

The results of the Karolinska Institute study demonstrated a substantial increase in available cancer drugs and costs between 1998 and 2007. This cost increment was not only related to the introduction of new drugs, as 68% of the costs in 2007 were from drugs approved before 1999. In 2007, drugs placed on the market between 1999 and 2002 accounted for 17% of the total costs; drugs placed between 2003 and 2005 accounted for 11%; and drugs placed between 2006 and 2007 accounted for 3%.

Innovations and breakthroughs

In this work the authors observed an increase in the global costs of cancer drugs which may be explained by the increased number of patients treated, new indications for treatment with previously approved drugs, and the placement of new drugs on the market. These results were in accordance to those published by the Karolinska Institute. Up to 2007, drugs placed on the national market before 1999 were responsible for cancer drug expenditure. Reasons for this may be new treatment indications for these drugs and loss of some drug

patents leading to a reduction in their price. Drugs available between 1999 and 2002 were second regarding expenditure, followed by drugs placed on the market between 2003 and 2005. Recently placed drugs (from 2006) accounted for a smaller percentage of the costs. However, after 2008 there was a reduction in costs for previously available drugs followed by a gradual increase in expenditure for new drugs.

Applications

The study results suggest that older drugs were responsible for most of the expenditure in cancer treatment, but costs of new cancer drugs are increasing.

Terminology

The burden of cancer to society can be measured by direct and indirect costs. Direct costs are related to prevention and treatment, while indirect costs include loss of production due to inability to work caused by disease, disability and death. The costs of chemotherapy drugs are related to expenditure for cytostatics, and costs for targeted therapy such as monoclonal antibodies, tyrosine kinase inhibitors and mammalian target of rapamycin inhibitors.

Peer review

The paper is interesting. It will benefit readers in the field of hospital economic.

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Prognostic value of preoperative serum tumor markers in gastric cancer

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Abstract

AIM: To evaluate the prognostic value of preoperative carcinoembryonic antigen (CEA), carbohydrate antigen (CA)19-9, and CA50 in patients undergoing D2 resection.

METHODS: We evaluated 363 patients with gastric cancer who underwent gastrectomy at our hospital from January 2006 to December 2009. Blood samples were obtained from each patient within 1 wk before surgery. The cut-off values for serum CEA, CA19-9, and CA50 were 5 ng/mL, 37 U/mL, and 20 U/mL, respectively. The correlation between preoperative tumor marker levels and prognosis was studied by means of univariate and multivariate analyses.

RESULTS: The preoperative serum positive rates of CEA, CA19-9 and CA50 were 24.0%, 18.9% and 24.5%, respectively. The positivity rate of serum CEA was significantly correlated with age ($P < 0.001$), sex ($P = 0.022$), tumor size ($P = 0.007$) and depth of invasion ($P = 0.018$); CA19-9 with tumor size ($P = 0.042$) and lymph node metastasis ($P < 0.001$); and CA50 only

with lymph node metastasis ($P = 0.001$). In multivariate analysis, tumor size, T category, N category, vascular or neural invasion, and adjuvant chemotherapy were independent prognostic factors for overall survival. CA19-9 had an independent prognostic significance in patients without adjuvant chemotherapy ($P = 0.027$).

CONCLUSION: Preoperative serum CEA, CA19-9 and CA50 are prognostic in patients with gastric cancer. Only CA19-9 is an independent prognostic factor after surgery without adjuvant chemotherapy.

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Key words: Gastric cancer; Carcinoembryonic antigen; Cancer antigen 19-9; Cancer antigen 50; Prognosis

Core tip: Recent researches have investigated the prognostic value of tumor markers in gastric cancer. The results were not conclusive and consistent. Most researchers did not account for some confounding factors, especially the use of adjuvant chemotherapy, so we investigated the prognostic value of carcinoembryonic antigen, carbohydrate antigen (CA)19-9 and CA50 in Chinese gastric cancer patients when considering the use of adjuvant chemotherapy. CA19-9 is an independent prognostic factor for gastric cancer patients after surgery without adjuvant chemotherapy.

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INTRODUCTION

Gastric cancer has shown a significant decline in incidence

Table 1 Previous studies reporting the association between preoperative tumor markers and oncologic outcomes

Ref.	CEA cut-off value (ng/mL)	CA199 cut-off value (U/mL)	CA50 cut-off value (U/mL)	Number of patients	Prognostic impact in overall survival (<i>P</i> value)				
					Univariate analysis			Multivariate analysis	
					CEA	CA19-9	CA50	CEA	CA19-9 CA50
Ishigami <i>et al</i> ^[10]	10	74	NA	549	< 0.0001	< 0.0001		0.040	0.150
Nakane <i>et al</i> ^[118]	10	NA	NA	865				0.001	
Kochi <i>et al</i> ^[6]	5	37	NA	434	< 0.01	< 0.01		0.044	0.169
Schauer <i>et al</i> ^[111]	NA	45	NA	120		0.007			
Marrelli <i>et al</i> ^[7]	5	37	NA	153	< 0.0005	< 0.0001		< 0.05	< 0.05
Park <i>et al</i> ^[8]	7	NA	NA	810	< 0.001			0.005	
¹ Liu <i>et al</i> ^[12]	10	37	20	273	0.000	0.000	0.000	0.000	0.000 0.006
Liu <i>et al</i> ^[13]	10	35	25	391	0.000	0.000	0.001	0.006	> 0.05 > 0.05
Gaspar <i>et al</i> ^[23]	5	35	NA	82				0.770	0.630
Ucar <i>et al</i> ^[19]	5	35	NA	95				0.500	0.600
Tachibana <i>et al</i> ^[14]	5	NA	NA	196	< 0.0001			0.000	
Duraker <i>et al</i> ^[15]	5	37	NA	168	0.003	0.014		0.145	0.174
Tocchi <i>et al</i> ^[16]	3	37	NA	59	< 0.03	< 0.05		0.000	0.014
Lai <i>et al</i> ^[9]	5	37	NA	196				0.867	0.230
Dilege <i>et al</i> ^[17]	5	33	NA	75	> 0.05	> 0.05			

¹Prognostic significance of tumor markers in T4a gastric cancer. NA: Not available; CEA: Carcinoembryonic antigen; CA: Carbohydrate antigen.

and mortality over the past decades, but due to its poor prognosis, it is still the second leading cause of cancer-related death worldwide^[1]. Surgery is the main approach for gastric cancer, and the most important prognostic factor of gastric cancer is tumor node metastasis (TNM) classification^[2]. However, it is difficult to obtain complete data preoperatively. For this reason, it may be important to find some other preoperative prognostic factors for evaluating the outcome of gastric cancer patients.

Tumor markers, including carcinoembryonic antigen (CEA), carbohydrate antigen (CA)19-9 and CA50, are not applied to TNM staging according to the American Joint Committee on Cancer (AJCC) 7th edition, but they have been applied in clinical practice for several decades and shown to have prognostic value in gastric cancer. CEA, a member of the immunoglobulin superfamily, was originally used as a serum marker for colorectal cancer^[3]. Now, it is widely used in the diagnosis and monitoring of gastric cancer. CA19-9 is one of the antigens of the Lewis family and has been reported to be elevated in the sera of patients with gastrointestinal cancer, particularly in pancreatic cancer^[4]. CA50 is a glycolipid antigen that plays an important role in cell growth and differentiation and can also be observed in a variety of malignancies, especially gastrointestinal cancers^[5]. According to previous studies of the association between preoperative tumor markers and outcome of gastric cancer (Table 1), a high preoperative CEA or CA19-9 level is associated with high tumor recurrence^[6-9] and poor survival rates^[6-8,10-17]. Some studies have suggested that preoperative CEA, CA19-9 or CA50 level is an independent prognostic factor of gastric cancer^[6-8,10,12-14,18]. However, some of these studies did not calculate the disease-free survival (DFS) time, which could indirectly reflect tumor recurrence. Moreover, most studies did not account for other factors, especially the use of adjuvant chemotherapy, which may be expected to be associated with long-term oncological outcomes.

In this retrospective study, we investigated the relationship between preoperative serum levels of CEA,

CA19-9 and CA50 and clinicopathological features, and the prognostic value of these three tumor markers in patients who underwent D2 resection for gastric cancer with adjuvant chemotherapy.

MATERIALS AND METHODS

Patient data

We included 363 patients who underwent D2 surgical resection at the First Affiliated Hospital of Nanjing Medical University between January 2006 and December 2009. The patient inclusion criteria in this study were as follows: (1) pathological diagnosis of gastric cancer; (2) D2 surgical resection; (3) Stage I - III cancer; (4) adjuvant chemotherapy regimen of cisplatin or oxaliplatin combined with 5-fluorouracil; (5) death due to gastric cancer; and (6) availability of follow-up data. The end of follow-up was April 2013. The study protocol was approved by the Ethics Committee of this hospital. Patients were staged according to the criteria of the AJCC 7th edition. CEA, CA19-9 and CA50 were assayed by enzyme linked immunosorbent assay. Blood samples were obtained from each patient within 1 wk before surgery. CEA, CA19-9, and CA50 were assayed with magnetic particle enzyme immunoassay in UniCel™ DxI 800 Access immunoassay system (Beckman Coulter Inc. Miami, United States). The cut-off values for serum CEA, CA19-9 and CA50 were 5 ng/mL, 37 U/mL and 20 U/mL, respectively, according to the manufacturer's instructions. These patients were monitored every 3 mo for the first 2 years after surgery, and every 6 mo thereafter. Follow-up examinations included physical examination, ultrasonic inspection, chest radiography, computed tomography, positron emission tomography, magnetic resonance imaging, endoscopy, and histological biopsy. Recurrence was determined by clinical and radiological examinations or by histological confirmation.

Statistical analysis

The χ^2 test was used to evaluate the associations between

Table 2 Correlation between tumor makers and major clinicopathologic characteristics of 363 patients with gastric cancer *n* (%)

Patient characteristics	Cases	CEA (+)	<i>P</i>	CA19-9 (+)	<i>P</i>	CA50 (+)	<i>P</i>
Age (yr)			< 0.001		0.213		0.134
< 60	169 (46.6)	26 (15.4)		36 (21.7)		40 (28.4)	
≥ 60	194 (53.4)	61 (31.4)		31 (16.5)		31 (20.8)	
Gender			0.022		0.800		0.375
Male	266 (73.3)	72 (27.1)		48 (18.6)		48 (23.1)	
Female	97 (26.7)	15 (15.5)		19 (19.8)		23 (28.0)	
Tumor size (cm)			0.007		0.042		0.051
< 6	243 (66.9)	48 (19.8)		38 (16.0)		40 (20.9)	
≥ 6	120 (33.1)	39 (32.5)		29 (25.0)		31 (31.3)	
Tumor location			0.463		0.737		0.662
Cardia or fundus	117 (33.1)	33 (28.2)		21 (18.6)		20 (22.0)	
Corpus or angulus	131 (37.1)	31 (23.7)		22 (17.2)		26 (25.2)	
Antrum	103 (29.2)	21 (20.4)		22 (21.8)		24 (28.2)	
Whole stomach	2 (0.6)	1 (50.0)		0 (0.0)		0 (0.0)	
Differentiation			0.65		0.671		0.701
Well/moderate	61 (16.8)	16 (26.2)		10 (16.9)		10 (22.2)	
Poorly	302 (83.2)	71 (23.5)		57 (19.3)		61 (24.9)	
T category			0.018		0.058		0.308
pT1-pT2	48 (13.2)	5 (10.4)		4 (8.7)		7 (17.9)	
pT3-pT4	315 (86.8)	82 (26.0)		63 (20.5)		64 (25.5)	
N category			0.109		< 0.001		0.001
pN0	61 (16.8)	14 (23.0)		10 (16.7)		10 (21.3)	
pN1	73 (20.1)	10 (13.7)		5 (6.9)		10 (16.9)	
pN2	100 (27.5)	29 (29.0)		12 (12.5)		10 (13.5)	
pN3	129 (35.5)	34 (26.4)		40 (31.7)		41 (37.3)	
Vascular/nerves invasion			0.997		0.950		0.763
Negative	171 (47.1)	41 (24.0)		31 (18.8)		30 (23.6)	
Positive	192 (52.9)	46 (24.0)		36 (19.0)		41 (25.2)	
AJCC stage			0.347		0.084		0.187
I	2 (0.6)	0 (0.0)		0 (0.0)		0 (0.0)	
II	97 (26.7)	19 (19.6)		11 (11.7)		13 (17.6)	
III	264 (72.7)	68 (25.8)		56 (21.7)		58 (27.1)	

AJCC: American Joint Committee on Cancer; CEA: Carcinoembryonic antigen.

tumor markers and the existing prognostic factors. Univariate survival analysis was performed using the Kaplan-Meier method. Survival curves were compared with the log-rank test. Multivariate analysis was performed using the Cox proportional hazards regression model. $P < 0.05$ was considered significant. All statistical analyses were performed using SPSS version 18.0.

RESULTS

Patient characteristics

The characteristics of 363 patients are presented in Table 2. These patients had a median age of 60.67 ± 11.91 years (range: 24-93 years) and included 266 (73.3%) men and 97 (26.7%) women. Only two patients (0.6%) had Stage I cancer, 97 (26.7%) were Stage II, and 264 (72.7%) were Stage III. Poorly differentiated tumors were observed in 302 patients (83.2%), and moderately and well-differentiated tumors in 61 (16.8%). And 261 patients (71.9%) received platinum-based adjuvant chemotherapy.

Association between clinicopathological features and tumor markers

Preoperative serum CEA levels were assayed in all 363 patients, however, for some unknown reasons, CA19-9 was assayed for 354 patients and CA50 for 290. The

preoperative serum positive rates of CEA, CA19-9 and CA50 were 24.0%, 18.9% and 24.5%, respectively. As shown in Table 2, positivity rate of CEA was significantly correlated with age ($P < 0.001$), sex ($P = 0.022$) and tumor size ($P = 0.007$), while CA19-9 was correlated with tumor size ($P = 0.042$). Compared with CA19-9 and CA50, CEA showed a more significant difference in depth of invasion ($P = 0.018$). In contrast, lymph node metastasis was significantly more frequent in patients with elevated levels of CA19-9 ($P < 0.001$) and CA50 ($P = 0.001$). Nevertheless, the tumor location, tumor differentiation, and vascular or neural invasion did not influence the positivity of the three tumor markers.

Survival and tumor markers

Overall survival (OS) was recorded for all patients, and DFS was recorded for 160. At the end of follow-up in May 2013, 167 (46.0%) patients were still alive.

On univariate analysis, by Kaplan-Meier method with the log-rank test, the OS of all patients was lower in those with elevated CEA, CA19-9 and CA50 compared to those with normal tumor marker levels ($P = 0.023$, $P = 0.009$ and $P = 0.004$, respectively). DFS showed no significant difference between elevated tumor marker levels and normal ones (Table 3). We divided these patients into two groups: those with and those without adjuvant che-

Table 3 Univariate model for disease-free survival and overall survival by log-rank test

Tumor markers	DFS (P)	OS (P)
CEA (+)	0.197	0.023
CA19-9 (+)	0.236	0.009
CA50 (+)	0.335	0.004
CEA (+) and CA19-9 (+)	0.236	0.026
CA50 (+) and CEA (+)	0.236	0.100
CA19-9 (+) and CA50 (+)	0.236	0.021
CEA (+) and CA19-9 (+) and CA50 (+)	0.397	0.751
CEA (+) or CA19-9 (+) or CA50 (+)	0.302	0.002

CEA: Carcinoembryonic antigen; CA: Carbohydrate antigen; DFS: Disease-free survival; OS: Overall survival.

Table 4 Univariate model for disease-free survival and overall survival by log-rank test when considering the adjuvant chemotherapy

Tumor markers	DFS (P)		OS (P)	
	Adjuvant chemotherapy	Non-adjuvant chemotherapy	Adjuvant chemotherapy	Non-adjuvant chemotherapy
CEA (+)	0.878	0.116	0.297	0.017
CA19-9 (+)	0.961	0.116	0.179	< 0.001
CA50 (+)	0.828	0.182	0.069	0.001
CEA (+) and CA19-9 (+)	0.961	0.116	0.304	0.004
CA50 (+) and CEA (+)	0.991	0.132	0.383	0.065
CA19-9 (+) and CA50 (+)	0.991	0.132	0.303	< 0.001
CEA (+) and CA19-9 (+) and CA50 (+)	0.991	0.132	0.867	0.298
CEA (+) or CA19-9 (+) or CA50 (+)	0.742	0.196	0.107	< 0.001

CEA: Carcinoembryonic antigen; CA: Carbohydrate antigen; DFS: Disease-free survival; OS: Overall survival.

mothy (Table 4). In the non adjuvant chemotherapy group, patients with elevated CEA, CA19-9 and CA50 had a significantly worse prognosis than patients with normal tumor marker levels. The adjuvant chemotherapy group did not show similar results. To evaluate combination assays of serum CEA, CA19-9 and CA50 levels, cumulative survival was compared among five groups: all three tumor markers elevated (CA50⁺/CEA⁺/CA19-9⁺), at least two markers elevated (CEA⁺/CA19-9⁺, CA50⁺/CEA⁺, CA19-9⁺/CA50⁺), and at least one marker elevated (CEA⁺, CA19-9⁺ or CA50⁺). As shown in Tables 2 and 4, the CEA⁺/CA19-9⁺, CA19-9⁺/CA50⁺ and CEA⁺, CA19-9⁺ or CA50⁺ groups displayed poor OS rates in all patients and in the adjuvant chemotherapy group.

On multivariate analysis, tumor size, T category, N category, vascular or neural invasion, and adjuvant chemotherapy were independent prognostic factors for OS. Meanwhile, only tumor size was a significant risk factor for DFS. Preoperative serum CEA, CA19-9 and CA50 were not independent prognostic factors for OS (Table 5), but CA19-9 was an independent prognostic factor

in patients without adjuvant chemotherapy ($P = 0.027$) (Table 6).

DISCUSSION

Tumor markers are often used to determine the prognosis of cancer patients after radical surgery, but the role of tumor markers in gastric cancer is still controversial. α -Fetoprotein, CEA, CA19-9, CA50 and CA72-4 were considered as relatively specific markers for gastric cancer in some studies^[12]. In our hospital, we began to use the preoperative serum levels of CEA, CA19-9 and CA50 to evaluate the prognosis of gastric cancer patients several years ago. That is the reason why we chose these three tumor markers in the present retrospective study.

The preoperative rate of positivity for serum CEA was 24.0%, which is similar to other studies using the same cutoff value^[7,19,20]. The corresponding proportion of patients with elevated serum CA19-9 and CA50 levels was 18.9% and 24.5%, respectively, which was also similar to previous studies^[6,9,12]. Some authors have reported that tumor marker positivity is associated with tumor stage^[6]. However, no such correlation was found in our study. The reason may be that most of our samples were Stage II or III tumors, and there was no patient with Stage IV disease. Nevertheless, we found that CA19-9 and CA50 were associated with pN stage, and CEA with pT stage, which indicated that the positive rates of tumor markers increased as the tumor progressed. Our analysis showed that the positive rate of CEA was higher in male and elderly patients. The proportion of patients with elevated serum CEA and CA19-9 was significantly higher in those with large tumors. Also, there was a tendency for CA50 to be a marker for tumor size. It has been reported in animal studies that the elevation of serum CEA was caused by the increase in weight of primary cancer, as well as the increase in CEA production in cancer tissues^[21]. It has also been reported that elevated CEA levels are related to the degree of differentiation^[22]. However, we did not find any correlation between the tumor markers studied and the degree of differentiation. Similar to our findings, tumor location has previously been noted to have no association with tumor marker positivity^[23].

On the basis of our univariate analysis, patients positive for CEA, CA19-9 and CA50 had significantly poorer OS than those who were the marker negative. We found that the correlations between CEA, CA19-9, CA50 and OS were consistent with previous studies^[6,7,10,13,15,16]. These correlations reflected the worse prognosis in patients with positive values for tumor markers. Marrelli *et al*^[7] observed that the combined assay of CEA and CA19-9 provided more useful prognostic information than CEA or CA19-9 alone. Combination of these three markers, with positivity for at least two, resulted in a significant difference in OS, and proved to be a better prognostic indicator with respect to the three markers used alone. This finding suggests the complementary role of the three markers, which is also supported by the increase in overall sensitivity obtained with their concomitant use.

Table 5 Independent prognostic factors for predicting disease-free survival and overall survival by multivariate analysis using Cox model

Factors	DFS			OS		
	HR	95%CI	P	HR	95%CI	P
Age	1.028	0.825-1.770	0.331	0.932	0.667-1.303	0.682
Gender	1.072	0.634-1.814	0.795	0.791	0.543-1.151	0.220
Tumor size	1.718	1.159-2.546	0.007	1.998	1.433-2.785	< 0.001
Differentiation	1.398	0.685-2.853	0.357	0.714	0.398-1.281	0.258
T category	0.796	0.213-2.978	0.734	6.042	2.190-16.668	0.001
N category	1.014	0.836-1.231	0.884	1.734	1.429-2.014	< 0.001
Vascular/nerves invasion	0.930	0.606-1.426	0.739	1.515	1.045-2.195	0.028
Adjuvant chemotherapy	0.931	0.614-1.411	0.736	0.618	0.425-0.898	0.012
CEA	3.045	0.401-23.157	0.282	0.902	0.618-1.316	0.592
CA19-9	0.392	0.045-3.384	0.394	0.836	0.449-1.556	0.571
CA50	1.058	0.546-2.050	0.868	1.470	0.842-2.569	0.176

CEA: Carcinoembryonic antigen; CA: Carbohydrate antigen; DFS: Disease-free survival; OS: Overall survival.

Table 6 Multivariate analysis of overall survival of patients without chemotherapy

Factors	OS		
	HR	95%CI	P
Age	1.525	0.765-3.041	0.230
Gender	1.229	0.559-2.701	0.608
Tumor size	4.758	2.339-9.678	< 0.001
Differentiation	0.784	0.259-2.379	0.668
T category	4.566	0.570-36.569	0.153
N category	2.096	1.506-2.916	< 0.001
Vascular/nerves invasion	2.861	1.247-6.566	0.013
CEA	1.329	0.670-2.637	0.415
CA19-9	5.077	1.203-21.427	0.027
CA50	0.671	0.183-2.463	0.547

CEA: Carcinoembryonic antigen; CA: Carbohydrate antigen; OS: Overall survival.

On the contrary, there was no significant difference in OS when all three markers were positive. This may have been due to the limitations of the small sample size and that the levels of the three markers in the patients were not high in our analysis. We found that when CA50 was positive, there was a 77.5% chance for at least one of the other two markers to be positive. This revealed that all three markers were sensitive in gastric cancer. Also, there may be similarities in the mechanism of generation of the three markers. We also intended to compare cumulative survival in patients who were positive for only one of the tumor markers, but the numbers of these patients were too small to reach statistical significance. Not many previous studies considered the confounding factors such as the use of adjuvant chemotherapy. Therefore, to exclude the potential bias of adjuvant chemotherapy, we observed the oncological outcomes in patients without chemotherapy after surgery. As a result, these patients who were positive for CEA, CA19-9 or CA50 had a poor prognosis by univariate analysis, while there was no association between tumor markers and oncological outcomes in patients with adjuvant chemotherapy. This may have been because adjuvant chemotherapy after surgery improved the prognosis of gastric cancer patients. In our multivariate analysis, adjuvant chemotherapy was an in-

dependent prognostic factor. To evaluate whether CEA, CA19-9 and CA50 could provide information about tumor recurrence, we compared cumulative survival curves for DFS. Choi *et al*^[22] reported that patients with an elevated tumor marker were at higher risk for recurrence. However, in our study, we did not find any correlation between recurrence and tumor markers.

In order to clarify the value of tumor markers in prognosis of gastric cancer patients, we performed multivariate analysis using a Cox proportional hazards model. The results showed that tumor size, T category, N category, vascular or neural invasion, and adjuvant chemotherapy had independent prognostic value for OS. However, the three tumor markers that we studied did not provide independent predictive value for recurrence and OS. Nevertheless, Tocchi *et al*^[16] reported that preoperative serum CEA and CA19-9 were independent prognostic factors in gastric cancer patients. Liu *et al*^[12] showed that CA50 had prognostic significance in T4a gastric cancer. Other studies did not show similar results^[13]. This may have been because of the heterogeneity of patients included in these studies. Our study revealed that adjuvant chemotherapy played an important role in prognosis of gastric cancer patients. When excluding the impact of chemotherapy, increased preoperative CA19-9 level was associated independently with oncological outcomes in patients without adjuvant chemotherapy. Duraker *et al*^[24] assessed CEA when investigating adjuvant chemotherapy in patients with colon cancer, but found no difference in patients with or without chemotherapy. Thus, we suppose that increased preoperative CA19-9 level could be a reason for initiating adjuvant chemotherapy in gastric cancer patients after surgery.

There were some limitations to our study. We did not obtain sufficient details about recurrence, and just calculated the DFS, which might not provide sufficient information about the correlation between tumor markers and recurrence. Also, not all three tumor markers were assayed in all the patients, so there was loss of some data. Further research with a more complete patient data is needed to obtain definitive results.

In conclusion, preoperative serum levels of CEA,

CA19-9 and CA50 can provide prognostic information in patients with gastric cancer. Furthermore, only CA19-9 is an independent prognostic factor for gastric cancer patients after surgery without adjuvant chemotherapy.

COMMENTS

Background

Gastric cancer is the second leading cause of cancer-related death worldwide. To predict the outcome of patients before surgery, it is important to find some preoperative prognostic factors. Tumor markers have prognostic significance in several neoplasms. This retrospective study evaluated the prognostic value of preoperative carcinoembryonic antigen (CEA), carbohydrate antigen (CA)19-9 and CA50 in patients who underwent D2 resection for gastric cancer.

Research frontiers

Many tumor markers are widely used clinically. CEA, CA19-9 and CA50 are relatively important in gastric cancer. The preoperative levels of these markers may indirectly reflect tumor load and prognosis. Therefore, it is becoming more important to explore the prognostic value of these tumor markers. Especially for clinicians, these markers can help them to choose appropriate treatment strategy.

Innovations and breakthroughs

Many studies have investigated the prognostic value of tumor markers, but most researches did not account for the confounding factors, especially the use of adjuvant chemotherapy. The authors investigated the prognostic value of CEA, CA19-9 and CA50 in gastric cancer patients when considering the use of adjuvant chemotherapy. To eliminate the influence of chemotherapy, the authors divided the patients in non-adjuvant chemotherapy and adjuvant chemotherapy groups for subgroup analysis.

Applications

CA19-9 is an independent prognostic factor for gastric cancer patients after surgery without adjuvant chemotherapy. The authors suggest that patients with a high preoperative level of CA19-9 may relapse earlier than those with a normal level; therefore, for patients with a high level of markers, more frequent physical examinations are needed.

Terminology

Tumor markers, including CEA, CA19-9 and CA50, have been applied in clinical practice for several decades and shown to have prognostic value in gastric cancer. Adjuvant chemotherapy: it is additional treatment given after surgery to lower the risk of the cancer recurrence.

Peer review

The authors evaluated the prognostic value of preoperative CEA, CA19-9 and CA50 in patients who underwent D2 resection for gastric cancer. They concluded that CA19-9 is an independent prognostic factor for gastric cancer patients after surgery without adjuvant chemotherapy. This study was important and interesting.

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Twenty-five years of research on the effects of exercise training in breast cancer survivors: A systematic review of the literature

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exercise training with statistical analyses performed in at least one of the following outcome measurements: Cardiorespiratory function, body composition, muscular strength, fatigue, depression, and overall quality of life. Five reviewers independently identified the studies that met the criteria for the review and discrepancies were resolved by consensus among all authors.

RESULTS: Fifty-one studies were included in this review with 5 from the period between 1989-1999, 11 from 2000-2006, and 35 from 2007-2013. The evolution of study designs changed from aerobic only exercise training interventions (1989-1999), to a combination of aerobic and resistance training (2000-2006), to studies including an arm of resistance training or examining the effects of resistance training as the main mode of exercise (2007-2013). Overall, the benefits of exercise showed improvements in cardiorespiratory function, body composition, strength, and patient reported outcomes including fatigue, depression, and quality of life.

CONCLUSION: Exercise training appears to be safe for most breast cancer patients and improvements in physiological, psychological, and functional parameters can be attained with regular participation in moderate intensity exercise.

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Key words: Breast cancer; Exercise training; Complementary Alternative Medicine; Oncology; VO_{2peak} ; Patients reported outcomes

Core tip: The purpose of this systematic literature review was to investigate the role of exercise training the past 25 years on major physiological-psychological outcomes studied thus far in this patient population. Exer-

Abstract

AIM: To investigate the role of exercise training the past 25 years on major physiological-psychological outcomes studied thus far in this patient population.

METHODS: PubMed, MedlinePlus, the Cochrane Library, Web of Science, SportDiscus, Embase, Scopus, and Google Scholar were searched from September to November 2013 to identify exercise training studies that used objective measurements of fitness and/or patient reported outcomes assessed pre and post-

cise training appears to be safe for most breast cancer patients and improvements in physiological, psychological, and functional parameters can be attained with regular participation in moderate intensity exercise.

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INTRODUCTION

Despite being the most commonly diagnosed cancer among women and the second most diagnosed type of cancer overall in the world (approximately 1.7 million diagnosed cases in 2012, 11.9% of all cancer cases) the incidence of breast cancer continues to rise with a concerning increase of 20% of newly diagnosed cases since 2008^[1]. This worldwide increase in new breast cancer cases has been attributed, in part, to changes in lifestyle in developing countries due to economic growth and societal changes similar in nature to those of developed industrialized nations^[1]. These lifestyle changes are often associated with poor dietary choices and lack of physical activity; both considered major risk factors for the development of the disease^[2]. However, in developed countries, new diagnostic tools that allows for faster detection and technological advances in anti-cancer treatments, have been credited as the main factors for the increased longevity observed in oncology patients, especially in the breast cancer population^[2,3].

Living longer with a history of the disease often comes with the deleterious effects of treatments, which regrettably are associated with reduced quality of life in the patients. Marked reductions in physical capacities (*i.e.*, reduced cardiac function and muscular strength), negative body composition alterations (*i.e.*, increase in body mass, reduction in muscle mass, and increases in fat mass), and detrimental patients reported outcomes (PROs) (*i.e.*, increased levels of fatigue, depression, and anxiety) are among the common side-effects of cancer treatments that negatively impact the overall quality of life of these patients and also put them at a greater risk for the development of other co-morbidities^[4]. If not addressed, these side effects can increase the likelihood for the development of secondary cancers as well as diminish life expectancy^[2].

During the past 3 decades, complementary therapies known as Complementary Alternative Medicine, have received more attention by the medical community due to its ability to mitigate some of the side-effects commonly experienced by cancer patients during and post-completion of cancer treatments. One intervention that has gained increasingly more attention due its efficacious

ability to positively impact many physiological systems that are altered during cancer treatment, its non-invasive nature, and relatively low cost, is exercise.

The effects of exercise training in breast cancer patients began to be examined in the late 1980's. The first study examining the effects of exercise in breast cancer patients was published in 1989^[5] and demonstrated that the patients tolerated exercise training and significant improvement in cardiorespiratory functional capacity [represented by the variable $\text{VO}_{2\text{max}}$ (L/min)] was observed in the exercise-training group. Since then, an ever-growing number of studies examining the effects of exercise in breast cancer patients has continued to be one of the most studied populations in this relatively new and exciting area of exercise oncology research. The results of the evaluation of these studies are promising and affirming, but clearly warrant further and more extensive investigations.

The purpose of this systematic review of the literature on the effects of exercise training in breast cancer patients was to present and evaluate the results of studies conducted in this area of research during the past 25 years, divided into the following time periods: (1989-1999, 2000-2006, 2007-2013). Most specifically, this review focuses on presenting and discussing the results of the effects of exercise training on the most commonly evaluated outcomes in the area including physiological parameters of cardiorespiratory function, body composition, and muscular strength, and PROs that evaluated fatigue, depression, anxiety, and overall quality of life. For organizational purposes a chronological description of studies results, studies common characteristics, and commentaries on changes observed in this area of research over the past 25 years are discussed.

MATERIALS AND METHODS

A systematic review of the literature using PubMed, MedlinePlus, the Cochrane Library, Web of Science, SportDiscus, Scopus, Embase, and Google Scholar was conducted from September to November 2013 to identify exercise-training studies in breast cancer patients. The MeSH terms and text word used for the search of studies included: exercise and cancer, exercise oncology, breast cancer, breast cancer and exercise, oncology, exercise training, exercise therapy, neoplasms, and malignancies. All references relevant to the topic were hand-searched.

Studies selection criteria

Included in the review were all exercise training studies that used aerobic training or aerobic in combination with resistance exercise training, or had a study arm evaluating the effects of aerobic training or aerobic in combination with resistance exercise training. These studies objectively assessed the effects that these exercise training modes had in breast cancer patients on at least one of the following fitness and/or patient rated outcomes variables including: cardiorespiratory function, body composition,

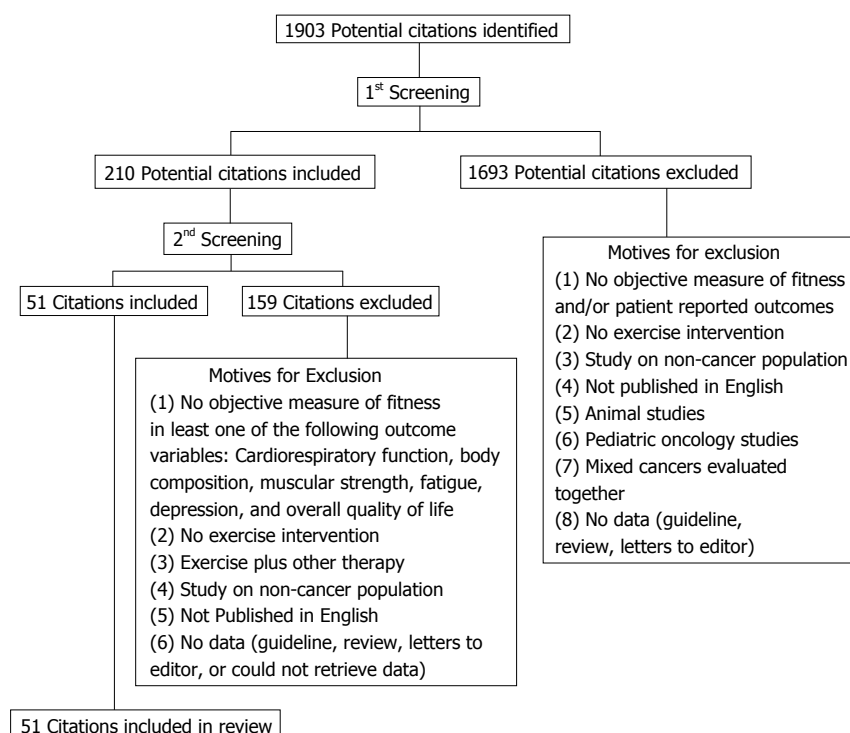


Figure 1 Literature review citation selection flow chart.

muscular strength, fatigue, depression, and overall quality of life. These outcome variables were chosen because they have been the most commonly evaluated variables in the area of exercise oncology during the past 25 years. Studies excluded from the review involved those conducted in pediatric patients (age below 18 years); that used non-conventional exercise prescriptions (*i.e.*, Yoga, aqua aerobics, Tai Chi, *etc.*); not published in English; used traditional exercise training programs in combination with other interventions (*i.e.*, dietary manipulations, psychosocial therapies, counseling, *etc.*); secondary articles from the same research team with data previously published on initial original article of outcome variables of interest of this review; studies that evaluated the results of the exercise intervention in breast cancer patients mixed with other types of cancers; studies with non-retrievable incomplete data sets, review articles, guidelines articles, letters to editor, and animal based studies. Since very few studies have used resistance training as the main mode of exercise, a separate evaluation summarizing the results of studies that objectively measured muscular strength using the 1 maximum repetition (1-RM) and presented data on at least one of the major outcomes mentioned above are also presented. Five reviewers (Mills RC, Phillips BL, Lee JT, Story CE, Nascimento MGB) independently identified the studies that met the criteria for the review mentioned above and discrepancies were resolved by consensus among all authors.

Statistical analyses

Descriptive statistics were used in the form of mean and standard deviations for the presentation of main out-

come variables data at baseline and post-exercise intervention. Comparisons between baseline and post-exercise training data were conducted using dependent samples *t* tests for both the exercise and control (non exercise training) groups. For studies where there was more than one exercise arm (*i.e.*, self-directed and supervised exercise), when no differences were observed for changes between groups, both groups were included in the analyses. An alpha of 0.05 set a priori was used for all tests. No alpha level adjustment was performed for the multiple comparisons. Due to differences of instruments used to evaluate the main outcome variables included in this review, only variables evaluated by at least two independent studies were included in the analyses. For variables evaluated by only one study, means, standard deviations, and change from baseline to post-intervention values were provided when data was retrievable.

RESULTS

Between September and November of 2013, 1903 potential citations for inclusion in the review were identified. After an initial screening of all of 1903 potential citations, 210 citations were included in a second screening for further evaluation of inclusion eligibility criteria. After the second screening, 51 citations were deemed eligible. The literature review citation selection process flow chart is presented in Figure 1.

For organizational purposes a chronological description of studies results conducted in this area of research during the past 25 years was arbitrarily divided into the following time periods: (1989-1999, 2000-2006, 2007-2013).

Table 1 1989-1999 study results summary

Measure	No. of studies	N	Baseline		Post-intervention		Change		P
			Mean	SD	Mean	SD	Mean	95%CI	
Cardiorespiratory function									
Bike VO _{2peak} (L/min)									
Exercise	1	45	1.02	-	1.45	-	0.43	-	-
Control	0								
12-min walk test (m)									
Exercise	2	25	1123.7	7.1	1216.7	42.3	93.1	-632	0.166
Control	2	16	1052.6	76.2	965.5	30.1	-87.1	-2476	0.536
Body composition									
Body mass (kg)									
Exercise	1	22	66.57		-		-		
Control	1	24	62.16		-		-		
Body fat (%)									
Exercise	1	12	-		-		-0.51		
Control	1	12	-		-		2.19		
Lean body mass (kg)									
Exercise	1	12	-		-		2.04		
Control	1	12	-		-		-1.26		
Quality of life									
QOL Index, 0-100									
Exercise	1	27	61.4	-	56.8	-	-4.60	-	-
Control	0	-	-	-	-	-	-	-	-
Fatigue									
SympASF, 0-100									
Exercise	2	31	12.5	2.1	22.0	5.7	9.50	-63.5	0.164
Control	2	29	19.5	7.8	41.5	5.0	22	-50.8	0.058
Depression									
SymptASD, 0-100									
Exercise	2	31	11.5	6.4	11.5	2.1	0.00	-76.2	1.000
Control	2	29	9.5	2.1	14.5	9.1	5.00	-127	0.500

Lower scores reflect lower fatigue and lower depression. VO_{2peak}: Peak oxygen uptake; QOL: Quality of life; SympASF: Symptom Assessment Scale-Fatigue; SymptASD: Symptom assessment Scale-Depression.

1989-1999

During the decade between 1989 and 1999, only 5 studies met the inclusion criteria for this review^[5-9]. All 5 studies were conducted while patients were receiving active treatment including chemotherapy and/or radiation therapy and the average age was 46.4 ± 1.5 for the patients who participated in the exercise interventions and 46.6 ± 3.4 for patients in the control groups. Four of the studies were randomized controlled trials and more than half of the studies were home-based interventions ($n = 3$)^[7-9]. The summary of the results of the studies is presented in Table 1.

Only 1 study^[6] assessed the effects of exercise training on cardiorespiratory function using a direct measurement through a cardiopulmonary exercise test (CPET) on a cycle ergometer with gas exchange; 2 evaluated overall physical function using the 12-min walk test; 1 focused on the effects of exercise training on body weight and body composition^[5]; the other 3 studies focused on patient reported outcomes^[7-9]. The overall exercise prescription used in these 5 initial studies included 100% aerobic exercise training prescriptions, with two studies using an interval-training methodology^[5,6]. The frequency of training ranged from 3-5 sessions per week (average of 3.7 sessions per week), with training sessions of 15-45 min (average of 27 min). The only modes of exercise used on these studies included either walking or cycle ergometry

with an exercise training program length varying from 6-24 wk of training (average of 11 wk). For the studies that used heart rate for the prescription of exercise intensity, 60%-85% of heart rate reserve was used. The majority of studies that used walking as the mode of exercise, the description of exercise intensity was subjective and included terms such as brisk walk, self-paced, low to moderate, or incremental exercise.

No statistical significance was observed for changes in any outcome variable analyzed. However, within the results the most noticeable effect of the exercise training was observed for the variable fatigue. A trend towards significance was observed for the variable fatigue in the control group with increased values from baseline to post-exercise training of 22 points in a scale from 0-100 representing a 22% increase in fatigue levels. This increase was of clinical relevance since the exercise group showed a much lower fatigue response. No data on body composition was analyzed, however, a trend towards a reduction in body fat % and gain in lean body mass was observed in the exercise group while the control group experienced the opposite effect. Even though no statistical analysis was performed on the variable cardiorespiratory function (only one study), an increase of VO_{2max} of 0.43 L/min was observed, representing a clinically relevant improvement on this variable with exercise training. No adverse events resulting from the exercise training

was reported in any study.

2000-2006

From 2000 to 2006, 11 studies^[10-20] met the review inclusion criteria with 6 of them being randomized controlled trials. More than half of the studies during this time period were conducted in patients who had completed chemotherapy and/or radiation therapy ($n = 6$, 54.5%) while all of the other studies (45.5%) were conducted with patients undergoing treatment ($n = 4$), and 1 study including patients in and off-treatment as part of the study design^[12]. The average age for patients that participated in the exercise interventions was 51.09 ± 4.6 and 51.03 ± 4.9 for patients in the control groups. The majority of studies used an exercise prescription that combined aerobic with resistance training (68.8%) with only 31.2% of the studies using aerobic training only. The major modes of aerobic exercise included walking, swimming and cycling. No studies during this time period used resistance training exclusively as the major mode of exercise. On average, aerobic exercise was prescribed 2-4 d/wk for approximately 30 min, with intensities varying from 50%-80% of peak heart rate, 50%-75% of heart rate reserve, rate of perceived exertion between 11-15 with overall training duration between 8-14 wk. The resistance training portion of the exercise prescription lasted approximately 40 min (37 ± 17 min) and consisted of exercises targeting major muscles groups performed for 2-3 sets, with approximately 10-12 repetitions using weight training machines, dumbbells, and rubber bands. The description of exercise intensity used in the 2000-2006 studies that included resistance training as a portion of the exercise intervention varied significantly and clear descriptions of the intensities used were often missing. For example, one study used the patient's own body weight to induce a training response^[14] while one study would increase the resistance between 5%-10% of the previous load as soon as patients were able to achieve the maximum number of repetitions prescribed^[19]. All other studies that used resistance training did not describe the intensity used during the resistance portion of the exercise intervention. The duration of training in studies that used aerobic or a combination of aerobic and resistance training was approximately 11 wk (10.9 ± 6.78). As mentioned, six studies were randomized controlled trials^[11,13,16,17,19,20]. The primary mean for prescribing and monitoring exercise intensity was heart rates, which varied from 70%-75% of $\text{VO}_{2\text{peak}}$, 40%-70% of estimated $\text{VO}_{2\text{max}}$, 60%-90% of predicted maximum heart rate, and 60%-70% of heart rate reserve. This heterogeneity in prescribing exercise intensity is troublesome since significant differences in the determination of exercise intensity from using one method versus the other can produce significantly different prescriptions. Eleven studies were performed between 2000-2006^[10-20]. Seven were conducted in a supervised setting^[11,12,13,15,16,19,20], while 3 were home-based interventions^[10,14,17,18]. One study used a supervised exercise session plus 2 self-monitored exercise

sessions at home per week^[14], while 1 study used a period of two weeks familiarizing subjects to the exercise intervention in a supervised setting followed by a home-based self-monitored exercise training for the remaining of the study^[18]. The summary of the results of the studies conducted between 2000-2006 is presented in Table 2.

Cardiorespiratory function, when assessed directly using maximal CPET with gas exchange *via* cycle ergometer, showed significant improvements in patients who underwent exercise training ($P < 0.0001$). An improvement corresponding to an approximately 9% (2.14 mL/kg per minute) from baseline testing was observed for the exercise group while the control group showed no significant changes^[11,13,14,18,19]. When cardiorespiratory function was indirectly measured (*i.e.*, using submaximal/estimated $\text{VO}_{2\text{max}}$ testing protocols), no significant changes in either the exercise or control groups were observed, however, a trend towards improvements in the exercise group was observed. Conversely, patients in the control group did not change or end up decreasing cardiorespiratory function from baseline to completion of study protocols^[6]. Due to the small number of studies that have assessed some strength parameter and the lack of a control group in studies conducted during the period of 2000-2006, it is not possible at this time to provide an evaluation on the effects of a combined aerobic and resistance training exercise intervention on measurements of strength in breast cancer patients.

The only body composition parameter that significantly changed from baseline was body fat % in the exercise group (change of 1.51%). No significant changes in any other body composition parameter was observed in either exercise or control groups. However, a trend towards positive changes for the exercise group (*i.e.*, reduction in body mass and fat mass) and some negative changes in the control group such as increases in body mass, fat mass, and body fat %, suggesting that exercise training may be a promising intervention in alleviating negative body composition changes in breast cancer patients warranting further research^[5].

Significant improvements in overall quality of life assessed using the Functional Assessment of Cancer Therapy-Breast was observed in the exercise group ($P = 0.027$) with no changes in the control group. No other PROs were significant in either group; however, trends of reductions in fatigue and depression were observed in the exercise but not in the control group^[9]. A few adverse events were reported during 2000-2006 and included lymphedema (3 cases), gynecologic complications (1 case), and influenza (1 case)^[13].

2007-2013

From 2007 to November 2013, 35 exercise studies involving breast cancer patients were conducted^[21-55]. Twenty-seven (77%) of all studies conducted during this time period were randomized controlled trials. Out of all 35 exercise studies, 22 examined the effects of exercise training in patients undergoing treatment (62.8%), 1

Table 2 2000-2006 study results summary

Measure	No. of studies	N	Baseline		Post-intervention		Change		P
			Mean	SD	Mean	SD	Mean	95%CI	
Cardiorespiratory function									
Bike VO _{2peak} (mL/kg per minute)									
Exercise	5	81	23.26	2.95	25.40	2.75	2.14	-4.28	0.000
Control	2	36	23.10	6.08	21.95	5.30	-1.15	-13.98	0.284
Bike VO _{2peak} (L/min)									
Exercise	1	22	1.67	0.35	1.81	0.37	0.14	-	-
Control	1	19	1.6	0.36	1.63	0.35	0.03	-	-
Est. Treadmill. VO _{2peak} (mL/kg per minute)									
Exercise	1	40	30.58	-	35.20	-	4.62	-	-
Control	0		-		-				
VO _{2peak} (L/min)									
Exercise	1	22	1.67	-	1.81	-	0.14	-	-
Control	1	19	1.6	-	1.63	-	0.03	-	-
mCAFT VO _{2peak} (mL/kg per minute)									
Exercise	1	82	25.7	0.28	26.45	0.49	-0.75	-	-
Control	1	41	25.1	6.10	25.10	6.10	0.00	-	-
12-min walk test (m)									
Exercise	2	56	1032.35	8.98	1264.8	126.15	232.45	-2428.16	0.248
Control	2	37	1045.2	59.11	944.8	194.03	-100.4	-2424.34	0.484
6-min walk test (m)									
Exercise	1	9	672.9	-	776.03	-	103.13	-	-
Control	0								
1 mile walk test (min)									
Exercise	1	43	17.45	-	16.34	-	-1.11	-	-
Control	1	43	17.65	-	17.85	-	0.20	-	-
Body composition									
Body mass (kg)									
Exercise	9	248	69.47	10.36	68.43	9.54	-1.04	-2.83	0.131
Control	7	183	75.80	6.46	76.64	6.31	0.84	-2.62	0.166
Fat mass (kg)									
Exercise	4	75	25.95	9.73	23.93	8.57	-2.03	-7.57	0.187
Control	3	73	32.90	8.18	33.20	6.52	-0.30	-8.44	0.789
Body fat (%)									
Exercise	8	218	33.82	8.25	32.31	8.03	1.51	0.37-2.66	0.017
Control	6	165	36.48	9.29	36.66	8.96	0.18	-2.03	0.673
Strength									
1RM UB composite (kg)									
Exercise	1	21	9.1	1.93	10.16	2.08	0.96	-	-
Control	1	15	8.77	1.75	8.59	1.29	-0.18	-	-
1RM UB (kg)									
Exercise	2	67	22.63	10.15	30.23	13.39	7.61	-58.33	0.187
Control	0								
1RM LB (kg)									
Exercise	3	76	99.87	31.29	133.19	57.10	33.32	-141.42	0.180
Control	0								
Psychosocial Measures									
FACT-G									
Exercise	2	37	78.75	9.55	87.60	5.23	8.85	-77.50	0.211
Control	2	38	83.90	6.93	82.70	9.33	-1.20	-43.20	0.609
FACT-B									
Exercise	3	47	100.3	9.30	111.03	7.42	10.73	-15.37	0.027
Control	2	38	105.75	13.79	105.05	15.20	-0.70	-25.42	0.611
SF-36									
Exercise	1	42	76.30	0.28	94.75	8.98	18.45	-	-
Control	1	41	83.50	-	102.4	-	18.90	-	-
WHO-QOL BREF									
Exercise	1	27	8.2	0.92	8.8	0.78	0.60	-	-
Control	0								
PFS									
Exercise	2	22	5.07	0.24	3.47	0.47	-1.61	0.50-4.81	0.194
Control	1	10	4.87	2.50	4.62	2.50	-0.25	-	-
SCFS									
Exercise	1	44	10.70	3.75	10.20	6.00	-0.50	-	-
Control	1	27	10.80	0.00	13.40	5.60	2.60	-	-
LAS									
Exercise	1	43	42.47	23.54	27.08	21.41	-15.39	-	-
Control	1	43	41.66	25.04	42.28	26.20	0.62	-	-

BDI										
Exercise	1	40	5.84	4.90	3.92	4.00	-1.92	-	-	
Control	0									
HRSD										
Exercise	1	40	9.46	6.00	6.10	5.00	-3.36	-	-	
Control	0									

VO_{2peak}: Peak oxygen uptake; mCAFT: Modified Canadian Fitness Test; UB composite: Upper body combined strength measures; UB: Upper body strength test; LB: Lower body strength test; EORTC: European Organization for Research and Treatment of Cancer; FACT-G: Functional Assessment of Cancer Therapy-General; FACT-B: Functional Assessment of Cancer Therapy-Breast; SF-36: Short-Form-36; WHO-QOL BREF: The World Health Organization-BREF Quality of Life Instrument; PFS: Piper Fatigue Scale; SCFS: Schwartz Cancer Fatigue Scale; LAS: Linear Analog Scale; BDI: Beck Depression Inventory; HRSD: The Hamilton Rating Scale for Depression; Est. Treadmill VO_{2peak}: VO_{2peak} estimated *via* submaximal CPET; CPET: Cardiopulmonary exercise test.

study studied patients in and off treatment^[40] and 1 study examined the effects of exercise in patients receiving neo-adjuvant therapy^[55]. The average age for patients that participated in the exercise interventions was 58.0 ± 3.9 and 53.7 ± 4.5 for patients in the control groups. Most of the studies were conducted in a supervised setting ($n = 27$, 77% of all studies conducted between 2007 and November 2013). Sixteen studies used aerobic exercise as the main mode of exercise training, 12 used a combination of aerobic and resistance exercise training, while only 7 used resistance exercise training as the main mode of exercise training or had a study arm that examined the effects of resistance training on outcome measurement/variables of interest to this review. The modes of exercise for the aerobic exercise training interventions included walking, jogging, treadmill, cycle ergometer, elliptical, low-impact aerobics, stepping, arm ergometer, rowing ergometer, mini-trampolines, step-up blocks, and circuit training. For studies that used resistance training as their major mode of exercise or as the mode of a study arm, weight machines, free weights, elastic bands, tubing, therapeutic balls, and resistance-training circuits were used. The average frequency of training was 3 d/wk ranging from 2-5 training sessions per week, with overall training duration average of 23 wk (range of 3-48 wk). Each training session lasted on average 46 min, with training sessions ranging from 15-90 min. The intensity for aerobic training varied from 40%-85% of maximum heart rate, 40%-90% of VO_{2peak}, while the intensity for resistance training ranged from 55%-85% of 1RM. Minimal adverse events due to exercise were reported; when reported, events such as lightheaded, hypotension, nausea, and weakness during exercise testing were basically the extent of the complications with patients recovering quickly afterwards.

Most studies assessed cardiorespiratory function using estimated measurement protocols. When using estimated measurements derived from a submaximal treadmill testing protocol, a significant improvement in cardiorespiratory function was observed for the exercise group (improvement from baseline of 3.54 mL/kg per minute, $P < 0.0005$). When cardiorespiratory function was measured directly using a cycle ergometer maximal oxygen uptake test protocol with gas exchange, improvement from baseline approached significance in the exercise group (improvement from baseline of 2.38 mL/kg per minute, $P = 0.057$). Significant decrease in meters walked

during a 6 min and 2 km walk tests was observed in the control group (-9.52 m, -0.61 m, $P = 0.008$ and $P = 0.010$ respectively) while improvements in a 12 min walk test approached significance in the exercise group (improvement of +162 m, $P = 0.062$). A summary of the results of the effects of exercise on cardiorespiratory function is presented in Table 3.

Positive body composition changes with exercise training were observed in the exercise group, with the most notorious changes in significant decrease in body fat % ($P = 0.037$) and increase in lean body mass ($P = 0.002$). The control group experience significant increases in body fat % ($P = 0.009$) and a trend toward an increase in overall body mass ($P = 0.065$). Table 4 provides a summary of studies that evaluated changes in body composition outcomes.

Quality of life significantly improved in the exercise group when the Functional Assessment of Cancer Therapy-General was used (improvement of +6.90 points, $P = 0.022$). Also, when using the Beck Inventory, the effects of exercise training significantly reduced depression levels in the exercise group (reduction of -3.40 points, $P = 0.037$). Even though many of the analyses performed on other PROs showed no significant changes in either group, there is a clear trend towards greater improvement in quality of life, reduction in fatigue and depression in the exercise group when compared to the control group. The summary of the analyses performed on reported PROs is presented in Table 5.

Resistance training

Due to the limited number of studies that used resistance training as the main mode of exercise training^[42,46,52,54], and those that used resistance training as an arm part of the study design^[27,29,43], we were unable to conduct analyses to evaluate the effects of resistance training on cardiorespiratory function measured directly through CPET with gas analyses. However, the effects of resistance training on cardiorespiratory function evaluated using a 12-min walk test, showed improvement in the exercise group of 35 m while a decline of 91 m was observed for the control group. Regarding the effects of resistance training on changes in body composition, a significant increase in body mass and lean body mass was observed in the exercise group (increase of 1.20 and 0.65 kg, $P = 0.016$ and $P = 0.049$, respectively). Upper body strength significantly increased in the exercise group (increase of +5.31

Table 3 2007-2013 cardiorespiratory function study results summary

Measure	No. of studies	N	Baseline		Post-intervention		Change		P
			Mean	SD	Mean	SD	Mean	95%CI	
Cardiorespiratory function									
Treadmill VO _{2peak} (mL/kg per minute)									
Exercise	1	71	25.20	-	25.70	-	0.5	-	-
Control	1	73	24.80	-	23.50	-	-1.3	-	-
Treadmill VO _{2peak} (L/min)									
Exercise	1	71	1.72	-	1.77	-	0.05	-	-
Control	1	73	1.76	-	1.68	-	-0.08	-	-
Est. Treadmill VO _{2peak} (mL/kg per minute)									
Exercise	9	265	23.51	4.56	27.05	5.12	3.54	-4.84-2.26	< 0.0005
Control	5	88	24.45	6.08	25.02	6.80	0.57	-2.85	0.328
Bike VO _{2peak} (mL/kg per minute)									
Exercise	2	37	22.11	3.68	24.49	3.38	2.38	-5.47	0.057
Control	2	33	21.98	6.34	20.83	6.82	-1.15	-8.77	0.185
Bike VO _{2peak} (L/min)									
Exercise	1	10	1.41	-	1.59	-	0.18	-	-
Control	1	10	1.34	-	1.20	-	-0.14	-	-
Est. Bike VO _{2peak} (mL/kg per minute)									
Exercise	1	18	21.07	-	23.88	-	2.81	-	-
Control	0	0	-	-	-	-	-	-	-
6-min walk test (m)									
Exercise	2	30	463.23	56.37	482.57	49.84	19.34	-117.4	0.149
Control	2	32	450.14	33.57	440.62	33.40	-9.52	8.05-10.98	0.008
12-min walk test (m)									
Exercise	3	129	931.00	102.93	1093.0	160.18	162	-364.7	0.062
Control	3	124	921.03	148.50	888.13	132.92	-32.9	-258.0	0.387
2 km walk test (min)									
Exercise	3	313	18.05	0.44	17.38	0.42	-0.67	-1.60	0.070
Control	3	280	17.94	0.33	17.33	0.23	-0.61	0.34-0.86	0.010

VO_{2peak}: Peak oxygen uptake; Treadmill VO_{2peak}: VO_{2peak} obtained directly through CPET with gas exchange; Est. Treadmill VO_{2peak}: VO_{2peak} estimated *via* sub-maximal CPET; CPET: Cardiopulmonary exercise test; Est.: Estimated.

Table 4 2007-2013 body composition study results summary

Measure	No. of studies	N	Baseline		Post-intervention		Change		P
			Mean	SD	Mean	SD	Mean	95%CI	
Body composition									
Body mass (kg)									
Exercise	10	550	74.66	3.94	74.25	3.84	-0.41	-0.38 to 1.20	0.268
Control	9	527	73.59	4.63	74.72	4.69	+1.13	-2.35 to 0.09	0.065
Fat mass (kg)									
Exercise	1	262	25.73	-	26.43	-	+0.70	-	-
Control	1	236	24.45	-	25.12	-	+0.67	-	-
Body fat (%)									
Exercise	13	609	38.00	5.03	37.15	5.80	-0.85	0.06 to 1.64	0.037
Control	11	536	38.50	4.41	39.00	4.12	+0.50	-0.84 to -0.16	0.009
Lean body mass (kg)									
Exercise	3	330	43.91	0.32	44.29	0.31	+0.38	-0.45 to -0.31	0.002
Control	3	308	43.45	0.37	43.27	0.56	-0.18	-0.49 to 0.87	0.358
Lean body mass (%)									
Exercise	1	10	71.00	-	74.10	-	+3.10	-	-
Control	1	10	69.10	-	68.90	-	-0.20	-	-

kg, $P = 0.005$) while for lower body, strength significantly increased in both, exercise and control groups ($P = 0.25$, $P = 0.008$ respectively) with a greater increase observed in the exercise group (increase of +17.82 kg *vs* +5.42 kg, respectively). No significant changes in quality of life were observed in either the exercise and control groups. The summary of the results of the effects of resistance training on outcomes included in this review is presented in Table 6.

DISCUSSION

During the past 25 years, studies examining the effects of exercise training in breast cancer survivors have steadily increased. Based on the inclusion criteria adopted by this systematic review, from 5 initial studies published between 1989-1999, 35 studies were published during the last 7 years. The increase in published studies in this area of research is a testament of the growing interest

Table 5 2007-2013 patient reported outcomes study results summary

Measure	No. of studies	N	Baseline		Post-intervention		Change		P
			Mean	SD	Mean	SD	Mean	95%CI	
Quality of life									
EORTC, 0-100									
Exercise	2	296	74.05	13.36	79.95	7.71	+5.90	-101.64	0.379
Control	1	236	80.90	-	84.30	-	3.40	-	-
FACT-G, 0-104									
Exercise	4	165	78.98	3.30	85.88	4.22	6.90	-10.05	0.022
Control	4	178	79.74	6.89	79.63	10.88	-0.11	-21.22	0.976
FACT-B, 0-104									
Exercise	5	99	103.92	6.75	110.32	4.25	6.40	-17.44	0.111
Control	5	82	108.76	5.72	106.53	9.98	-2.23	-22.82	0.616
SF-36 physical function, 0-100									
Exercise	1	25	47.41	-	50.80	-	3.39	-	-
Control	0	0	-	-	-	-	-	-	-
Fatigue ¹ piper, 0-10									
Exercise	3	140	3.88	1.05	3.08	0.41	-0.80	-4.34	0.250
Control	2	45	3.78	0.17	3.91	0.35	0.13	-3.30	0.500
FSI, 1-10									
Exercise	1	11	3.40	-	3.40	-	0.00	-	-
Control	1	9	3.25	-	3.30	-	0.05	-	-
FACIT-F									
Exercise	4	373	37.85	2.95	39.58	5.87	1.73	-10.49	0.372
Control	4	351	40.11	4.07	40.08	3.73	-0.03	-6.12	0.975
Depression ² BDI, 0-63									
Exercise	2	93	11.75	0.07	8.35	0.35	-3.40	0.86-5.94	0.037
Control	2	102	10.96	2.89	11.21	0.42	0.25	-44.48	0.910
R-Beck Inventory									
Exercise	2	295	8.53	7.11	4.60	1.98	-3.93	-92.24	0.475
Control	2	271	7.15	5.15	6.67	5.18	-0.48	0.23-0.73	0.027
CES-D, 0-24									
Exercise	1	71	12.80	-	9.70	-	-3.10	-	-
Control	1	73	13.90	-	10.80	-	-3.10	-	-
HADS, 0-21									
Exercise	1	30	4.17	-	2.70	-	-1.47	-	-
Control	1	28	4.79	-	4.64	-	-0.15	-	-

¹Lower scores reflect lower fatigue; ²Lower scores reflect lower depression. BDI: Beck Depression Inventory; EORTC: European Organization for Research and Treatment of Cancer; FACT-G: Functional Assessment of Cancer Therapy-General; FACT-B: Functional Assessment of Cancer Therapy-Breast; SF-36: Short-Form-36; FSI: The Fatigue Symptom Inventory; FACIT-F: Functional Assessment of Chronic Illness Therapy-Fatigue; BDI: Beck Depression Inventory; R-Beck Inventory: R-Beck Depression Inventory; CES-D: Center for epidemiologic studies depression scale (short-form); HADS: Hospital anxiety and depression scale.

by health care professionals and the medical community on exploring complementary therapies that have the potential to improve the overall care and health of breast cancer patients while promoting alleviation of cancer treatment-related side-effects. The evolution of the science examining the effects of exercise in breast cancer patients has progressed from studies using simpler exercise prescriptions (*i.e.*, aerobic exercise only prescriptions) to more complex designs incorporating prescriptions that combined aerobic and resistance training exercises. Interestingly, however, of the studies conducted during first decade^[5-9], all examined the effects of exercise while patients were undergoing major cancer treatments (*i.e.*, surgery, chemotherapy and/or radiation therapy) and the majorities were home-based interventions^[7-9]. Besides some limitations presented by these initial studies^[5-9] (including relatively small sample sizes and lack of more rigorous exercise prescriptions) the overall results were promising. Breast cancer patients, who exercised while undergoing cancer treatments, were not only able

to tolerate the exercise prescriptions but also presented a more favorable trend towards alleviation of decrements in functional capacity, fatigue levels, and depression when compared to patients who did not exercise. Also, and very importantly, no adverse events were reported and patients seemed to have no problems engaging in regular exercise training. Although limited data were available for a more precise evaluation of these initial studies, the results were promising and served as the basis for subsequent trials conducted with breast cancer patients.

The most noticeable differences between the initial studies conducted between 1989-1999 and those between 2000-2006 were the use of aerobic exercise combined with resistance training in the majority of the studies (68.8%) and the fact that over 50% of these studies were conducted in patients who had completed their major cancer treatments. Furthermore, the heterogeneity of exercise prescriptions and a variety of different measurements used to assess the major outcome variables included in this review makes it impossible for direct com-

Table 6 2007-2013 resistance exercise study results summary

Measure	No. of studies	N	Baseline		Post-intervention		Change		P
			Mean	SD	Mean	SD	Mean	95%CI	
Cardiorespiratory fitness									
Treadmill VO _{2peak} (mL/kg per minutes)									
Exercise	1	77	25.5	-	24.20	-	-1.30	-	-
Control	1	73	24.8	-	23.50	-	-1.30	-	-
Treadmill VO _{2peak} (L/min)									
Exercise	1	77	1.73	-	1.67	-	-0.06	-	-
Control	1	73	1.76	-	1.68	-	-0.08	-	-
Est. Treadmill VO _{2peak} (mL/kg per minutes)									
Exercise	1	9	23.46	-	24.22	-	0.76	-	-
Control	0	0	-	-	-	-	-	-	-
12-min walk test (m)									
Exercise	1	21	1020.00	-	1055.00	-	35.00	-	-
Control	1	23	1035.00	-	944.00	-	-91.00	-	-
Body composition									
Body mass (kg)									
Exercise	3	136	72.53	2.96	73.73	2.70	1.20	-1.32	0.016
Control	3	129	72.33	1.81	73.30	0.95	0.97	-4.29	0.192
Fat mass (kg)									
Exercise	2	59	28.10	3.25	28.70	3.11	0.60	-2.54	0.105
Control	2	56	26.70	3.25	27.50	2.12	0.80	-20.32	0.500
Body fat (%)									
Exercise	4	145	36.75	2.82	36.60	3.05	-0.15	-1.18	0.476
Control	3	129	37.27	2.57	37.97	2.15	0.70	-3.94	0.266
Lean body mass (kg)									
Exercise	2	59	44.35	1.34	45.00	1.41	0.65	-1.29 to -0.01	0.049
Control	2	56	44.70	0.00	45.15	0.07	+0.45	-1.28	0.070
Muscular strength									
1-RM upper body composite (kg)									
Exercise	1	43	32.93	-	39.38	-	6.45	-	-
Control	1	19	31.45	-	32.30	-	0.85	-	-
1-RM upper body single test (kg)									
Exercise	5	166	26.37	5.03	31.68	4.78	5.31	-7.94 to -2.69	0.005
Control	4	152	27.60	3.79	28.65	3.15	+1.05	-2.27	0.059
1-RM lower body single test (kg)									
Exercise	5	200	70.58	29.80	88.40	38.01	17.82	-32.06 to -3.58	0.025
Control	5	171	68.19	25.36	73.61	27.74	+5.42	-8.51 to -2.33	0.008
Quality of Life									
SF-36 physical function, 0-100									
Exercise	2	79	47.80	3.54	50.28	2.02	+2.48	-27.31	0.261
Control	2	50	49.00	3.96	48.85	4.88	-0.15	-16.52	0.856
Fatigue ¹ SCFS, 6-36									
Exercise	1	36	9.90	-	10.10	-	0.20	-	-
Control	1	31	9.30	-	9.00	-	-0.30	-	-
FACIT-F									
Exercise	1	77	34.30	-	36.30	-	2.00	-	-
Control	1	73	34.60	-	34.90	-	0.30	-	-
Depression ² CES-D, 0-24									
Exercise	1	77	13.80	-	10.60	-	-3.20	-	-
Control	1	73	13.90	-	10.80	-	-3.10	-	-

¹Lower scores reflect lower fatigue; ²Lower scores reflect lower depression. VO_{2peak}: Peak oxygen uptake; Est. Treadmill VO_{2peak}: VO_{2peak} estimated *via* sub-maximal CPET; CPET: Cardiopulmonary exercise test; Est.: Estimated; RM: Repetition Maximum; SF-36: Short-Form-36; SCFS: Schwartz cancer fatigue scale; FACIT-F: Functional assessment of chronic illness therapy-fatigue; CES-D: Center for Epidemiologic Studies Depression Scale (short-form).

parison to be made between studies. Importantly during 2000-2006, a significant increase in number of breast cancer patients studied and overall improvements in the quality of study methodologies, marked a new era in this area of research with significant improvements observed in physiological (*i.e.*, improved VO_{2max}, decreases in body fat %) and overall quality of life in the exercise group, while the control patients continued to present not so favorable outcomes during and post completion of cancer treatments. Out of studies that utilized exercise prescrip-

tions following a training progression (*i.e.*, considered by the exercise physiology scientific community as efficacious training methodologies in the promotion of more pronounced training responses) a study conducted by Courneya *et al*^[14], showed a remarkable improvement in cardiorespiratory function using a prescription derived from the results of a gold standard evaluation of cardiopulmonary function (CPET with gas analyses). Not only did Courneya's study show an improvement of 17.4% in cardiorespiratory function in patients randomized

into the exercise group, the patients in the control group decreased their cardiopulmonary function by 3.4%. Furthermore, these authors provided a better description of the sample, exercise testing procedures, and exercise prescription, along with the reporting of adverse events that occurred during the study improved the scientific rigor in this area, which was later followed by many groups around the world.

Overall, the results of all studies conducted during 2000-2006 continued to show promising results, mainly on cardiorespiratory function, body composition, and overall quality of life of patients, increasing even more the interest of the medical community for the use of exercise as an intervention to alleviate treatment-related side effects. With improved science approaches, the challenges faced by investigators around the globe began to surface even more. The heterogeneity in exercise prescriptions characteristics, different instruments that were used to evaluate major outcome variables, different treatment regimens, and testing of different exercise interventions designed to provide the most benefit to patients were among major issues that preclude one's ability to evaluate the real benefit of exercise in breast cancer patients.

During the last 7 years of research evaluating the effects of exercise in breast cancer patients, more pronounced improvements were observed in all major outcome variables included in this review when compared to previous years. Significant improvements in cardiorespiratory function of 2.38 mL/kg per minute, when measured directly using cycle ergometry and slightly higher values (3.54 mL/kg per minute) observed when cardiorespiratory function was estimated used submaximal testing protocols were reported. The larger improvement in cardiorespiratory function can be explained, in part, by the more rigorous exercise prescriptions as well as due to the inclusion of the resistance training as a component of training. Furthermore, more pronounced improvements were also observed in various body composition parameters. Significant decreases in body fat % and increases in lean body mass were observed in the exercise group while significant increase and no change in body fat % and lean body mass were observed for the control group. Furthermore, a trend of overall weight gain was more noticeable in the control group than the exercise group. Again, the inclusion of resistance training on study design of a large portion of the studies conducted between 2007-2013 may help explain the more pronounced positive changes observed in the exercise groups. Lastly, significant decrease in depression along with improvements in quality of life observed in the exercise group (PROs), while no change in quality of life and slight increases in depression in patients on the control group, once again support the idea that exercise during breast cancer helps patients to live a higher overall quality of life than patients that do not engage in regular exercise training.

The inclusion of resistance training on the study designs during 2007-2013 as a study arm or even as the main exercise training mode, has been utilized with the

objective of producing more effective changes in overall functionality and to alleviate the negative alterations in body composition commonly observed in breast cancer patients during the entire cancer continuum (*i.e.*, increases in body mass, with concomitant increase in fat mass and loss of muscle mass; condition known as sarcopenic obesity). The inclusion of this type of training has also provided for variety in the training prescription of cancer patients, which results in increased adherence; a key factor to promote more robust physiological adaptations. A few studies did not meet the inclusion criteria for this review, however, those studies examined the effects of exercise on biological markers associated with the negative changes in body composition. This area of study is highly significant due to the influence negative changes in body composition may have on the development of secondary cancers, mortality and co-morbidities (*e.g.*, sarcopenic obesity development consequences)^[2]. Recently, an exercise-related model proposed by our group on the alleviation of negative changes in body composition and its potential associations with biomarkers of inflammation and androgenic hormones has been developed and initial investigations are promising^[56].

Very little data available today allow for a good understanding on the effects of resistance training on the outcomes included on this review. However, as expected, due to the known effects of resistance training on muscle mass gain and muscular strength, significant improvements were observed on lean body mass and upper and lower body strength on patients who participated in exercise training and were objectively measured on these outcomes.

Unfortunately, due to the small number of studies that met the rigorous criteria for inclusion in this review, the data on effects of strength training on PROs are very limited and no definite conclusion can be drawn at this time regarding the effects of resistance training on fatigue, depression, and overall quality of life in breast cancer patients. More research is desperately needed in this area of study.

There are limitations within this systematic review on the effects of exercise in breast cancer survivors that must be considered when evaluating the overall results; including a small number of studies per outcome variable included in this review, the heterogeneity of tools used to evaluate the outcome variables of interest, significant differences in the characteristics of the exercise prescription utilized, and to a much lesser extent, the evaluation of patients in and off treatment included together in the analyses of the data. The latter could impose difficulty in interpreting the results, however, a priori evaluation of the overall changes in the parameters included in this review indicated very little clinical difference between results of studies that evaluated patients undergoing treatment and those off treatment. Therefore, we decided that for a chronological overview of the current literature in the topic, the clinical difference observed between patients in and off treatment would not drastically under

or overestimate the evaluation of the outcome variables studied. Since this is a relatively new area of research and until larger randomized trials are completed (controlling for many variables that can confound study results such as type, frequency, intensity of exercise, different types of cancer treatments, age of patients, previous fitness levels, and other co-morbidities that can further diminish the tolerability for exercise participation) definite conclusions and more precise exercise summary guidelines can be presented.

Despite these methodological limitations and our inability to currently provide specific exercise guidelines for breast cancer patients, general-generic exercise guidelines^[2] are available and can be used to guide safer participation in exercise programs. It is recommended by the exercise guidelines set forth by the American College of Sports Medicine^[2], that patients should engage, whenever possible, in 150 min/wk of moderate-intensity exercise spread throughout the week, that the exercise prescription should include aerobic and resistance modes of exercise, and that the prescription should be individualized taking into consideration the limitations of each patients.

In conclusion, order to improve the knowledge in the area of research examining the effects of exercise in breast cancer patients more studies are necessary. These studies should include larger samples sizes, involve randomized clinical controlled trials, provide more detailed descriptions of the sample studied (*i.e.*, presence of other co-morbidities, previous physical activity levels, amount of therapies received, *etc.*) and all testing and exercise protocols (extremely important consideration for improvement in the quality of the science) in their published reports, and provide better reporting on adverse events due to exercise testing and training. Furthermore, studies examining the mechanisms involved in the plasticity of different physiological systems due to different exercise training protocols in breast cancer patients are critical for continued progress of this area of research. Nevertheless, based on the current data available in this area of research, exercise training appears to be safe for most patients and improvements in physiological, psychological, and functional parameters can be attained with regular participation in moderate intensity exercise.

COMMENTS

Background

An ever-growing number of studies in the area of exercise oncology, especially those examining the effects of exercise training in breast cancer survivors, speak loudly to the increased interest by the medical community in exploring complementary interventions that can alleviate treatment-related side effects and improve quality of life of cancer patients.

Research frontiers

Most specifically, this review focuses on presenting and discussing the results of the effects of exercise training on the most commonly evaluated outcomes in the area including physiological parameters of cardiorespiratory function, body composition, and muscular strength, and patients reported outcomes that evaluated fatigue, depression, anxiety, and overall quality of life.

Innovations and breakthroughs

For organizational purposes a chronological description of studies results, stud-

ies common characteristics, and commentaries on changes observed in this area of research over the past 25 years are discussed.

Peer review

The arm of this study was to review the effect of exercises for patients with breast cancer in the past 25-years. It is fairly well designed and the statistical analyses appear reasonable. It is worthy of recommending for publication.

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Meta-regression of treatments for metastatic colorectal cancer: Quantifying incremental benefit from 2000 to 2012

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Abstract

To evaluate the overall effectiveness of treatments for metastatic colorectal cancer, a meta-regression was undertaken in which randomized studies from 2000 to 2012 were evaluated and the temporal trend for both overall survival (OS) and progression-free survival (PFS) was determined. Our literature search was essentially based on PubMed but information sources were scanned. Trials were included if a fluoropyrimidine regimen was given to at least one arm and information on PFS and OS was available. Medians for OS and PFS were our end-points. Covariates included temporal trend, arm allocation and Kirsten rat sarcoma status. In analyzing 130 treatment arms identified through our literature search, meta-regression showed an improvement with time for both OS ($P < 0.001$) and PFS ($P < 0.001$). The increase in median OS was from 14.9 mo in 2000 to 18.8 mo in 2012. Likewise, the improvement in PFS was from 5.7 to 8.1 mo. Multivariate analysis confirmed these findings. A post-hoc multivariate analysis was focused on patient arms treated with bevacizumab ($n = 17$) or without bevacizumab ($n = 113$); the multivariate-adjusted improvement attributable to bevacizumab was 1.66 mo for OS ($P = 0.071$) and 1.59 mo for PFS ($P = 0.002$). Overall, our results indicate

that OS and PFS have improved from 2000 to 2012 but the extent of this improvement is small and seems to have quite a questionable clinical relevance.

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Key words: Metastatic colorectal cancer; Chemotherapy; 5-Fluorouracil; Chemotherapy; Irinotecan; Oxaliplatin; Bevacizumab; Meta-analysis; Meta-regression

Core tip: We conducted out a meta-regression in which randomized studies from 2000 to 2012 were evaluated and the temporal trend was analyzed [end points: overall survival (OS), and progression-free survival (PFS)]. Our literature search identified all trials employing a fluoropyrimidine regimen. Covariates included temporal trend, arm allocation and Kirsten rat sarcoma status. According to meta-regression of 130 treatment arms, the improvement between 2002 and 2012 was from 14.9 to 18.8 mo for OS ($P < 0.001$) and from 5.7 to 8.1 mo for PFS ($P < 0.001$). Our study indicates that OS and PFS have improved from 2000 to 2012 but the extent of this improvement is small.

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TO THE EDITOR

It is widely accepted that treatments for metastatic colorectal cancer (mCRC) have considerably improved over the past decade^[1-7], but quantitative estimates of increased benefits with time are scarce and have rarely

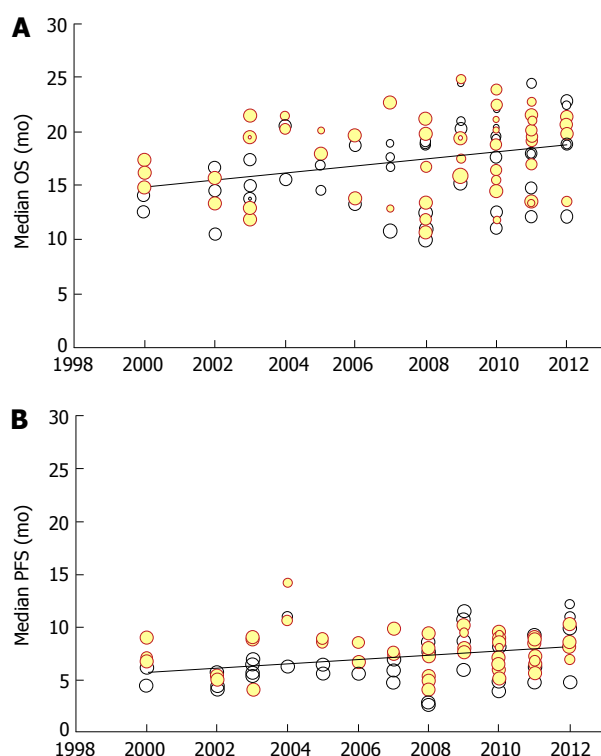


Figure 1 Meta-regression: temporal trend of medians for overall survival (A) and progression-free survival (B). These graphs refer to 130 treatment arms from 52 randomized studies. The regression lines were the following: A: Median (mo) = $0.331 \times \text{yr} - 674.138$ ($P < 0.001$); B: Median (mo) = $0.2026 \times \text{yr} - 399.538$ ($P < 0.001$). Symbols: each study is represented by a circle, the area of which is proportional to its statistical weight. Experimental arms are depicted in red while control arms are in black. OS: Overall survival; PFS: Progression-free survival.

relied on meta-analysis techniques. The temporal trend of this therapeutic improvement has mostly been based on narrative reviews or, in some cases, on traditional meta-analyses focused on hazard ratios^[8-10]. No attempt has been made to apply meta-regression. Furthermore, the only studies available in this area^[8,9] have generally preferred relative indexes (*e.g.*, hazard ratios) rather than absolute end-points like median values of survival^[11].

We describe the results of a meta-regression of randomized studies conducted from 2000 to 2012, in which we determined the temporal trend of overall survival (OS) and progression-free survival (PFS). The literature search for our analysis was nearly identical to that of Sidhu *et al.*^[8], although we extended our time horizon up to December 2012. Randomized trials were included if they met two simple criteria: fluoropyrimidine-based regimens in at least one arm and data available on PFS and OS. Our statistical methods were the same as those described previously^[12-15]. Temporal trends (focused on medians as end-point) were determined for OS and PFS. Time as covariate was defined as publication year for each trial. The statistical model was random effect. Both univariate and multivariate analyses were performed; in the latter case, two additional covariates were tested (arm = experimental group versus controls; Kirsten rat sarcoma (KRAS) status = wild-type, not reported, or mutant type). After

completing our literature search, 130 treatment arms were found to meet our inclusion criteria. Most of these trials employed oxaliplatin or irinotecan; bevacizumab was given to a total of 17 treatment arms.

On the basis of these 130 treatment arms, our meta-regression (univariate analysis) found a significant improvement with time for both end-points of OS and PFS (Figure 1). From 2000 to 2012, median OS increased from 14.9 to 18.8 mo. Likewise, median PFS improved from 5.7 to 8.1 mo. The absolute improvement for every 10 years was 3.3 and 2.0 mo for OS and PFS, respectively.

At multivariate analysis, our results for OS showed that the difference between the experimental groups and the control groups was not statistically significant ($P = 0.24$). Although this result seems to be surprising, one should recall that, over this long time period, there has been a progressive adoption of more and more effective treatments firstly in the experimental groups and then in the control groups. In other words, treatments showing a small but significant incremental benefit when given to the experimental arms have then been incorporated as new standards for the control groups. So, this fact has contributed in this analysis to reduce the magnitude of the overall benefit (expressed with reference to the comparison of experimental arms *vs* control arms). On the other hand, a significant effect at meta-regression was found for publication year ($P < 0.001$; direction of the benefit: improvement with time) and KRAS type ($P = 0.014$; direction of the benefit: improvement with wild-type). The multivariate adjusted improvement in OS was 3.2 mo per decade (nearly identical to that of the unadjusted analysis).

As regards PFS, our multivariate analysis showed that KRAS type was not statistically significant ($P = 0.23$), whereas significance was achieved by publication year ($P < 0.001$; improvement with time) and arm ($P = 0.033$; improvement for experimental arm *vs* controls). The improvement in PFS per decade was identical to that of the unadjusted analyses. Further details on our meta-regression are reported in the appendix.

Our results highlight the profound difference in this area between statistical significance and clinical relevance. While an improvement in OS (and also in PFS) has clearly occurred from 2000 to 2012, the extent of this improvement is small and so the effect of innovation in this area seems to be scarcely relevant from a clinical standpoint.

In a post-hoc multivariate analysis, we tested whether the patient arms treated with bevacizumab ($n = 17$) had better outcomes than those not given this antibody ($n = 113$); this analysis included all previous covariates except “experimental *vs* control arm” (omitted to avoid confounding with the bevacizumab-based covariate). The multivariate-adjusted improvement with bevacizumab was 1.66 mo for OS ($P = 0.071$) and 1.59 mo for PFS ($P = 0.002$); the corresponding univariate (unadjusted) values were 2.21 mo ($P = 0.023$) and 1.74 mo ($P = 0.001$), respectively.

Estimating the magnitude of incremental benefits expected from treatments for mCRC has important implications both for this specific therapeutic area and from a more general perspective. For example, from a cost-effectiveness viewpoint, the prolongation of about 3 mo in OS observed in these mCRC patients does not justify the increased expenditure of more than 20000 euros that is frequently claimed for targeted agents; thresholds in this area are in fact at around 5000 euros per month gained.

This approach of evidence synthesis focused on temporal trends deserves to be tested in as many therapeutic areas as possible because a systematic application can be helpful to decision-makers, in particular to balance costs and benefits across different treatments. Finally, the main strength of our study is that a formal analytical tool was employed to determine the temporal trend of outcomes; quantifying these trends through meta-regression can generate more reliable results than those obtained through a purely narrative approach. The limitations of our study include the lack of patient-level data and the small number of studies focused on bevacizumab.

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Towards optimal treatment of ductal carcinoma *in situ*

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is treated with mastectomy (\pm immediate reconstruction). RT may be safely omitted in some patients with adequately excised low risk DCIS.

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Abstract

Ductal carcinoma *in situ* (DCIS) is a non-obligate precursor of invasive breast cancer with a variable biological behavior which is difficult to accurately predict using the current clinico-pathological parameters. Randomized controlled trials have demonstrated that adjuvant radiotherapy (RT) reduces the risk of local recurrence after adequate local excision of DCIS. Tamoxifen may be considered as an adjuvant endocrine treatment in patients with high risk estrogen receptor positive disease. There is however a growing consensus that RT can be safely omitted in a subgroup of patients with favorable biological features in order to avoid overtreatment. The sentinel node biopsy is not routinely indicated but should be considered in women undergoing mastectomy for DCIS. The discovery of molecular signatures that accurately predict the biological behavior of this common malignancy will facilitate a personalized treatment approach in the future.

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Key words: Ductal carcinoma *in situ*; Treatment; Radiotherapy; Tamoxifen

Core tip: Localized ductal carcinoma *in situ* (DCIS) is treated with adequate local excision followed by radiotherapy (RT) in most cases whereas extensive disease

INTRODUCTION

Ductal carcinoma *in situ* (DCIS) represents an intra-ductal epithelial proliferation of malignant cells and is considered to be a non-obligate precursor of invasive breast cancer. It currently accounts for approximately one fifth of newly-diagnosed breast cancers and its incidence has been rising due to the wider adoption of screening mammography and the introduction of high spatial resolution magnetic resonance imaging (MRI)^[1,2]. DCIS usually presents as mammographic micro-calcifications or non-mass enhancement (with segmental distribution) on MRI. The latter is more sensitive imaging modality than mammography in detecting intermediate and high grade DCIS and is more accurate in estimating the disease extent^[2]. Symptomatic DCIS is much less common nowadays and clinically presents as a palpable mass or nodularity, pathological nipple discharge or occasionally found as an incidental pathological finding during surgery for other reasons such as reduction mammoplasty. Furthermore symptomatic DCIS is associated with higher rates of local recurrence (LR) after treatment compared with screen-detected disease^[1].

The overall risk of DCIS progressing to invasive breast cancer has been reported to range from 14% to 75% depending upon the nuclear grade^[1]. This indicates that a significant proportion of DCIS cases are not life-threatening and do not require any treatment. The challenge however is to accurately identify such cases in order to avoid overtreatment. Unfortunately the current



Figure 1 Skin-sparing mastectomy technique (with or without nipple-areola preservation) facilitates immediate reconstruction with improved aesthetic outcomes in women opting to have immediate reconstruction.

clinico-pathological parameters used in clinical practice are unable to identify clinically less relevant disease and therefore all DCIS lesions require at least surgical excision.

SURGICAL TREATMENT

Optimal treatment of DCIS requires adequate surgical excision of the lesion with tumor-free surgical margins^[1]. The surgical treatment may consist of breast conservation surgery (BCS) or mastectomy with or without immediate breast reconstruction.

The sentinel node biopsy (SNB) is not routinely indicated for pure DCIS and should be reserved for patients undergoing mastectomy^[1]. There is a growing consensus that a 2 mm tumor-free margin represents an adequate surgical margin^[1]. The skin-sparing mastectomy (SSM) technique (with or without nipple-areola preservation) facilitates immediate reconstruction with improved aesthetic outcomes (Figure 1) in women opting to have immediate reconstruction^[3].

ADJUVANT TREATMENTS

Having addressed the issue of surgical treatment and the need for complete removal of DCIS lesions in the light of current knowledge, the next issue to address is the need for adjuvant treatments.

Patients undergoing mastectomy for DCIS have an excellent prognosis and do not usually require further treatment. Post-mastectomy radiation should be considered for extensive high grade DCIS with significant involvement of the surgical margins^[1]. If the DCIS is ER positive, then adjuvant endocrine therapy can be considered in such cases (extensive disease involving surgical margins) and in the context of chemoprevention of malignancy in the contra-lateral breast.

For women undergoing BCS, all randomized controlled trials (RTCs) have demonstrated that adjuvant radiotherapy (RT) reduces the risk of LR after adequate local excision of localized disease^[4-6].

A recent update from the NSABP B-17 and NSABP B-24 trials^[4] has demonstrated that adjuvant RT is as-

sociated with a significantly lower LR rate after a median follow up of 15 years. Approximately one half (54%) of the recurrences were invasive, and for these patients the overall survival (OS) was significantly lower [hazard ratio (HR) of death = 1.75, 95%CI: 1.45 to 2.96, $P < 0.001$].

The EORTC 10853 randomized trial also showed that RT reduced the risk of any LR by 48% (HR = 0.52; 95%, $P < 0.001$) after a median follow up of 15 years^[5].

The UK/ANZ DCIS trial investigated the effect of adjuvant treatment with tamoxifen after BCS and RT for DCIS^[6]. After a median follow-up of 12.7 years, tamoxifen use was associated with a significant reduction in LR and the incidence of contra-lateral breast cancer (HR = 0.71, 95%; $P = 0.002$).

A combined analysis of the UK/ANZ DCIS and B-24 trials revealed that the addition of tamoxifen to BCS and RT for DCIS reduced the risk of invasive LR and the incidence of *in situ* disease in the contralateral breast regardless of age with no improvement in OS^[7]. Taken together these studies suggest that tamoxifen is not routinely indicated after BCS for ER+ DCIS, but can be considered in selected cases at an increased risk of LR. The results of these trials should be communicated to patients in order to help them make informed decisions in the context of adverse effects of tamoxifen. The NSABP B-35 and IBIS II trials are currently evaluating whether aromatase inhibitors are more effective than tamoxifen as an adjuvant endocrine treatment after BCS for DCIS.

IS IT SAFE TO OMIT RT AFTER BCS?

It is clear that adjuvant treatments following BCS for DCIS significantly reduce the risk of LR which is invasive in 50% of LR cases. Although adjuvant therapy has not been shown to improve OS, it is reasonable to assume that reduction in invasive LR will translate into an OS benefit over time. The uncertainty remains however whether adjuvant treatments especially RT could be safely omitted in certain subgroups of patients at a low risk of LR.

Numerous prospective and retrospective studies have reported the lesion size, nuclear grade, patient's age, the presence/absence of necrosis, margin's width and the

comedo morphology to be significantly associated with the risk of^[8]. The use of these factors in combination can guide adjuvant treatment recommendations in order to minimize overtreatment in the context of multidisciplinary management. For example a woman who had complete excision of a low grade DCIS measuring less than 2 cm with a tumor-free margin of at least 1 cm does not require any further treatment, whereas a premenopausal woman who had BCS for a 3 cm high grade ER+ DCIS will benefit from RT and tamoxifen. The Van Nuys prognostic index includes tumor size, nuclear grade and margin width. Patients with low Van Nuys scores can avoid adjuvant treatment after BCS^[9].

There is a consensus however that the above predictive factors are crude and that there is a need to develop more accurate predictors of DCIS behavior based on the molecular profile of the tumor. The Oncotype-DX-DCIS genomic score has been recently introduced to guide RT recommendations after BCS^[10]. This multi-gene expression assay calculates a score based on 7 cancer-related genes and 5 reference genes. It was validated using formalin-fixed paraffin embedded tumor tissues and clinical outcome data from the ECOG 5194 trial which included patients treated with BCS with or without adjuvant RT^[11]. A low score (< 39) indicates a very low risk of LR and suggests that adjuvant RT can be safely omitted.

The expression of $\alpha v\beta 6$ on myoepithelial cells of DCIS has been recently reported to predict disease progression to invasive malignancy and LR. This protein up-regulates MMP-9 through TGF β causing tumor progression^[12].

Further research focused on molecular and biological profiling is likely to facilitate a personalized treatment approach to patients with newly-diagnosed DCIS in order to optimize the clinical outcome while minimizing harm from overtreatment. However such research is likely to be complicated by intra-tumor molecular heterogeneity and the role of the microenvironment in tumor development and progression^[13].

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WJCO 5th Anniversary Special Issues (1): Lung cancer**Paraneoplastic syndromes associated with lung cancer**

Nobuhiro Kanaji, Naoki Watanabe, Nobuyuki Kita, Shuji Bandoh, Akira Tadokoro, Tomoya Ishii, Hiroaki Dobashi, Takuya Matsunaga

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Abstract

Paraneoplastic syndromes are signs or symptoms that occur as a result of organ or tissue damage at locations remote from the site of the primary tumor or metastases. Paraneoplastic syndromes associated with lung cancer can impair various organ functions and include neurologic, endocrine, dermatologic, rheumatologic, hematologic, and ophthalmological syndromes, as well as glomerulopathy and coagulopathy (Trousseau's syndrome). The histological type of lung cancer is generally dependent on the associated syndrome, the two most common of which are humoral hypercalcemia of malignancy in squamous cell carcinoma and the syndrome of inappropriate antidiuretic hormone secretion in small cell lung cancer. The symptoms often precede the diagnosis of the associated lung cancer, especially when the symptoms are neurologic or dermatologic. The proposed mechanisms of paraneoplastic processes include the aberrant release of humoral mediators, such as hormones and hormone-like peptides, cyto-

kines, and antibodies. Treating the underlying cancer is generally the most effective therapy for paraneoplastic syndromes, and treatment soon after symptom onset appears to offer the best potential for symptom improvement. In this article, we review the diagnosis, potential mechanisms, and treatments of a wide variety of paraneoplastic syndromes associated with lung cancer.

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Key words: Paraneoplastic syndrome; Small cell lung cancer; Non-small cell lung cancer; Symptom; Diagnosis; Treatment; Endocrine; Neurologic; Hematologic; Trousseau's syndrome

Core tip: A wide variety of paraneoplastic syndromes are associated with lung cancer, including endocrine, neurologic, dermatologic, rheumatologic, hematologic, and ophthalmological syndromes, as well as glomerulopathy and coagulopathy (Trousseau's syndrome). The histological type of lung cancer is generally dependent on the associated syndrome, the two most common of which are humoral hypercalcemia of malignancy in squamous cell carcinoma and the syndrome of inappropriate antidiuretic hormone secretion in small cell lung cancer. The symptoms of paraneoplastic syndromes often precede the diagnosis of lung cancer. The early detection and treatment of the underlying lung cancer offers the best outcomes for paraneoplastic syndromes.

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INTRODUCTION

Paraneoplastic syndromes are a group of clinical disorders that are associated with malignant diseases and are

Table 1 Criteria for the diagnosis of paraneoplastic endocrine syndromes

Abnormal endocrine function without physiologic feedback regulation
The absence of metastasis in the respective endocrine gland
Deterioration with increasing tumor burden
Improvement in endocrine function with the treatment of the tumor
Evidence of the presence of hormones in the tumor or hormone synthesis by the tumor

not directly related to the physical effects of the primary or metastatic tumors^[1]. In the current understanding, these conditions arise from secretion of functional peptides or hormones from the tumor, or inappropriate immune cross-reaction between normal host cells and initially targeted tumor cells^[2]. Although paraneoplastic syndromes can be associated with many types of malignancies, they are most frequently associated with lung cancer^[3]. The histology of lung cancer influences the type of associated paraneoplastic syndrome. Paraneoplastic syndromes occur in approximately 10% of patients with lung cancer^[1], and two of the most common are humoral hypercalcemia of malignancy (HHM) in squamous cell carcinoma and the syndrome of inappropriate antidiuretic hormone secretion (SIADH) in small cell lung cancer. There is no relation between the severity of symptoms and the size of the primary tumor, and in some cases, paraneoplastic syndromes are manifested before the diagnosis of cancer^[1]. The early recognition of paraneoplastic syndromes may contribute to the detection of a highly treatable, early-stage tumor^[2]. At other times, the syndromes may occur late in the course of disease or may appear as the first sign of recurrence^[1]. Some syndromes, such as hypercalcemia and hematologic syndromes, are associated with a poor prognosis; others, such as onconeural antibody-related neurologic syndromes, may predict longer survival. This review provides an overview of a wide variety of paraneoplastic syndromes associated with lung cancer, with an emphasis on the diagnosis and treatment of the most frequently encountered syndromes. We searched the literature on PubMed (<http://www.ncbi.nlm.nih.gov/pubmed>) using the terms “lung cancer” and “paraneoplastic syndrome”. As a result, we found more than 1000 studies published in the 21st century, and we cited these studies with priority. However, we also cited some older studies when only a few studies associated with the syndromes had been published in the 21st century.

PARANEOPLASTIC ENDOCRINE SYNDROMES

Lung cancers have a potential to synthesize and secrete peptides or hormones that lead to a variety of endocrine syndromes^[4]. There are several criteria for diagnosing a paraneoplastic endocrine syndrome (Table 1)^[4]. However, all of these criteria may not be satisfied in a given patient^[4]. In particular, the presence of hormones in the tu-

Table 2 Paraneoplastic endocrine syndromes and causes of hypercalcemia associated with lung cancer

Paraneoplastic endocrine syndromes associated with lung cancer
Humoral hypercalcemia of malignancy
Syndrome of inappropriate antidiuretic hormone production
Cushing's syndrome
Hypoglycemia
Acromegaly
Carcinoid syndrome
Gynecomastia
Hyperthyroidism
Causes of hypercalcemia associated with lung cancer
Humoral hypercalcemia of malignancy
(1) Parathyroid hormone-related protein
(2) Parathyroid hormone
(3) 1,25-dihydroxyvitamin D
(4) Granulocyte colony-stimulating factor
Osteolytic activity at the sites of skeletal metastases

mor tissue is not always necessary for a clinical diagnosis. Several paraneoplastic endocrine syndromes have been reported (Table 2), and the three most common of which are HHM, SIADH, and ectopic Cushing's syndrome (ECS)^[4].

HHM

The incidence of hypercalcemia in patients with lung cancer ranges from 2%-6% at the initial diagnosis to 8%-12% throughout the course of the disease^[1]. The most common type of cancer related to hypercalcemia is squamous cell carcinoma, in which higher incidences up to 23% has been reported^[2,4]. The two major mechanisms of hypercalcemia in cancer patients are: (1) HHM; and (2) osteolytic activity at sites of skeletal metastasis (Table 2). HHM is considered a paraneoplastic syndrome and accounts for 46%-76% of hypercalcemia in lung cancers^[5]. Although four mechanisms of HHM have been described, the majority of HHM cases are caused by the secretion of parathyroid hormone (PTH)-related protein (PTHrP) from the tumor. Rare cases of HHM with ectopic PTH secretion by lung cancers have also been reported^[6-8]. Although ectopic 1,25-dihydroxyvitamin D secretion is frequently observed in patients with malignant lymphoma^[5], no cases of 1,25-dihydroxyvitamin D-producing lung cancer have been reported, to our knowledge. Another mechanism of HHM may be mediated by granulocyte colony-stimulating factor (G-CSF)^[9,10]. Long-term exposure to G-CSF results in the stimulation of osteoclastic bone resorption or an increase in osteoclast progenitors^[9,10].

The PTHrP has significant homology with PTH in the amino-terminal region^[11], and both PTH and PTHrP bind to a common PTH/PTHrP receptor^[12]. PTHrP and PTH exert equivalent actions in regulating bone resorption and renal calcium/phosphorus levels^[5]. However, unlike PTH, PTHrP does not increase 1-hydroxylase activity and 1,25-dihydroxyvitamin D production^[5]. In two lung squamous cell carcinoma xenograft models of hypercalcemia, the inhibition of autocrine epidermal

Table 3 Syndrome of inappropriate antidiuretic hormone secretion

Hyponatremia (serum sodium < 134 mEq/L)
Hypoosmolality (plasma osmolality < 275 mOsm/kg)
Inappropriately high urine osmolality (> 500 mOsm/kg)
Inappropriately high urinary sodium concentration (> 20 mEq/L)
Absence of hypothyroidism
Absence of adrenal insufficiency
Absence of volume depletion

growth factor receptor (EGFR) signaling has been shown to reduce plasma PTHrP and total calcium concentrations^[13]. Amphiregulin stimulation of EGFR resulted in high levels of PTHrP gene expression in squamous cell carcinomas^[14]. Furthermore, the reconstitution of the amphiregulin-EGFR signaling system in a squamous cell carcinoma line led to HHM and rapid osteolytic growth in animal models^[14].

HHM is usually found in patients with an increased tumor burden^[4], and therefore, the incidence increases late in the course of illness. A very poor prognosis has been reported in patients with hypercalcemia, with a median survival of 1-3 mo^[9,15]. The clinical features of hypercalcemia include circulatory effects (thirst, polyuria, dehydration, and renal failure), gastrointestinal effects (anorexia, nausea, vomiting, abdominal pain, and constipation), neurologic effects (fatigue, muscular weakness, confusion, lethargy, irritability, and coma), and psychiatric manifestations (depression, anxiety, and cognitive dysfunction)^[1,2,4,5]. The severity of the symptoms are influenced by the patient's baseline renal functions, neurologic conditions, and the rapidity of progression of hypercalcemia as well as the degree of hypercalcemia^[2,5,16]. Pancreatitis due to hypercalcemia is a less common but serious complication^[17].

In the absence of ionized calcium measurements, total calcium, which represents both bound and unbound calcium, should be corrected for the albumin concentration using the following formula: corrected Ca (mg/dL) = measured Ca (mg/dL) + [0.8 × (4.0 - albumin (mg/dL))]^[2]. It is important to measure the calcium and albumin concentrations at the same time because the albumin concentration is often lower than 4.0 mg/dL in patients with cancer. In addition, including the constant 0.8 in the calculation is important to avoid the overestimation of hypercalcemia when the serum albumin level is very low.

Although the optimal approach to paraneoplastic hypercalcemia is to treat the underlying tumor^[2], fluid replenishment with normal saline should be the initial treatment. This replenishment corrects the dehydration, increases the glomerular filtration rate, and also decreases renal calcium reabsorption^[2]. Loop diuretics should not be routinely used for all patients with hypercalcemia although these agents inhibit renal calcium reabsorption. However, these agents may be added after adequate fluid replenishment in order not to deteriorate the dehydration and hypercalcemia^[2,16]. Bisphosphonates such as zoledro-

nate and pamidronate have been widely used because of their inhibitory effect of bone resorption mediated by osteoclasts and less toxicity^[2]. The calcium levels in serum generally decrease within 2-4 d and reach a nadir 4-7 d after intravenous bisphosphonate administration, and the favorable efficacy usually continues for up to 3 wk^[2,16]. The time to achieve normocalcemia is positively correlated with the pretreatment level of PTHrP^[18]. The main adverse effects of bisphosphonates are renal dysfunction and osteonecrosis of the jaw^[2]. Osteonecrosis of the jaw is caused by reduced local blood flow and leads to pain, swelling, loosened teeth, and exposed bone^[2]. Calcitonin is a useful adjunctive initial therapy that inhibits bone resorption and increases the renal excretion of calcium^[4,5]. The efficacy of calcitonin appears rapidly, but the effect is often partial and temporary^[4,19]. Denosumab, a receptor activator of nuclear factor-kappa B ligand-targeted monoclonal antibody, was superior to zoledronic acid in delaying or preventing skeletal-related events in patients with bone metastases and was generally well tolerated^[20]. The efficacy of denosumab for treating HHM is currently being evaluated in a phase II clinical trial^[5].

SIADH

SIADH manifests as euvolemic hypoosmolar hyponatremia characterized by low serum osmolality and inappropriately high urine osmolality in the absence of diuretic treatment, adrenal insufficiency, heart failure, cirrhosis, or hypothyroidism (Table 3)^[21]. Clinical SIADH has been reported to occur in 7%-16% of SCLC cases^[22-24]. Approximately 70% of paraneoplastic SIADH cases are associated with SCLC^[25]. Non-small cell lung cancer (NSCLC) has also been reported as a rare cause of SIADH^[26-28]. The stage of SCLC is not related to the incidence of SIADH^[22,23]. Patients with SCLC with hyponatremia had shorter survival times than patients with normal serum sodium levels^[29,30]. In a study of 61 patients receiving two or more cycles of chemotherapy, the patients who did not fully regain normal serum sodium levels had poorer survival compared with the patients who did^[30].

Non-ADH-mediated causes of hyponatremia, including insufficient intake of sodium, sodium wasting because of drug nephrotoxicity, and infusion of hypotonic fluid, should be distinguished from SIADH in the differential diagnosis of hyponatremia^[21]. Paraneoplastic hyponatremia secondary to elevated atrial natriuretic peptide (ANP) has also been reported^[31]. Most SCLC cell lines have been shown to produce ANP^[32,33]. Of the 23 SCLC lines examined, 16 (70%) had elevated ANP levels^[33], whereas only two (8.7%) had elevated ADH levels, and these two also had elevated ANP levels^[33]. Of 11 cell lines derived from SCLC patients with hyponatremia, 9 produced ANP mRNA, 7 produced ADH mRNA, and 5 produced both ANP and ADH mRNAs^[29]. Other studies have found that the plasma levels of ANP were also elevated in SIADH^[34-37]. In addition, when hyponatremia was corrected *via* water restriction or demeclocycline administration, the plasma ANP levels decreased significantly into

the normal range^[35].

The symptoms of SIADH are affected by the development speed and the degree of hyponatremia^[2]. Headache, general fatigue, muscle weakness, and memory loss are common symptoms. Serum sodium levels less than 125 mEq/L, particularly if they develop within 48 h of hyponatremia onset, can lead to the alterations of mental and emotional status, loss of consciousness, seizures, and in some cases, even death^[2,38]. On the other hand, when hyponatremia develops slowly, neurologic complications are less likely to occur^[2,39].

The most effective long-term therapy for SIADH associated with SCLC is the treatment of the tumor itself^[4,22,23]. Chemotherapy for SCLC results in the improvement of more than 80% of cases of clinically manifested SIADH^[4,22,23]. However, with SCLC recurrence, 60%-70% of patients will experience a recurrence of SIADH as well^[4]. Rarely, chemotherapy-induced tumor lysis may be associated with the sudden onset of SIADH^[40]. In addition to the therapy directed to SCLC, other treatments are also required to normalize serum sodium levels. There are no evidence-based guidelines for managing SIADH^[21]; the recommended management is based on expert opinion^[21,39,41]. Free water restriction (< 1 L/d) is the first-line treatment for mild, asymptomatic SIADH. Adequate sodium intake, if necessary by the salt tablets, also contribute to correcting hyponatremia. In life-threatening or acute cases of severe (< 120 mEq/L), symptomatic hyponatremia, a hypertonic 3% saline infusion is administered at a rate of approximately 1 mL/kg per hour for the first several hours. In SIADH, the urine osmolality is often higher than that of normal saline (308 mOsm/kg), and in these cases, administration of normal saline will lead to the increase in volume of free water, which results in further deterioration of hyponatremia^[2]. Demeclocycline, an antibiotic in the tetracycline group, has been demonstrated to be effective in treating SIADH^[4,42]. Demeclocycline decreases the renal response to ADH, resulting in a dose-dependent and reversible decrease in the urine-concentrating ability of the kidney. Vasopressin (ADH) receptor antagonists, such as conivaptan, an intravenously administered agent, and tolvaptan, an oral agent, are also available for the treatment of SIADH^[2,43,44]. In the renal collecting ducts, these antagonists can block ADH to bind to the receptors, resulting in the urinary free water excretion rate^[39]. Although high sensitivity to tolvaptan in SIADH has been reported^[45], the United States Food and Drug Administration (FDA) announced restrictions on the use of tolvaptan in 2013 because of the risk of serious and potentially fatal liver injury. Other adverse effects of vasopressin receptor antagonists include nausea, vomiting, diarrhea, and infusion site reaction^[2]. These agents are usually administered only in the cases of the fluid restriction failure^[2]. When possible, medications that exacerbate SIADH, such as opioids, certain antidepressants, vinca alkaloids, and cisplatin, should be discontinued^[46].

ECS

The manifestations of ECS are due to hypercortisolism, which generally resulted from the uncontrolled secretion of adrenocorticotrophic hormone (ACTH) from nonpituitary tissue^[4,21]. ECS represents approximately 12% of all patients with CS^[47]. ECS caused by the production of corticotropin-releasing hormone (CRH) is rare, and only a few patients with ECS and SCLC have been reported^[48,49]. Approximately 50% of ECS cases are neuroendocrine lung tumors; carcinoid tumors and SCLC constitute 36%-46% and 8%-20% of ECS cases, respectively^[50-52]. ECS is clinically apparent in 1.6%-4.5% of SCLC cases^[53,54], although immunoreactive ACTH was found in almost all tissue extracts of lung cancer from patients without clinical evidence of CS^[55]. Rarely, ECS cases from NSCLC have also been reported^[56].

The clinical features of ECS include moon face, acne, purple striae, proximal muscle weakness, peripheral edema, hypertension, and metabolic alkalosis with hypokalemia^[21]. Almost all ECS patients show hypokalemia, and in the majority, hyperglycemia is observed^[53,54,57]. However, ECS secondary to SCLC rarely exhibits all of the classic signs of CS^[58]. One reason for this finding could be the brief duration of exposure to excessive ACTH due to the aggressive nature of SCLC^[4]. The poor prognosis of patients with ECS has been reported compared with that of patients without ECS^[57,59].

If the clinical features of CS are present in a patient with lung cancer, iatrogenic causes of CS, such as exogenous glucocorticoid use, must be excluded^[21]. The clinical practice guidelines of the Endocrine Society recommend the initial use of one of the following first-line tests with high diagnostic accuracy, based on the suitability for a given patient: (1) at least two measurements of 24-h urinary free cortisol (greater than the normal range); (2) two measurements of late-night salivary cortisol (at bedtime or between 23:00 and 00:00, greater than 145 ng/dL); and (3) the 1-mg overnight dexamethasone suppression test (the administration of dexamethasone at 23:00 or 00:00 combined with blood cortisol measurements at 08:00 or 09:00 revealing a concentration greater than 1.8 µg/dL) or, in certain populations, the 2-mg 48-h dexamethasone suppression test^[60,61]. If one of these tests produces abnormal results, further evaluation by an endocrinologist is recommended and should include one of the above tests or, in some cases, a serum midnight cortisol or dexamethasone-CRH test^[61]. For the high-dose (8 mg) dexamethasone test, urinary or serum cortisol suppression greater than 50% is considered indicative of Cushing's disease with a sensitivity of 84%-89%^[62-65]. Based on the high-dose dexamethasone test, 6%-31% of ECS patients also exhibit the suppression of serum or urinary cortisol or 17-OHCS^[65-67]. Computed tomography (CT) scanning is useful for identifying ectopic sources of cortisol. Although ¹¹¹In-octreotide scintigraphy (Octreoscan) generally does not reveal a source that is undetectable on CT, it can provide supportive functional data^[68,69].

The ideal treatment for ECS is the radical excision of the tumor^[68]. In addition to the treatment of the underlying lung cancer, the direct inhibition of cortisol secretion is the considerable treatment for ECS^[2]. Ketoconazole, metyrapone, etomidate, mitotane, and mifepristone can be used to reduce circulating glucocorticoids^[21]. Among these agents, ketoconazole might have the best tolerance in spite of having some toxicity such as nausea and liver injury^[2,70]. An additional option is octreotide, which blocks the release of ACTH, although it is not universally effective^[4,71,72]. When these medications have unsuccessful result, bilateral adrenalectomy might be considered^[4].

The prognosis of patients with ECS is influenced by tumor histology and by the severity of hypercorticolesmia because both factors affect mortality and morbidity^[68]. Most patients with ECS due to SCLC present at an advanced stage and have a poor response to chemotherapy^[1,4].

Hypoglycemia

Tumor-associated hypoglycemia is rare and insulin-producing islet cell tumor is the best known cause. Nonpancreatic tumors may also cause recurrent hypoglycemia, known as non-islet cell tumor hypoglycemia (NICTH). NICTH is usually caused by the production of insulin-like growth factor (IGF)-2 from the tumor cells^[2]. NICTH is suspected when low levels of serum insulin (usually < 1.44 IU/mL) and C-peptide (usually < 0.3 ng/mL) in addition to low levels of blood glucose with acute episodes. Moreover, the characterization of NICTH includes low levels of growth hormone (GH) and IGF-1 and normal or elevated levels of IGF-2^[2]. The IGF-2 produced by nonpancreatic tumors is usually incompletely processed or unprocessed, referred to as “big” IGF-2 because its molecular weight is 10-17 kDa, in contrast to mature IGF-2, which has a molecular weight of 7.5 kDa^[73]. Big IGF-2 impairs the formation of the heterotrimeric, 150 kDa IGF-binding protein (IGFBP) complex, which consists of IGF-1, IGF-2, IGFBP-3, and an acid-labile subunit^[73,74]. In normal individuals, most circulating IGF is bound to this complex and is prevented from displaying its insulin-like potential^[73]. When the formation of the 150-kDa complex is impaired, IGF is sequestered in a binary complex with IGFBP-3^[73,75]. In the latter complex, the bioavailability of IGF is increased and its insulin-like potential is unmasked, leading to hypoglycemia^[73,75]. Rarer cases of NICTH of the lung due to the production of insulin or IGF-1 have also been reported^[73,76].

The treatment of the underlying tumor is the optimal approach to NICTH^[2]. However, the first-line treatment should be the maintenance of adequate blood glucose levels. In the acute situation, parenteral dextrose administration is usually performed although oral intake can also be considered if possible. Intravenous administration of one 50 mL ampule of 50% dextrose fluid results in a rapid elevation in blood glucose levels^[2]. Chronic and/or recurrent hypoglycemic episodes have also been observed

in NICTH, and continuous infusion or oral intake of dextrose may be necessary. Other longer-term management includes glucagon, GH, or corticosteroids^[73,74,77,78].

Acromegaly

In the vast majority of cases, acromegaly is caused by a pituitary adenoma. Ectopic acromegaly is rare (< 1% of cases) and is usually caused by ectopic growth hormone-releasing hormone (GHRH) or, less frequently, by ectopic GH secretion from the tumors^[79,80]. The most common tumors that secrete ectopic GHRH or GH are bronchial carcinoids and pancreatic islet cell tumors^[79,81]. Other types of lung cancer associated with acromegaly have also been reported, including SCLC, bronchioloalveolar carcinoma, and epidermoid carcinoma^[82-84]. In some cases, IGF-I might play a role in the development of acromegaly^[83,85].

The routine evaluation of circulating GHRH in acromegalic patients may enable the early recognition of overproduction of GHRH because plasma levels greater than 0.3 ng/mL are virtually diagnostic of a GHRH-producing tumor^[79]. In contrast, the dynamics of ectopic GH secretion in ectopic acromegaly are characterized by high basal GH levels, which are not suppressed by glucose load, and by low or undetectable plasma GHRH levels^[80].

Complete surgical resection of the tumor is the best treatment for ectopic acromegaly, and it usually leads to the normalization of GH levels and the regression of acromegalic features^[80,86,87]. Residual, recurrent, and inoperable lesions have been successfully treated with octreotide and other somatostatin analogs^[88-91].

PARANEOPLASTIC NEUROLOGICAL SYNDROMES

Paraneoplastic neurological syndromes (PNSs) are neurological disorders caused by the remote effects of cancer and are not caused by the tumor itself, its metastasis, infection, ischemia, or metabolic disruption^[92]. In response to the development of a tumor, some antibodies against the tumor can be generated, that are known as onconeural antibodies^[2]. These onconeural antibodies and the associated onconeural antigen-specific T lymphocytes inadvertently attack both the components of the nervous system and tumor cells^[2]. Fewer than 50% of patients with PNS harbor known antibodies although many types of antibodies have been reported^[92]. Therefore, the absence of known antibodies does not rule out a diagnosis of PNS^[92]. In 80% of cases, the neurological disorder develops before the cancer becomes clinically overt, and the patient is referred to a neurologist for the identification of a neurological disorder as paraneoplastic^[92].

In 2004, an international panel of neurologists advocated two levels of evidence for the diagnosis of a PNS: “definite” and “possible”^[93]. They defined “classical” syndromes and “well-characterized” onconeural antibodies,

Table 4 Classical and non-classical paraneoplastic neurological syndromes^[93]

Syndromes of the central nervous system
Encephalomyelitis ¹
Limbic encephalitis ¹
Brainstem encephalitis
Subacute cerebellar degeneration ¹
Opsoclonus-myoclonus ¹
Optic neuritis
Cancer-associated retinopathy
Melanoma-associated retinopathy
Stiff person syndrome
Necrotizing myelopathy
Motor neuron diseases
Syndromes of the peripheral nervous system
Subacute sensory neuropathy ¹
Acute sensorimotor neuropathy
Guillain-Barre syndrome
Brachial neuritis
Subacute/chronic sensorimotor neuropathies
Neuropathy and paraproteinaemia
Neuropathy with vasculitis
Autonomic neuropathies
Chronic gastrointestinal pseudo-obstruction ¹
Acute pandysautonomia
Syndromes of the neuromuscular junction and muscle
Myasthenia gravis
Lambert-Eaton myasthenic syndrome ¹
Acquired neuromyotonia
Dermatomyositis ¹
Acute necrotizing myopathy

¹Classical syndromes.

and determined each level by combining the criteria with the evidence of cancer status (presence or absence)^[93]. PNS can affect the central nervous system (CNS), the peripheral nervous system, and the neuromuscular junction and muscles. The classical and non-classical syndromes identified by the panel are shown in Table 4. It should be noted that these syndromes are also seen in non-paraneoplastic^[2]. Particularly, more than 70% of patients with subacute sensory neuropathy and limbic encephalitis (LE) are not associated with cancer^[92]. Well-characterized and partially characterized onconeural antibodies determined by the panel are shown (Table 5). A study of 200 patients with SCLC reported prevalence rates of onconeural antibodies for Hu, CRMP5, amphiphysin, Yo, Ri, and Ma2 of 22.5%, 5.0%, 2.5%, 0.5%, 1.5%, and 1%, respectively^[94]. These antibodies may be detected in individuals without neurologic symptoms^[2,93]. In particular, a high frequency of anti-Hu antibody has been reported, including 16% of 196 SCLC patients without PNS in one study^[93]. In this regard, anti-Hu antibody titers may be important because none of the 109 SCLC patients without PNS had high titers of anti-Hu, whereas 44% of 57 patients with PNS had high titers (> 1:10000). Antibodies that occur in both cancer- and non-cancer-associated syndromes were not defined as onconeural antibodies, which include anti-acetylcholine receptor (AChR), anti-nicotinic AChR, anti-voltage-gated calcium channel (VGCC), anti-voltage-gated potassium channel (VGKC), anti-NR1/NR2 subunits

Table 5 Onconeural antibodies

Well-characterized onconeural antibodies
Anti-Hu (ANNA1)
Anti-Yo (PCA1)
Anti-CV2 (CRMP5)
Anti-Ri (ANNA2)
Anti-Ma2 (Ta)
Anti-amphiphysin
Partially characterized onconeural antibodies
Anti-Tr (PCA-Tr)
ANNA3
PCA2
Anti-Zic4
Anti-mGluR1
Other antibodies
Anti-acetylcholine receptor
Anti-nicotinic AChR
Anti-voltage-gated calcium channel
Anti-voltage-gated potassium channel
Anti-NR1/NR2 of N-methyl-D-aspartate
Anti-glutamic acid decarboxylase

ANNA: Anti-neuronal nuclear antibodies.

of the N-methyl-D-aspartate (NMDA) receptor, and anti-glutamic acid decarboxylase (GAD) antibodies^[2,93,95]. The diagnostic criteria for PNS described by the panel are presented in Table 6. When cancer and a classical syndrome develop, the diagnosis of a PNS can be made without the presence of onconeural antibodies. The time period of five years determined in the criteria was based on previous work demonstrating that cancers almost always developed in five years after the occurrence of neurological symptoms^[93]. Importantly, even in the absence of a detected cancer, a neurological syndrome accompanied by the presence of well-characterized onconeural antibodies leads to the diagnosis of definite PNS. However, false-positive cases can exist in this set of criteria, in which cancer will never develop, although a very small number of cases are applicable^[93]. The most plausible explanation is the elimination of cancer mediated by the host immune systems^[96]. The panel emphasized that in cases of both definite and possible PNS, all other causes that might lead to the neurological symptoms should be excluded on the diagnosis of a PNS, even if onconeural antibodies are detected^[93]. In fact, neurological alterations arise from many conditions other than PNS in the patients with cancer: brain metastasis, leptomeningeal disorders, nerve root or spinal cord invasion or compression, uncontrolled electrolytes and blood glucose, and adverse effects due to treatments such as irradiation and cytotoxic agents, including vinca alkaloids, taxanes, and platinum^[2]. Opioids such as morphine, phentanyl, and oxycodone, which are often administered during cancer treatment, can also cause neurologic and psychiatric changes.

Although PNSs are rare and occur in fewer than 1% of cancer patients overall, up to 3%-5% of patients with SCLC develop PNSs^[95,97]. The irreversible destruction of neurons and nervous systems by the inflammation con-

tributes to the severity in most PNSs^[92,98,99]. PNSs are usually progressive, and they debilitate patients deeply within weeks to months in many cases^[92]. In general, a paraneoplastic case is highly suspected when symptoms are progressive in a subacute course and disability remains severe^[92]. SCLC accounts for more than 90% of cases of PNS that are positive for anti-Hu antibody [also known as type 1 anti-neuronal nuclear antibodies (ANNA1)]^[1]. The Hu antigen is normally found in neurons. However, healthy adults do not have anti-Hu antibodies because the developing CNS is sequestered from the immune system by the blood-brain barrier^[1]. Most SCLCs express the Hu antigen, and approximately 20% of patients with SCLC have detectable levels of circulating anti-Hu antibodies, although PNS will not develop in all of these patients^[1,96]. Because many types of PNS are associated with anti-Hu antibody, the term “anti-Hu syndrome” has been used as an independent entity^[21]. The clinical manifestations of anti-Hu syndrome may include encephalomyelitis, LE, brainstem encephalitis, cerebellar degeneration, opsoclonus-myoclonus, sensory neuropathy, and chronic gastrointestinal pseudo-obstruction (CGP)^[21,93]. In several patients with anti-Hu antibody-positive SCLC, the spontaneous regression of SCLC without treatment has been reported, suggesting a host immune response directed against both cancer and the nervous system^[100-102]. T and natural killer (NK) cell-mediated autoimmunity may play a role in the pathogenesis of the neurologic damage caused by SCLC^[21,103-105].

Successful treatment of SCLC favorably affects the course of PNS^[106]. Limited experience suggests that immunosuppressive therapy with a combination of IV immunoglobulin (IVIg), methylprednisolone, and cyclophosphamide may transiently stabilize the PNS; however, these treatments cannot improve PNS over the long term^[98].

Encephalomyelitis

In response to the obstacle of various levels of the CNS, patients with encephalomyelitis exhibit relevant clinical dysfunction^[93]. The terms “encephalomyeloneuritis” and “encephalo neuropathy” have also been used to describe this entity^[93]. The main clinical syndromes observed in encephalomyelitis vary and include the following: subacute cerebellar degeneration, myelitis, LE, brainstem encephalitis, and even several peripheral nervous system dysfunction such as subacute sensory neuropathy^[93]. However, if the clinical signs and symptoms are elucidative by a single level dysfunction of the CNS, the term “encephalomyelitis” should not be used, and the above described syndromes should be chosen according to the prominent symptoms^[93].

LE

LE is clinically suspected with the acute or subacute progression, over the period of several days to three months, and symptoms including personality changes, irritability, depression, seizures, memory loss, confusion, and some-

times dementia^[93,107]. Twenty percent of LE cases are associated with neoplasms^[92]. In a study of 50 paraneoplastic LE patients, 50% had lung cancer, including 40% of the SCLC patients and 10% of the NSCLC patients^[107]. Anti-Hu antibody is present in 36-50% of patients with paraneoplastic LE^[107,108]. Anti-Ma2 antibody (Ta) was detected in 20% of patients with paraneoplastic LE, typically those with testicular cancer^[107]. Anti-CRMP5 (CV2) and anti-amphiphysin antibodies have been reported less frequently^[95]. The presence of anti-VGKC antibodies has been reported in idiopathic and paraneoplastic LE^[109,110]. The electroencephalographic findings include focal or generalized slow activity, and epileptic activity in the temporal lobes^[111]. The magnetic resonance imaging (MRI) findings reveal high signal intensities in unilateral or bilateral temporal lobes in 70%-80% of the patients using with T2 emphasized or fluid-attenuated inversion recovery (FLAIR) image^[107,111]. CSF analysis has been reported to show evidence of inflammation, including pleocytosis, elevated protein, elevated IgG, and oligoclonal bands, in 80% of LE patients and may support the clinical diagnosis^[93,107]. CSF analysis is also important to exclude carcinomatous meningitis.

Unlike most paraneoplastic syndromes of the CNS, paraneoplastic LE is well known for its favorable response to therapy^[112]. Therapy for underlying SCLC is the best treatment for paraneoplastic LE, although immunosuppressive therapy is sometimes encouraged for paraneoplastic LE. The treatment of the tumor was found to improve the neurological syndrome in 73% of patients^[107]. Chemotherapy improved neurologic symptoms accompanied by regression of the tumor^[113] or the resolution of the temporal lobe signal abnormalities^[114].

Subacute cerebellar degeneration

This syndrome has often been described using other terms, such as paraneoplastic cerebellar degeneration (PCD) or cerebellar ataxia^[2,92]. To define subacute cerebellar degeneration (SCD), the following criteria are required: no evidence of significant cerebellar atrophy more than the expected on MRI, subacute development of the symptoms within three months, and a severity of at least 3 (moderate disability; requires some help) on the Rankin scale^[93]. Approximately 50% of SCD cases have a paraneoplastic origin^[92].

Purkinje cells represent one of the most common targets of the immune response in patients with cancer, and the death of Purkinje cells results in SCD^[95]. In an analysis of 50 patients with antibody-associated SCD, the following antibodies were detected: anti-Yo (19 patients), anti-Hu (16 patients), anti-Tr (7 patients), anti-Ri (6 patients), and anti-mGluR1 (2 patients)^[115]. In contrast to limbic encephalitis, the frequency of the anti-Hu antibody in cerebellar degeneration is low (18%-31%)^[115-117]. The underlying tumor is associated with the types of antibodies present: gynecological and breast cancers are associated with anti-Yo and anti-Ri, lung cancer with anti-Hu, and Hodgkin's lymphoma with anti-Tr and anti-

Table 6 Criteria for the diagnosis of paraneoplastic neurological syndromes^[93]

Definite PNS
A classical syndrome and cancer that develops within five years of the diagnosis of the neurological disorder
A non-classical syndrome that resolves or improves significantly after cancer treatment without concomitant immunotherapy, provided that the syndrome is not susceptible to spontaneous remission
A non-classical syndrome with onconeural antibodies (well characterized or not) and cancer that develops within five years of the diagnosis of the neurological disorder
A neurological syndrome (classical or not) with well-characterized onconeural antibodies and no cancer
Possible PNS
A classical syndrome, no onconeural antibodies, and no cancer, but a high risk of an underlying tumor
A neurological syndrome (classical or not) with partially characterized onconeural antibodies and no cancer
A non-classical syndrome, no onconeural antibodies, and cancer present within five years of the diagnosis

PNS: Paraneoplastic neurological syndromes.

mGluR1^[115]. In fact, 14 (88%) of 16 anti-Hu-positive SCD patients had lung cancer^[115]. Lung cancers associated with anti-Ri^[118] or anti-Tr^[119] have also been reported, with the most common being SCLC, although NSCLC has also been reported^[119-121]. It has been reported that P/Q-type VGCC antibodies, which are thought to be associated with the development of Lambert-Eaton myasthenic syndrome (LEMS), were present in 20%-44% of SCD cases^[116,122]. A recent study found that the intrathecal injection of P/Q-type VGCC antibodies from PCD led to ataxia in mice, suggesting a pathogenic role in the development of PCD^[123]. It is also interesting that LEMS was reported to be present in 16%-40% of SCD cases associated with lung cancer^[116,122]. Serum from a patient with SCD without typical onconeural antibodies showed reactivity to protein kinase C of the Purkinje cells^[121], which may suggest another immune response-mediated mechanism. In addition, ZIC antibodies were identified in 15% of patients with SCD and SCLC^[124], although the role of ZIC antibodies in the development of SCD is not fully understood.

The clinical presentation of SCD includes ataxia, diplopia, nystagmus, dysphagia, dysarthria, vertigo, dizziness, nausea, and vomiting^[2,92]. Nystagmus and positional vertigo have also been reported^[118,125]. In contrast to LEMS, the treatment of the tumor and/or immunomodulation have not been reported to alter the course of SCD^[122]. This finding might be due to a difference in the reversibility of VGCC-induced damage to terminal neurons and Purkinje cells in the cerebellum. However, early treatment after onset has the potential to improve these symptoms^[126,127].

LEMS

LEMS is a presynaptic disorder of neuromuscular transmission characterized by impaired quantal release of acetylcholine, which causes proximal muscle weakness,

Table 7 Criteria for the diagnosis of Lambert-Eaton myasthenic syndrome

Clinical features
(1) Proximal muscle weakness
(2) Autonomic symptoms
(3) Reduced tendon reflexes
Anti-voltage-gated calcium channel antibodies
Repetitive nerve stimulation abnormalities
(1) Low compound muscle action potential
(2) Decrease > 10% at low frequency (1-5 Hz)
(3) Increase > 100% after maximum voluntary contraction or at high frequency (50 Hz)

depressed tendon reflexes, and post-tetanic potentiation, as well as autonomic changes^[128]. Approximately 50% of patients with LEMS have a tumor^[129,130]. The tumor type that occurs in these patients is almost always SCLC, although there have been a few reports of NSCLC^[130,131]. Higher frequencies of limited disease in patients with SCLC-LEMS have been reported than in patients with SCLC without LEMS (66% *vs* 40%), most likely because of early detection^[132]. In almost all patients (96%), SCLC was found within 1 year of LEMS diagnosis^[132]. In the most patients, a symptom of LEMS comes first, and then SCLC is diagnosed. In only 7% of SCLC-LEMS cases, the diagnosis of SCLC preceded the recognition of LEMS^[132].

The diagnosis of LEMS is based on clinical symptoms and signs, electrophysiological studies, and the detection of autoantibodies (Table 7)^[133]. In 80% of patients, the first symptom is muscle weakness of proximal legs^[134]. The muscle weakness usually spreads proximal to distal regions, involving the feet and hands, and caudal to cranial regions, finally reaching the oculobulbar regions^[133]. It has been reported that the rapidity of development is more significant in SCLC-LEMS compared with LEMS without SCLC^[134]. In contrast to myasthenia gravis (MG), limited muscle weakness of the external eye regions is rarely observed^[133]. Characteristically, the impaired reflexes and the muscle weakness can improve after a brief isometric muscle contraction (post-exercise facilitation)^[135]. However, a lack of facilitation does not exclude a diagnosis of LEMS^[135]. Autonomic dysfunction is also found in more than 80% of patients with LEMS^[129,130,134,136]. The most common symptom of autonomic dysfunction is dry mouth, and others include constipation and erectile dysfunction in men^[133]. Even if a patient does not volunteer the presence of these symptoms, it is important to specifically inquire about them^[135].

On electrodiagnostic study, the first compound muscle action potential (CMAP) amplitude is low in the basal condition, and it becomes even lower at low-frequency stimulation (2-5 Hz)^[133]. This phenomenon is due to a lack of release of Ach molecules at the junction. In contrast, post-exercise stimulation or high-frequency stimulation (50 Hz) results in an increase in CMAP amplitude. The easiest and most reliable means of repairing the transmission defect is to provide a brief isometric

voluntary muscle contraction and to measure the CMAP amplitude before and after exercise^[133]. In typical patients with LEMS, the amplitude increases significantly, by greater than 100%, which establishes the presynaptic blockage of the neuromuscular transmission^[135]. Although comparable sensitivity has been reported at high-frequency stimulation, this test should be avoided if possible because it is very painful^[133]. A greater than 100% increase in post-exercise stimulation or high-frequency stimulation has a sensitivity of 78%-85% and a specificity of 100% for LEMS^[137,138]. A cut-off of a 60% increase showed a sensitivity of 97% for LEMS and a specificity of 99% for excluding MG^[137]. In practice, an increase in the amplitude to greater than 60% in several muscles can be used to confirm the diagnosis^[135]. Higher diagnostic sensitivity with the 10-second exercise compared with 30-second exercise has been reported^[139].

Antibodies against the P/Q-type VGCC are elevated in more than 95% of patients with SCLC-LEMS, whereas antibodies against the N-type VGCC are elevated in approximately 40% of these patients^[135]. These antibodies are also elevated in approximately 70% of patients with LEMS without tumors^[135]. Patients who are positive for N-type VGCC antibodies usually have the P/Q-type VGCC. However, two cases with only N-type VGCC antibodies have also been reported in patients with squamous cell lung carcinoma^[140]. Although it has been reported that P/Q-type VGCC antibodies are very specific for LEMS, they have also been detected in 1%-4% of patients with SCLC without any neurological dysfunction^[133]. SOX1 is the antigen recognized by anti-glial nuclear antibody-positive sera^[117]. SOX1 antibodies were present in 64% of patients with LEMS and SCLC but in no patients with idiopathic LEMS^[117]. In another report that assessed several types of SOX, SOX antibodies had a sensitivity of 67% and a specificity of 95% for discriminating between LEMS with SCLC and non-tumor LEMS^[141].

The treatment of LEMS is threefold, including treatment of the underlying cancer, symptomatic treatment, and immunotherapy^[135]. After the detection of cancer, the patient should be treated accordingly. It is important to remember that two-thirds of patients with SCLC-LEMS have a limited form of the disease, in which effective treatment could lead to sustained clinical remission^[132,142,143]. The 3,4-diaminopyridine has been used as the first-line treatment for the patients with symptomatic LEMS^[133]. This agent blocks VGKC, and prolongs the opening time of VGCC and the action potentials at terminals of the motor nerves^[135,144]. As a result, there is an increase in the influx of calcium into the nerve terminal, which is subsequently followed by a release of Ach^[135]. A recent study showed that aminopyridines could target the VGCC β subunit leading to enhancement of synaptic and neuromuscular transmission^[145]. Four controlled trials of 3,4-diaminopyridine compared with placebo in a total of 54 patients with LEMS reported significant improvements in the primary outcome, muscle strength score,

or myometric limb measurement for hours to one week following treatment and significant improvements in the resting CMAP amplitude following 3,4-diaminopyridine administration^[128,146-149]. Adverse events reported for 3,4-diaminopyridine treatment during the trials include epigastric discomfort, brief perioral tingling, digital paresthesia, and insomnia^[128,146-149]. The starting dose is 5-10 mg 3-4 times per day, and a maximum dose is 60-80 mg per day^[135,150]. The risk of seizures increases in a dose-dependent manner although it is mostly reported by administration of approximately 100 mg per day^[133,146,148,150]. In addition, treatment with prednisolone and azathioprine for long time might be chosen if some symptoms remain^[133]. In 46 of 104 (44%) patients with SCLC-LEMS, prednisolone was required, and in many patients, azathioprine was also administered for the management of LEMS^[133]. However, the effects of corticosteroids and immunosuppressive agents have not been tested in randomized controlled trials. A single cross-over trial reported a significant improvement in myometric limb strength with intravenous immunoglobulin (IVIg) compared to placebo^[151]. IVIg treatment resulted in an improvement in resting CMAP amplitudes compared with placebo, but this difference did not reach statistical significance^[151]. However, a combination of 3,4-diaminopyridine, prednisolone, and azathioprine could lead to the satisfactory management in most patients with SCLC-LEMS, in addition to chemotherapy^[133].

CGP

Pseudo-obstruction as a paraneoplastic manifestation of SCLC was first reported in 1975^[152]. An autopsy of a patient who had abdominal pain and obstipation revealed autonomic neuropathy limited to the gastrointestinal tract, which was considered to be a remote effect of carcinoma^[152]. CGP is now a well-known autonomic neuropathy^[153]. This syndrome is characterized by severe gastrointestinal dysmotility without evidence of mechanical obstruction, leading to chronic pseudo-obstruction accompanied by abdominal pain, nausea, vomiting, and, more frequently, severe constipation^[92,153]. Gastroparesis is a disorder of the stomach caused by delayed gastric emptying in the absence of mechanical obstruction^[154]. Gastroparesis has been described as a complication of several malignancies, including lung cancers, although diabetes mellitus is the most common identifiable cause of benign gastroparesis^[154]. This "malignant gastroparesis" includes postvagotomy syndrome, cancer invasion to the autonomic nervous system, and paraneoplastic dysmotility of the stomach as a subtype of CGP^[154].

In an analysis of 162 anti-Hu antibody-positive patients, 142 patients (88%) had cancer^[155]. Of these 142 patients, SCLC was proven in 132 (93%). Although sensory neuropathy was the most common neurologic syndrome, 23% of patients had gastrointestinal symptoms^[155]. In another report, anti-Hu antibody was detected in 8 of 9 patients with CGP associated with SCLC (one patient was not tested)^[156]. CGP is often the first

manifestation of the malignancy^[155,157]. Although SCLC is most common in CGP, NSCLC has also been described as an underlying malignancy^[155,156].

Histopathology has shown marked mononuclear cell infiltration of the myenteric plexus with less involvement of the submucosal plexus and a decrease in the number of myenteric plexus neurons^[158]. Immunohistochemical staining showed infiltration by a mixture of B-cell and T-cell lymphocytes and plasma cells, and sparse and disorganized interstitial cells of the Cajal network^[158,159]. The inflammatory/immune response in enteric ganglionitis leads to neuronal dysfunction and degeneration over time and sometimes results in the complete loss of enteric neurons^[160]. The early treatment of cancer appears to offer the greatest chance for the improvement of CGP^[161]. A case of successful treatment with octreotide, a somatostatin analog, has also been reported^[153].

Polymyositis /dermatomyositis

Polymyositis (PM)/dermatomyositis (DM) is an autoimmune-based systemic inflammatory myopathy. In the case of DM, the characteristic cutaneous manifestations include scaly and erythematous plaques on the dorsal sides of the both hands (Gottron's sign), edematous rash of the eyelids and periorbital (heliotrope rash), photosensitive poikilodermatous eruptions, and periungual telangiectasia^[92]. In a review of 2439 patients with DM, 574 patients (24%) have been reported to be associated with malignancy, and 97 (10.2%) of 947 patients with PM had an associated malignancy^[162]. A wide variety of malignancies has been associated with DM and are significantly influenced by the patient ethnicity^[162]. Carcinomas of the nasopharynx (21%), breast (15%), lung (15%), ovary (9%), and colon (5%) have been reported as the most common malignancies associated with DM^[162-166]. In an analysis of population-based cohorts derived from the Caucasian populations of Sweden, Denmark, and Finland, 115 of 618 patients (18.6%) with DM developed malignancies after the diagnosis of DM^[167]. The types of malignancy that increase in relative risk significantly were carcinomas of ovary (standardized incidence ratio 10.5), lung (5.9), pancreas (3.8), stomach (3.5), and non-Hodgkin's lymphoma (3.6)^[167]. In a report of 138 Chinese patients with DM and cancer, the most common malignancies were carcinomas of nasopharynx, breast, and the lung^[168]. More severe signs and symptoms in skin and muscle often indicate an underlying malignancy in patients with DM^[162]. The characteristic cutaneous manifestations associated with malignancy include cutaneous leukocytoclastic vasculitis^[169], bullous DM^[170], cutaneous necrosis^[164,165], periungual erythema^[165], and necrotizing myopathy^[171]. Regarding myositis-specific antibodies, a low frequency of anti-Jo-1 antibody has been reported in cancer-associated myositis, whereas anti-p155/140 antibodies have been identified in 50% of cancer-associated myositis cases, much higher than in non-cancer-associated myositis (4.1%)^[172].

In PM/DM associated with lung cancer, the com-

mon histological types were SCLC (29%) and squamous cell carcinoma (21%)^[173]. PM/DM associated with lung adenocarcinoma appears to be rare^[171,174,175]. A case of PM with bronchopulmonary carcinoid has also been reported^[176]. The onset of PM/DM is frequently observed before the diagnosis of lung cancer^[173].

The role of internal malignancies in the development of DM remains controversial. Several case reports have highlighted a causal role of cancer on development of DM because DM significantly improved after the successful therapy of lung cancer^[162,174,177]. Furthermore, among 22 patients with cancer-associated DM who received effective antitumor therapy, 16 patients (73%) experienced remission of DM^[178]. In contrast, the improvement of DM after treatment for cancer has been observed only in 18%-37% of cases^[179-183]. It has been reported that myositis-associated autoantigens, Jo-1 and Mi-2, were expressed at high levels in myositis muscle, particularly in regenerating muscle fibers, and in adenocarcinomas of the lung and breast, but not in the corresponding healthy tissues^[162,184]. Thus, a model of "cross-over" immunity was proposed in which an initial cellular immune response directed at tumor cells overexpressing antigens can also target muscles commonly expressing antigens in patients with myositis^[162,184]. In this setting, a causative role of malignancy in the development of DM can be considered. However, malignancy may also trigger the development of DM. In a case report, DM developed in a patient with SCLC, and the DM went into remission after the successful treatment of SCLC. The DM recurred 10 years later without any malignancy^[180]. In this case, internal malignancy was not a prerequisite for DM relapse; the patient had had an occult autoimmune mechanism of DM. SCLC triggered the expansion of an autoreactive T cell clone as an autoantigen, and DM resulted^[180]. Because of the aging of the immune system, the autoreactive clone gradually increased over 10 years and induced DM again^[180].

A combination of corticosteroids and tumor resection or chemotherapy can improve the symptoms of DM associated with lung cancer^[173,180]. It has also been reported that an epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor (TKI), gefitinib, resulted in the dramatic recovery of a patient with DM associated with adenocarcinoma^[174].

Opsoclonus-myoclonus

Opsoclonus-myoclonus (OM), or opsoclonus-myoclonus-ataxia (OMA), is a unique movement disorder characterized by conjugate, randomly directed, rapid eye movements (opsoclonus) and by myoclonus occurring in the face, head/neck, trunk, and limbs^[185]. Some patients also exhibit cerebellar dysfunction with dysarthria and truncal ataxia, and a few become confused or even comatose^[186]. Various etiologies have been implicated in OM, including infectious, toxic, metabolic, degenerative, and paraneoplastic disorders^[185]. An underlying malignancy accounts for 20% of cases with OM^[187]. In

a Spanish survey in which infectious, toxic, or metabolic disorders were excluded, 14 (58%) of 24 patients with OM were paraneoplastic, and the remaining 10 cases were idiopathic^[186]. Among tumors associated with OM, the most common were SCLC (9 patients; 64%) and breast cancer (2 patients; 14%). Rarely, NSCLC has also been reported^[186,188-191]. Antineural antibodies have been found in only a few patients with paraneoplastic OM^[186]. Antineural antibodies detected in lung cancer include anti-Ri^[190,192], anti-Hu^[186,191], anti-amphiphysin^[186], and P/Q-type VGCC^[191] antibodies. A case of OM associated with SCLC with a high anti-mitochondrial antibody titer has also been reported^[193].

In contrast to most of the other PNSs, paraneoplastic OM may remit either spontaneously, following the treatment of the tumor, or in association with clonazepam or thiamine treatment^[194]. Paraneoplastic OM has been reported to exhibit a more severe clinical course than idiopathic OM, despite treatment with IVIg or corticosteroids^[186]. When underlying tumors were treated, however, a complete or partial neurological recovery could be observed^[186]. With appropriate treatment for SCLC, approximately half of reported patients have experienced improvements in neurologic function^[195]. Tumor control is essential for the successful long-term management of paraneoplastic OM.

Subacute sensory neuropathy

A diagnosis of subacute sensory neuropathy (SSN) could be performed when the following criteria are all fulfilled: subacute development in less than 12 wk, severity of at least 3 (moderate disability) assessed by a Rankin score, onset of numbness with often pain and sensory disturbance, involvement of arms and legs (so-called gloves and stockings area), and often asymmetry at onset. Furthermore, electrophysiological findings have shown the significant sensory fibers disturbance and, in at least one sensory nerve, the absence of sensory nerve action potentials sensory nerve^[93]. The motor nerves may also be minimally disturbed^[196]. Tendon reflexes are depressed or absent^[194], and patients often exhibit autonomic, cerebellar, or cerebral abnormalities^[196]. SSN occurs in approximately 75% of patients with paraneoplastic encephalomyelitis, is predominant in 50%, and is clinically pure in 25%^[194,197,198]. In a review of 26 patients with paraneoplastic SSN, 19 (73%) had SCLC^[196]. There was a striking predominance of females (20:6)^[196]. SSN usually predates the diagnosis of cancer, with a median delay of 3.5-4.5 mo^[194,197,198].

SSN in patients with SCLC is typically associated with anti-Hu antibody^[194,199]. Less frequently, SSN in patients with SCLC is associated with anti-CV2 (CRMP-5), anti-amphiphysin, or anti-Yo antibodies^[194,199]. A patient with SSN associated with SCLC who was positive for ganglionic neuronal acetylcholine receptor (nAChR) antibody has been reported^[200].

The early treatment of the underlying tumor appears to offer the best chance of stabilizing the neurological

symptoms^[198]. The complete response of SCLC appears to produce an improvement in neurological function^[100,106,201]. Several cases of spontaneous SCLC regression and SSN progression have been reported, suggesting a host immune response directed at both the cancer and the nervous system^[100-102]. However, immunotherapy consisting of corticosteroids, plasma exchange, and IVIg is ineffective in most cases^[194,201,202].

PARANEOPLASTIC DERMATOLOGIC SYNDROMES

The paraneoplastic dermatologic syndromes associated with lung cancer are presented in Table 8. Several dermatologic syndromes represent very specific conditions that suggest the presence of associated lung cancer. In the paraneoplastic cases, the responsiveness to the therapy is generally weaker compared with the non-paraneoplastic equivalents^[2]. Dermatomyositis is involved in neurologic, dermatologic, and rheumatologic syndromes. We describe dermatomyositis as a PNS because both the neuromuscular system and the skin are affected. Generally, paraneoplastic dermatologic syndromes improve in response to the treatment of the underlying neoplasm; the efficacy of ectopic local treatments is partial or insufficient.

Acrokeratosis paraneoplastica (Bazex syndrome)

The typical features of acrokeratosis paraneoplastica, also called Bazex syndrome, are erythematous scaly lesions on the extremities, ears, and bridge of the nose in association with a malignancy^[203]. The most common malignancies are squamous cell carcinomas of the laryngopharyngeal region or of the esophagus, tongue, or lung^[203,204]. Other histological types of lung cancer, such as adenocarcinoma and SCLC, have also been reported, although squamous cell carcinoma is the most common^[204-207]. In a review of 109 patients, psoriasiform lesions preceded the diagnosis of the associated malignancy in 73 (67%) patients, whereas cutaneous manifestations followed the diagnosis of the neoplasm in only 16 (15%) of 109 patients^[204]. The cutaneous lesions are erythematous to violaceous in color with an associated scaling eruption, and the most common sites of involvement are the ears, nose, hands, and feet, including the nails^[204,208]. These lesions are typically non-pruritic, have ill-defined margins, and are symmetric^[209,210]. The psoriasiform dermatitis begins in the fingers, toes, nose, and helix of the ears and progresses to involve the palms, soles, and cheeks, finally extending centripetally to the arms, legs, scalp, and trunk^[205,211].

The underlying mechanisms of acrokeratosis paraneoplastica have not been elucidated. One theory proposes that antibodies against the tumor cross-react with keratinocyte or basement membrane antigens^[210]. Alternatively, a T cell-mediated immune response to tumor-like antigens in the epidermis has been proposed^[210]. Squamous cell carcinoma lines have been shown to synthesize and secrete growth factors, such as transforming growth fac-

Table 8 Paraneoplastic dermatologic syndromes and paraneoplastic rheumatologic syndromes associated with lung cancer

Paraneoplastic dermatologic syndromes associated with lung cancer
Polymyositis/dermatomyositis
Acrokeratosis paraneoplastica (Bazex syndrome)
Acanthosis nigricans
Tripe palms
The sign of Leser-Trélat
Erythema gyratum repens
Cutaneous leukocytoclastic vasculitis
Pityriasis rubra pilaris
Rhinophyma
Eosinophilic cellulitis
Herperiformis pemphigus
Hypertrichosis lanuginosa acquisita
Erythema elevatum diutinum
Paraneoplastic rheumatologic syndromes associated with lung cancer
Polymyositis/dermatomyositis
Vasculitis
Cutaneous leukocytoclastic vasculitis
Henoch-Schönlein purpura
Hypertrophic pulmonary osteoarthropathy
Remitting seronegative symmetrical synovitis with pitting edema
Polymyalgia rheumatica

tor (TGF)-1, which is active in autocrine growth^[212,213]. The excessive expression of growth factors by the tumor might lead to epidermal hyperplasia and cancer cell proliferation.

The treatment of the underlying neoplasm often significantly improves the cutaneous symptoms^[210]. For some patients, cutaneous lesions, most commonly nail dystrophy, may persist despite successful tumor eradication^[210].

Acanthosis nigricans, tripe palms and the sign of Leser-Trélat

The characteristic manifestations of acanthosis nigricans (AN) are thickening and hyperpigmentation of the skin, and develop predominantly in the neck to axilla^[2]. Although AN is usually observed in benign conditions accompanied by endocrinopathies, such as insulin resistance, obesity, erythema nodosum, or medications such as sex hormones or nicotinic acid^[2,214], a paraneoplastic form has also been documented. Among paraneoplastic AN cases, the most common histologic type is adenocarcinoma, of which 70%-90% are located intra-abdominally and 55%-61% are gastric adenocarcinomas^[215]. Less commonly, paraneoplastic AN is associated with NSCLC^[216-218]. The major features of AN are darkening and thickening of the skin (hyperkeratosis), which occurs mainly in the folds of the skin in the axilla, groin, and back of the neck^[216]. Oral lesions, observed in 50% of cases, are generally located on the lips, tongue, and buccal mucosa^[219]. Gross and eruptive AN with florid cutaneous papillomatosis and palmar/plantar keratoderma are typical features of AN associated with malignancy^[215]. Rapid onset is also typical in paraneoplastic dermatoses^[219].

“Tripe palms” is also known as acanthosis palmaris,

pachydermatoglyphy (PDG) or palmar hyperkeratosis^[220]. This condition is characterized by a yellowish rugose hypertrophy of the palms and sometimes the soles, leading to an exaggeration of the skin lines^[218,221]. Tripe palms are usually associated with AN. More than 90% of cases are associated with neoplasms; in the majority of these cases, lung and gastric cancers are the underlying malignancies^[218,221].

The sign of Leser-Trélat (LT) refers to the eruption of numerous seborrheic keratoses^[215]. A rapid onset, rather than the number of seborrheic keratoses, suggests the presence of an underlying malignancy^[219]. Although gastric adenocarcinoma is the most common malignancy^[215,219], NSCLC has also been reported^[217,222]. In patients with tripe palms, the coexistence of AN (72%) and LT (10%) has been reported^[219,221].

The pathogenesis of AN is poorly understood. However, one possibility is that interactions between excessive amounts of circulating insulin with insulin-like growth factor receptors on keratinocytes and dermal fibroblasts lead to the development of AN^[223]. An increased production of TGF- α by the tumor could be responsible for an increased proliferation of keratinocytes and the development of paraneoplastic AN^[217,218]. No specific treatment has been established for AN. However, the treatment of the underlying malignancy usually improves AN as well^[215].

Erythema gyratum repens

Erythema gyratum repens (EGR) is rare, and the primary lesion is a serpiginous macular, occasionally popular, erythema^[210]. Most of the body is covered in numerous serpiginous bands arranged in a parallel configuration of concentric red swirls or a wood-grain pattern resembling a knotty cypress board^[210,224]. *Gyratum* means “coiled or winding around a central point”, and *repens*, from the Latin, means “to crawl or creep”; the name itself describes the classic eruption of concentric erythematous rings, which develop a trailing scale at their edges and advance at a rapid rate (≤ 1 cm per day)^[224]. All reported patients with EGR have been Caucasian^[210]. In a review of 49 cases of EGR, 41 (84%) were associated with a neoplasm, most commonly of the lung^[225]. Cases of NSCLC, including adenocarcinoma and squamous cell carcinoma, but not SCLC, have been reported^[224,226-228]. The skin symptoms usually disappear with therapy for the underlying lung cancer^[225,227,228].

Cutaneous leukocytoclastic vasculitis

Cutaneous leukocytoclastic vasculitis (CLV) is an inflammatory vascular disease characterized by the prominent involvement of the skin and the infiltration of the small blood vessels with polymorphonuclear leukocytes and the presence of leukocytosis, fibrinoid necrosis, and extravasation of red blood cells^[229]. In cases of paraneoplastic vasculitis, CLV (45%) and polyarteritis nodosa (36.7%) were the most frequently observed subtypes of vasculitis^[230]. In 71% of CLV cases, manifestations of vasculitis

appeared before or concurrent with the initial identification of the tumor or at the relapse of the tumor^[231]. The most common malignancies were hematologic malignancies and carcinomas of urinary organs, gastrointestinal tract, and lung (20%-26%)^[231,232]. The associated lung cancers were adenocarcinoma and squamous cell carcinomas^[229,231-233]. The mechanism by which neoplasms cause vasculitis has not been defined. However, it has been postulated that tumor antigens may induce immune complexes, which could be deposited on blood vessel walls, stimulating a direct autoimmune reaction against the host's vessels; alternatively, the tumor emboli or the tumors themselves may invade the vascular structures^[231,233].

The rash associated with CLV can present as a multitude of morphologic appearances, including urticaria, purpura, hemorrhagic vesicles, ulcers, nodules, livedo reticulares, infarcts, or digital gangrene; a skin biopsy is the gold standard for the diagnosis of CLV^[233,234]. The rash is usually symmetric and most commonly involves the lower extremities, often accompanied by pain or burning^[2,233]. Most patients demonstrate concordance of disease activity and treatment response for both cancer and vasculitis^[232]. Treatments for vasculitis include corticosteroids and immunosuppressants, such as cyclophosphamide^[230]. Successful surgical resection of lung cancer has resulted in the complete improvement of the skin rash in a few cases^[229,233].

PARANEOPLASTIC RHEUMATOLOGIC SYNDROMES

Many rheumatologic syndromes, such as rheumatic arthritis and systemic lupus erythematosus occur without an association with malignancy^[2]. However, several conditions have been reported to be associated with lung cancer (Table 8). Dermatomyositis is a symptom of several conditions, such as neurologic, dermatologic, and rheumatologic syndromes. In addition to the standard treatments for the non-paraneoplastic cases, the therapy directed to the cancer is important for the management of paraneoplastic rheumatologic syndromes^[2]. The paraneoplastic cases are generally less responsive to therapy compared with their non-paraneoplastic cases^[2]. We describe hypertrophic pulmonary osteoarthropathy (TPO) as a rheumatic syndrome here, although it may also be classified as a skeletal syndrome^[1].

Hypertrophic pulmonary osteoarthropathy

Hypertrophic osteoarthropathy (HOA) is characterized by the abnormal proliferation of the cutaneous and osseous tissues at the distal regions of the extremities^[235,236]. The triad of clinical signs and symptoms includes clubbed fingers, symmetric polyarthritis, and periostitis of the long tubular bones^[237,238]. Hypertrophic pulmonary osteoarthropathy (HPO) is defined as HOA secondary to pulmonary disease^[239]; it is frequently described in association with lung cancer, cystic fibrosis, or right-to-left cardiac shunts^[235]. More than 70% of HPO cases are

associated with lung cancer^[237,239]. In a review of 2625 lung cancer cases, 19 patients (0.72%) were found to have HPO^[239]. Of 19 lung cancers, 10 (53%) were adenocarcinomas and 4 (21%) were squamous cell carcinomas^[239]. HPO differs from rheumatoid arthritis in several key ways: the presence of non-inflammatory synovial fluid, a lack of radiographic erosions of the joints, negative rheumatoid factor, and pain that involves both joints and bones^[238]. Periostitis is the hallmark of HPO. Most cases involve the tibiae and fibulae, and severe cases have included all the tubular bones, such as ulnae and femurs^[238]. Bone radiography reveals periosteal membrane thickening and periosteal new bone formation^[237,239]. Unlike osteoarthritis, HPO is not associated with joint space narrowing or subchondral sclerosis^[238]. Bone scintigraphy is a useful, widespread medical imaging procedure that facilitates the detection of HPO^[239]. Characteristic findings include symmetric bilateral increased uptake in the long bones^[238]. Although FDG-PET may also show diffusely increased FDG uptake in the periosteal lesions^[240,241], the reliability of FDG-PET in the diagnosis of HPO has not been established.

Although the exact mechanism of HOA remains unclear, several theories have been proposed^[237,238,242]. The most promising explanation involves megakaryocytes and platelet clumps. Megakaryocytes continually emerge from the bone marrow; they are trapped by the pulmonary capillary bed and fragment there into platelets^[242]. In disorders in which megakaryocytes or megakaryocyte fragments bypass the pulmonary capillary network, these large particles can enter the systemic circulation and reach the fingertips in axial vascular streams^[237,242]. These large megakaryocyte fragments then interact with endothelial cells, leading to the release of growth factors, including platelet-derived growth factor (PDGF), prostaglandin E, and vascular endothelial growth factor (VEGF)^[238]. These growth factors cause fibroblast proliferation, digital clubbing, vascular hyperplasia, edema, and new bone formation^[237,238,243]. Significantly higher levels of PDGF and VEGF have been reported in HOA patients compared with healthy controls or subjects with pulmonary diseases other than HOA^[244-246].

Conventional analgesic medications, including non-steroidal anti-inflammatory drugs, have limited effects on HPO. In contrast, the treatment of lung cancer often resolves HPO^[237,238,247-249]. In particular, the complete resection of lung cancer may result in long-term improvements in HPO^[248,249]. The successful treatment of HPO with gefitinib has also been reported in a case of lung adenocarcinoma^[250]. Recently, it was reported that bisphosphonates (pamidronate or zoledronic acid) could dramatically resolve pain and swelling related to HPO^[235,251,252]. Although the mechanism of bisphosphonate-mediated improvement of HPO is unclear, the anti-tumor and anti-inflammatory effects of these drugs might be beneficial. In this regard, it has been reported that pamidronate is a potent inhibitor of VEGF^[253]. In addition, the effectiveness of octreotide in relieving pain in patients with HPO

has been reported^[254,255]. Subcutaneous octreotide at a dose of 100 g twice daily resulted in complete pain relief within several days^[254,255]. Octreotide has been reported to indirectly exert anti-angiogenic activity by inhibiting growth factors, including VEGF, or *via* immunomodulatory effects^[256]. Based on a role of VEGF in the pathogenesis of HPO, an anti-VEGF antibody, bevacizumab, might also be a potent therapeutic option.

PARANEOPLASTIC HEMATOLOGIC SYNDROMES

Paraneoplastic hematologic syndromes are often asymptomatic and are usually detected after the diagnosis of cancer, typically in the advanced stage of the disease^[2]. There is no specific treatment for these conditions, and the successful therapy for lung cancer may often improve hematologic disorders as well. In general, patients with hematologic syndromes have been reported to have a poor prognosis.

Granulocytosis (neutrophilia)

An increased level of white blood cells (leukocytosis) is often found in patients with lung cancer, either at the time of diagnosis or during the course of the disease^[257]. Leukocytosis may be caused by one or more factors, such as concomitant infections or the administration of corticosteroids^[257]. When leukocytosis is observed in the absence of these conditions, tumor-related leukocytosis should be considered. Occasionally, extreme leukocytosis ($> 50000/\text{mm}^3$ or even more than $140000/\text{mm}^3$) has been reported^[258]. In an analysis of 227 patients with lung cancer, 33 patients (14.5%) were diagnosed with tumor-related leukocytosis ($> 10000/\text{mm}^3$)^[257]. The associated histology in 14 (42%) patients was adenocarcinomas, 12 (36%) squamous cell carcinomas, 6 (18%) large cell carcinomas, and only 1 (3%) SCLC. However, the highest frequency of association was observed in large cell carcinomas (54.5%; 6 of 11 patients). The majority of the patients had granulocytosis. Of 33 patients, 16 exhibited high serum G-CSF levels (47-1103 pg/mL), 4 patients had high serum GM-CSF levels (31-61 pg/mL), and 18 patients had high serum IL-6 levels (11-1060 pg/mL)^[257]. In addition, 12 specimens exhibited positive staining against anti-G-CSF antibody, suggesting a G-CSF-producing lung cancer. Bone marrow examinations revealed a massively increased and left-shifted granulopoiesis extending to the myeloblasts, as typically develops under G-CSF stimulation^[258]. In contrast to leukemic blasts, the mature neutrophils that characterize paraneoplastic leukocytosis do not cause hyperviscosity or vaso-occlusion, and therefore do not require specific therapy^[2]. Patients with tumor-related leukocytosis have been reported to have a poor prognosis^[9,257,259]. The survival of patients with hypercalcemia and leukocytosis (MST 1.5 mo) was significantly shorter than that of patients with hypercalcemia alone (MST 3.8 mo)^[9]. Hypercalcemia and leukocytosis can occur through a common mechanism, *i.e.*, long-

term exposure to G-CSF^[9]. “Hypercalcemia-leukocytosis syndrome” has been proposed as a clinical entity of paraneoplastic syndrome, and it may indicate a poorer outcome in lung cancer^[9].

Hypereosinophilia

Excessive eosinophilia (hypereosinophilia) is a phenomenon associated with a wide variety of allergic diseases, parasitic infections, certain forms of vasculitis, and medications. Excessive eosinophilia has also been associated with malignancies, especially hematologic malignancies, including malignant lymphomas. Although case reports have described patients with various solid tumors and excessive eosinophilia, this association is thought to be less common^[260]. Several reports have described excessive eosinophilia associated with lung cancer^[260-262]. Hypereosinophilia has been reported in all types of lung cancer, including large cell carcinoma, squamous cell carcinoma, adenocarcinoma, and SCLC^[260,261,263,264]. Although the exact pathogenesis of hypereosinophilia associated with lung cancer has not been fully elucidated, bone marrow stimulation *via* circulatory factors secreted by cancer cells is the most widely accepted theory^[260,261,265-267]. Interleukin-5 (IL-5) and GM-CSF are the most frequently implicated factors^[260,262,265-267]. IL-5 has emerged as a key cytokine that controls the production, activation, and recruitment of eosinophils^[260]. In a case report of large cell carcinoma with excessive eosinophilia, the serum IL-5 level was elevated, and immunohistochemical staining of the resected primary tumor revealed large amounts of intracellular IL-5^[260]. Both the eosinophil count and IL-5 levels normalized after the surgical removal of the tumor, suggesting that the eosinophilia was mediated by IL-5 produced by the cancer^[260]. IL-5 mediates the antitumor cytotoxicity of eosinophils induced by IL-2 against various human tumor cell lines^[268]. Nevertheless, hypereosinophilia is generally associated with tumor aggressiveness and poor prognosis^[261,265,267]. A poor prognosis may reflect extensive disease and dissemination^[260]. The exact functional roles of eosinophils in human cancers remain unclear^[260].

Thrombocytosis

Thrombocytosis is generally diagnosed when a platelet count is more than $400000/\text{mm}^3$ and is associated with various diseases, including chronic inflammatory diseases and infectious diseases. Thrombocytosis is also frequently observed in patients with various malignancies, including lung cancer^[2,269]. The reported prevalence of thrombocytosis at the time of lung cancer diagnosis is 13%-32%^[269-271]. Paraneoplastic thrombocytosis is thought to be caused by the production of cytokines from the tumor, and the most representative is IL-6^[2,272]. In an analysis of 100 patients with renal cell carcinoma, serum levels of IL-6 were positively correlated with platelet counts^[272]. In addition, anti-IL-6 antibody administration reduced platelet counts in all 12 patients tested^[272]. This finding suggests that the overproduction of IL-6 is responsible

Table 9 Criteria for the diagnosis of paraneoplastic glomerulopathy

No obvious alternative etiology other than malignancy
Existence of a time relationship between the diagnosis of glomerulopathy and malignancy
Clinical improvement after the complete surgical removal of the tumor or complete remission achieved by chemotherapy/radiotherapy
Deterioration of glomerulopathy associated with recurrence of the malignancy

for paraneoplastic thrombocytosis. Survival in patients with thrombocytosis has been reported to be significantly shorter than in those without thrombocytosis^[269-271]. A multivariate analysis of prognostic factors using the Cox proportional hazards model indicated that thrombocytosis was an independent prognostic factor^[269,271]. Although the reasons for poor survival in patients with thrombocytosis remain unclear, PDGF may play a role in cancer progression^[273,274].

PARANEOPLASTIC GLOMERULOPATHY

Paraneoplastic glomerulopathies are rare manifestations of neoplastic disease, which should be distinguished from iatrogenic renal damage. Criteria for the diagnosis of paraneoplastic glomerulonephropathy have been established (Table 9)^[275]. Optimally, a diagnosis of paraneoplastic glomerulopathy should include the establishment of a pathophysiologic link between the tumor and glomerulopathy, including the detection of tumor antigens and antitumor antibodies within subepithelial immune deposits^[276]. However, establishing such a connection would be always unnecessary for a clinical diagnosis. In an analysis of 600 patients with lung cancer, the prevalence of proteinuria and hematuria was 10% and 7%, respectively^[277]. This relatively high prevalence may be attributed to the study's low cut-off value for proteinuria (0.1 g/d) or the use of the qualitative dipstick test alone to detect hematuria, without an assessment of urinary sediment. However, a prospective case-control study reported a significantly higher prevalence of proteinuria and hematuria in patients with lung cancer compared with asthmatic patients^[277]. Regarding the histology of the glomerular diseases, solid tumors, including lung cancer, are preferentially associated with membranous glomerulonephropathy^[275,278,279]. Other types of glomerular disease associated with lung cancer have also been reported, including minimal change disease^[280], IgA nephropathy^[281], focal segmental glomerulosclerosis^[282,283], membranoproliferative glomerulonephritis^[284], and crescentic glomerulonephritis^[285]. These studies reported a variable histology of lung cancer associated with glomerulopathy, including SCLC and NSCLC.

Two thirds of MGN cases are idiopathic^[275]. The etiology of secondary MGN frequently includes infections, autoimmune diseases, and drug toxicity^[275,286]. In a cohort study of 240 patients with MGN, 24 patients (10%) had a

malignancy at the time of renal biopsy or within one year thereafter^[286]. Among the patients with cancer and MGN, 40%-45% clinically manifested the nephrotic syndrome prior to the diagnosis of the tumor^[276]. Simultaneous presentation occurred in approximately 40% of patients, and in the remaining 15%-20%, glomerular disease became apparent following the diagnosis of the tumor^[276]. Regarding the pathogenesis of paraneoplastic MGN, several reports have demonstrated the presence of tumor antigens, such as carcinoembryonic antigen (CEA), in the glomeruli of patients with paraneoplastic MGN^[287-289]. The eluate of the glomeruli was found to react specifically with the surface of the cancer cells from the same patient, and it reacted not only with the cancer cell extract but also with CEA^[289]. An IgG antibody eluted from the kidney reacted with gastric and colonic carcinomas, and the reactivity was blocked by preincubating the tumor substrate with CEA^[289]. These cross-reactions between eluates from glomeruli and tumor antigens provide evidence for a role of the immune complex in the pathogenesis of paraneoplastic glomerulopathy^[275,287,289].

PARANEOPLASTIC

OPHTHALMOLOGICAL SYNDROMES

Cancer-associated retinopathy

Cancer-associated retinopathy (CAR) is a rare paraneoplastic syndrome that is often associated with SCLC^[290]. More than 100 CAR cases have been reported since CAR was first reported in 1976^[291], although the precise frequency of CAR in SCLC patients remains unclear. CAR causes progressive visual loss within several months, and it is associated with a triad of symptoms: photosensitivity, ring scotomatous visual field loss, and attenuated retinal arteriole caliber^[292]. Retinopathy may occur either before or after the diagnosis of cancer^[290]. It is believed that CAR is autoimmune-mediated *via* autoantibodies against retinal photoreceptor proteins, such as recoverin, heat-shock cognate protein 70, enolase, and transient receptor potential cation channel, subfamily M, member 1 (TRPM1)^[293-296]. In certain patients with lung cancer, high-titer serum autoantibodies against recoverin trigger the development of CAR^[297,298], whereas low-titer serum antibodies of patients with lung cancer do not necessarily cause CAR^[299,300]. In addition, the frequencies of serum autoantibodies against recoverin in patients with SCLC or NSCLC without CAR were found to be 15% and 20%, respectively^[293]. These values are much higher than the frequency of the development of CAR, suggesting that serum autoantibodies against recoverin do not necessarily trigger the development of CAR. Aberrant expression of recoverin protein resulting from altered demethylation of the recoverin gene can also trigger the host immune response, followed by the development of CAR^[301]. Regarding treatment, corticosteroids, in addition to anticancer therapy, may be effective for the control of CAR. In a review of 15 SCLC patients with CAR who received corticosteroids, 13 patients (87.7%) recovered

visual function^[290]. In those 13 patients, the mean dose of corticosteroids administered at initial treatment was 57.1 mg (25-100 mg) of prednisolone with or without induction with high-dose methylprednisolone^[290]. Lower doses of corticosteroids might not be sufficient to improve visual function^[290,302]. In addition, treatment delay after visual symptom onset may lead to irreversible injury to the photoreceptor cells^[290,291,302]. An effect of the expressed recoverin on sensitivity to anti-cancer drugs has also been reported^[303]. Compared with recoverin-negative control cells, recoverin-transfected lung cancer cells were more sensitive to several anticancer drugs [Matsuo S, 2010]. Aberrantly expressed recoverin may regulate tumor cell proliferation and drug sensitivity to anticancer drugs in patients with CAR^[303].

Diffuse uveal melanocytic proliferation

Diffuse uveal melanocytic proliferation (DUMP) is a rare paraneoplastic syndrome characterized by: (1) multiple round or oval, subtle, red patches at the level of the retinal pigment epithelium in the posterior fundus; (2) a striking pattern of multifocal areas of early hyperfluorescence corresponding to these patches; (3) the development of multiple, slightly elevated, pigmented and non-pigmented uveal melanocytic tumors, as well as evidence of diffuse thickening of the uveal tract; (4) exudative retinal detachments; and (5) rapidly progressive cataracts^[304,305]. More than 30 cases of DUMP have been reported; almost all of the cases were bilateral, except for one report of unilateral DUMP associated with SCLC^[306]. The most common associated malignancies are ovarian carcinoma in women and lung and pancreatic cancers in men^[307]. On histology, NSCLC (adenocarcinoma, large cell carcinoma, and squamous cell carcinoma) and SCLC have been reported^[306-309]. In half of the cases, DUMP manifests before the diagnosis of an underlying malignancy^[310]. Cutaneous and/or mucosal focal melanocytic proliferation has also been observed in several cases^[310]. It is believed that protein release from the primary cancer may incite an antibody response, which may cross-react with the ocular tissue^[309]. Another pathogenic mechanism may include hormonal stimuli from the cancer, leading to ocular melanocytic proliferation^[309]. The treatment of DUMP with systemic corticosteroids and orbital radiotherapy is ineffective^[308]. However, the regression of DUMP can be achieved by resecting the underlying lung cancer^[308].

PARANEOPLASTIC COAGULOPATHY

Trousseau's syndrome

In 1865, Armand Trousseau reported that unexpected and migratory thrombophlebitis could be the first symptom of visceral cancer^[311]. Ironically, two years later, he found the thrombophlebitis on himself, and was died of development of gastric cancer. Similar descriptions were repeated and extended over the years. Sack *et al*^[312] reviewed 182 patients with chronic disseminated intravascu-

lar coagulopathy (DIC) and malignancy, in whom migratory thrombophlebitis, arterial emboli in various organs, and hemorrhage were frequently observed. Hematologic data showed abnormalities associated with intravascular coagulation, such as hypofibrinogenemia and thrombocytopenia. Other abnormalities included prolonged prothrombin time, increased fibrinogen-fibrin degradation products, microangiopathic hemolytic anemia, and verrucous endocarditis^[312]. More recently, the term "Trousseau's syndrome" is sometimes even applied to patients who already have an advanced malignancy and then develop some form of thrombosis^[313]. Multiple definitions of Trousseau's syndrome have been proposed, ranging from the classic "occurrence of migratory thrombophlebitis with visceral cancer" to simply "carcinoma-induced coagulopathy", "hypercoagulability syndrome associated with cancer", and "malignancy-related thromboembolism"^[313]. Multiple mechanisms of Trousseau's syndrome have also been described^[313]. Intravascular coagulation (thrombosis or DIC) has been most frequently associated with mucin-producing adenocarcinomas^[314]. Mucins can initiate intravascular coagulation by activating factor X^[314]. Injecting carcinoma mucins into mice generated platelet-rich microthrombi dependent on P- and L-selectin, but not thrombin^[315]. The release of tissue factor (TF) and cysteine protease from cancer *via* the activation of factor X has also been reported in the pathogenesis of Trousseau's syndrome^[313]. Hypoxia may increase the expression of genes that facilitate coagulation, including TF and plasminogen activator inhibitor-1 (PAI-1)^[316].

The incidence of thrombosis in 537 patients with lung cancer was assessed^[317]. A total of 39 venous thrombotic events (VTEs), including 17 deep venous thrombi, 15 pulmonary emboli, and 7 cases of both deep venous thrombi and pulmonary emboli, were observed over 879 person-years of follow-up, resulting in 44.4 venous thrombotic events per 1000 person-years^[317]. After adjusting for age and sex, the estimated thrombotic risk in lung cancer patients was 20-fold higher than in the general population^[317]. Patients with adenocarcinoma had a higher risk than patients with squamous cell carcinoma (66.7 and 21.2 per 1000 person-years, respectively)^[317]. Patients with distant metastasis had a 6-fold increased risk compared with patients with localized tumors^[317]. It should be noted that the risk of venous thrombosis increases 3-fold when chemotherapy is started compared with the time when no chemotherapy has been administered^[317].

Heparin has been widely used as the preferred treatment for Trousseau's syndrome^[312,318]. A therapeutic range of 1.5-2.5 times the baseline activated partial thromboplastin time (APTT) has been recommended^[319]. Heparin binds to antithrombin III, causing its activation and leading to the inactivation of thrombin and other proteases, such as factor Xa. Heparin prevents the binding of mucin to L- and P-selectins and mucin-induced microthrombi^[315]. Trousseau's syndrome is often resistant to warfarin, which inhibits fluid-phase coagulation but not selectins^[315]. A serious side effect of heparin is hep-

arin-induced thrombocytopenia (HIT), which involves an immunological reaction that causes platelets to be targeted by the immunological response and results in the degradation of platelets, resulting in thrombocytopenia. Recently, low-molecular-weight heparins (LMWHs) have become popular treatments for Trousseau's syndrome, in part because of their greater bioavailability, the ability to administer single daily doses, the reduced incidence of HIT, and possibly improved safety^[320,321]. Successful treatment with long-term LMWH therapy has been reported in lung cancer patients with Trousseau's syndrome^[322,323]. However, it should be noted that the ability of some LMWHs to mediate some of heparin's actions may not be equivalent^[313]. Some LMWHs are not as effective at blocking L- and P-selectins, even at comparable levels of anti-factor Xa activity^[313,324,325], which might be a disadvantage for the treatment of Trousseau's syndrome. There is no evidence of the efficacy of other drugs that have anti-factor Xa activity, such as danaparoid sodium, fondaparinux sodium, edoxaban tosilate hydrate, and rivaroxaban. In addition, there is no evidence of the efficacy of antiplatelet drugs, such as aspirin, dipyridamole, and cilostazol, in the treatment of Trousseau's syndrome. Some of these drugs can be taken orally, which is a greater advantage for long-term management than heparin if there is an efficacy on Trousseau's syndrome. In addition to heparin, the interruption of the inferior vena cava (IVC) with a filter can be performed to prevent life-threatening PE. Potential indications for this procedure include ilio caval thrombosis^[326]. Poor survival of patients with Trousseau's syndrome has been reported. Compared with patients who did not develop a VTE, patients who developed a VTE during the course of lung cancer had decreased survival time until death^[317].

CONCLUSION

The 21st century has witnessed significant progress in the understanding of some paraneoplastic syndromes associated with lung cancer, especially paraneoplastic neurological syndromes. The elucidation of the mechanisms by which these syndromes occur could lead to the development of new therapeutic strategies. Currently, treatment for these syndromes fundamentally consists of direct therapies aimed at the underlying lung cancer. In the era of molecular-targeted therapy, newly developed drugs have resulted in favorable outcomes in some cases. Remarkable progress is occurring in the development of molecular-targeted therapies for paraneoplastic syndromes.

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WJCO 5th Anniversary Special Issues (2): Breast cancer

Targeting autophagy in breast cancer

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Core tip: Autophagy is thought to be a tumor suppressor pathway. However, once a tumor is formed, it may contribute to tumor cell survival in response to metabolic stress or to therapy. On the other hand, it has also been suggested that autophagy could be induced during breast cancer therapy to kill cells that avoid apoptosis. Here, we discuss some of the recent findings relating autophagy and cancer with a particular focus on breast cancer therapy. We conclude that there are important unresolved questions that should be addressed before autophagy can be successfully targeted for breast cancer treatment.

Abstract

Macroautophagy (referred to as autophagy here) is an intracellular degradation pathway enhanced in response to a variety of stresses and in response to nutrient deprivation. This process provides the cell with nutrients and energy by degrading aggregated and damaged proteins as well as compromised organelles. Since autophagy has been linked to diverse diseases including cancer, it has recently become a very interesting target in breast cancer treatment. Indeed, current clinical trials are trying to use chloroquine or hydroxychloroquine, alone or in combination with other drugs to inhibit autophagy during breast cancer therapy since chemotherapy and radiation, regimens that are used to treat breast cancer, are known to induce autophagy in cancer cells. Importantly, in breast cancer, autophagy has been involved in the development of resistance to chemotherapy and to anti-estrogens. Moreover, a close relationship has recently been described between autophagy and the HER2 receptor. Here, we discuss some of the recent findings relating autophagy and cancer with a particular focus on breast cancer therapy.

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INTRODUCTION

The term autophagy describes the lysosomal degradation, or eating (phagy) of part of the cell itself (auto). Several forms of autophagy have been described. Among them, macroautophagy (referred to here as autophagy) is a ubiquitous process in eukaryotic cells in which cytoplasmic components are engulfed in a double membrane structure called the autophagosome which delivers its contents for degradation to the lysosome. This process occurs at basal levels in all cells during nutrient rich conditions providing tissues with a housekeeping mechanism of cytoplasmic turnover and removal of damaged organelles as well as protein aggregates. Autophagy is also up-regulated in response to physiological conditions, such as starvation and in response to diverse pathological stresses, including hypoxia, formation of protein aggregates

or infection, and many others, thus allowing the cells to adapt to environmental and developmental changes^[1].

Alterations in autophagy are involved in several diseases including cancer^[2]. In cancer, autophagy is thought to have both tumor suppressive and tumor promoting functions. These paradoxical effects may be explained because the role of autophagy in cancer depends on the context and on the stage of tumorigenesis. The tumor suppressive functions of autophagy are most apparent during tumor initiation. Autophagy has been found to limit inflammation, tissue damage and genome instability which are known promoters of cancer initiation. Thus, it has been suggested that autophagy stimulation could be beneficial for cancer prevention. On the other hand, in later stages of cancer development, when tumor cells are exposed to stresses encountered during progression, metastasis and cancer therapy, autophagy is thought to be a tumor promoting mechanism by enabling survival of tumor cells^[3,4]. In this setting, it has been proposed that autophagy should be inhibited during cancer treatment in order to improve therapy, especially in those cancer cells that have high levels of autophagy and that are dependent on autophagy for survival under metabolic stress. Nevertheless, it is important to note that other studies suggest that excessive levels of autophagy could lead to cell death especially in those cells that are apoptosis deficient, which is the case of many cancer cells. It has also been suggested that autophagy modulation could have an effect on anti-tumor immune response^[5,6]. In this review, we discuss some of the recent evidence regarding the role of autophagy in cancer initiation, progression and therapy with a special emphasis on breast cancer.

AUTOPHAGIC PROCESS

The proteins mediating the formation of the autophagosome are known as Atg (autophagy-related) proteins. More than 30 Atg proteins have been identified in yeast and many have mammalian orthologs. The autophagic process (Figure 1 and Table 1) involves a series of steps which start with the initiation of the autophagosome, in which the phagophore (a cup-shaped structure that will later develop into the autophagosome) is formed. It is mediated by the Atg1/ULK kinase complex and is negatively regulated by mTORC1 (mammalian target of rapamycin complex 1), which integrates signals from growth factors, amino acids, glucose and energy status and allows autophagy to be induced in the absence of these stimuli. During nucleation, the Atg proteins are hierarchically recruited to the phagophore assembly site. This process is mediated by a complex integrated by beclin 1, hVps34/class III phosphatidylinositol 3-kinase (PI3K) and several other beclin 1 binding proteins (Figure 2). The elongation of the autophagosomal membrane is controlled by two ubiquitin-like protein conjugation systems: Atg12-Atg5 and Atg8/LC3. Autophagosomes may acquire membrane from multiple sources, including the endoplasmic reticulum (ER), the mitochondrial outer membrane, the plasma

membrane and the Golgi apparatus. Additionally, Atg8/LC3 also recruits adaptor proteins such as p62 and NBR1 to the autophagosomes mediating selective autophagy of different cellular structures, protein aggregates and microorganisms. Finally, autophagosomes move along microtubules towards the microtubule organizing center where lysosomes are enriched. Autophagosomes fuse with endosomes-lysosomes to form autolysosomes and are degraded together with their luminal content^[1,2,7].

At its basal rate, autophagy exercises quality control of the cytoplasm by removing damaged organelles and protein aggregates. Also, autophagy responds to a range of stimuli and in most cases protects the cell against stressful situations. In response to starvation, autophagy is important for lysosomal recycling of metabolites to the cytoplasm, where they are reused either as a source of energy or to provide building blocks for the synthesis of new macromolecules^[8]. Although the pro-survival functions of autophagy have been demonstrated at the cellular and organismal level in different contexts, it is also believed that autophagy can induce cell death. This has shown to be true particularly in lower eukaryotes, where autophagy seems to be directly involved in cell demise. Examples include *Dictyostelium discoideum*, a soil amoeba and midgut cell death occurring during development in *Drosophila melanogaster*^[9,10]. However, the existence of autophagic cell death in mammalian cells remains controversial. The bulk of the available data suggest that autophagic cell death occurs in specific *in vitro* models^[11-13] particularly in cells that are defective in apoptosis, or contributes to the induction of apoptosis or necrosis rather than being directly responsible for cell killing^[14,15]. Nevertheless, a recent report described a form of autophagic cell death, which the authors name autosis that is induced by an autophagy inducing peptide and is regulated by the Na⁺, K⁺-ATPase. Dead cells with morphological characteristics consistent with this type of cell death were found in a subpopulation of cells dying by starvation and in hippocampal neurons exposed to hypoxia-ischemia^[16], providing evidence of autophagic cell death in mammalian cells *in vivo*.

Several signaling molecules and cascades modulate autophagy in response to numerous cellular and environmental cues. The best characterized modulator of autophagy is mTORC1. It negatively regulates autophagy by inhibiting the ULK1 complex through direct phosphorylation and is inhibited by rapamycin, which induces autophagy. The activity of mTORC1 is stimulated by a variety of anabolic inputs, including cellular energy status as well as the presence of amino acids and growth factors. Conversely, mTORC1 is inhibited when amino acids are scarce, when growth factor signaling is reduced (*e.g.*, decreased insulin receptor signaling as shown in Figure 1) and/or ATP concentration decreases, resulting in a de-repression of autophagy. In mammalian cells, ULK1 is also directly phosphorylated and activated by the AMP-activated protein kinase (AMPK) in response to energy restriction. The class III PI3K complex is another impor-

Table 1 Some autophagy-related proteins and other proteins implicated in autophagy mentioned in the text and their autophagy-independent functions

Protein	Role in autophagy	Autophagy-independent roles
ULK1/2	Protein kinase involved in autophagy induction and phagophore biogenesis ^[105]	
Atg2	Interacts with Atg18, possibly involved in phagophore biogenesis ^[105]	Regulation of lipid droplet morphology and dispersion ^[106]
Atg3	E2-like enzyme for Atg8/LC3 ubiquitin-like conjugation system. Involved in phagophore expansion ^[105]	Atg12-Atg3 complex formation does not affect starvation-induced autophagy, increases mitochondrial mass and inhibits cell death mediated by mitochondrial pathways ^[107]
Atg4	Cysteine protease involved in Atg8/LC3 processing (removal of amino acid residues to expose the C-terminal glycine for lipidation) and in lipid removal from Atg8/LC3-PE (lipidated LC3 or LC3 II). Involved in phagophore expansion ^[105]	
Atg5	Atg12-Atg5 conjugation system, involved in phagophore expansion ^[105]	Its expression increases during DNA damage induced by chemotherapy. It induces cell cycle arrest and mitotic catastrophe ^[108]
Atg6/beclin 1	Component of the class III PI3K complex, involved in the induction of autophagy and phagophore biogenesis ^[105]	Interacts with Bcl-2 family proteins. Regulates the stability of USP10 and USP13 thus controlling p53 levels ^[92]
Atg7	E1 (ubiquitin-activating)-like enzyme for the two ubiquitin-like conjugation systems (Atg12-Atg5 and Atg8/LC3) ^[105]	Binds p53 and regulates cell cycle arrest upon metabolic stress ^[109]
Atg8/LC3	Ubiquitin-like protein that is conjugated to PE. It is involved in cargo recruitment into, biogenesis of autophagosomes and phagophore expansion ^[105]	
Atg9	Transmembrane protein that may act as lipid carrier for phagophore expansion ^[105]	
Atg10	E2 (ubiquitin-conjugating)-like enzyme for the Atg12-Atg5 ubiquitin-like conjugation system ^[105]	
Atg12	Ubiquitin-like protein that gets covalently linked to Atg5 in the Atg12-Atg5 conjugation system. Involved in phagophore expansion ^[105]	Atg12-Atg3 complex formation does not affect starvation-induced autophagy, increases mitochondrial mass and inhibits cell death mediated by mitochondrial pathways ^[107]
Atg13	Binding partner and regulator of ULK1/2, involved in induction of autophagy and phagophore biogenesis ^[105]	
Atg14/ATG14L	Component of the class III PI3K complex, involved in induction of autophagy and phagophore biogenesis ^[105]	
Atg16	Associates with Atg12-Atg5 and acts as an E3 ligase to direct LC3 lipidation, involved in phagophore expansion ^[105]	
Atg17/FIP200	Binding partner and regulator of ULK1/2 involved in induction of autophagy and phagophore biogenesis ^[105]	
Atg18/WIPs	PI3P-binding proteins possibly involved in phagophore biogenesis ^[105]	Retrograde transport from the vacuole to the Golgi complex. Regulation of PI(3,5)P ₂ synthesis ^[105]
mTORC1	Serine/threonine kinase rapamycin-sensitive complex, main down-regulator of autophagy that responds to growth factor and nutrient availability ^[105]	Regulates cell growth (accumulation of cell mass) through coordination of protein anabolism, nucleotide biosynthesis, lipogenesis, glycolysis and autophagy ^[110]
Vps34	Class III PI3K, produces PI3P and allows recruitment of PI3P-binding proteins WIP1/2 and of the two ubiquitin-like conjugation systems ^[105]	Regulation of vesicular trafficking in the endosomal/lysosomal system. Regulates signaling by recruiting proteins that bind PI3P ^[111]
Vps15	Regulatory kinase subunit of the class III PI3K ^[105]	
p62	Selective substrate of autophagy that functions as an adaptor protein that links ubiquitinated proteins to LC3 ^[105]	Serves as a scaffold to promote NFκB signaling in TNFR, IL-1βR or NGFR signaling by binding RIP1 or TRAF6. Can bind caspase-8 and stimulate apoptosis. Binds mTORC1 and Rag GTPases on the lysosomal surface to signal amino acid availability ^[112] . Activates transcription factor Nrf2, which drives expression of antioxidant and detoxifying enzymes by competitively binding to Keap1, its ubiquitin ligase ^[113]
NBR1	(Neighbor of BRCA1 gene 1), selective substrate of autophagy with structural similarity to p62 ^[105]	Negatively regulates receptor tyrosine kinase endocytic traffic ^[114]
AMPK	(AMP-activated protein kinase), a sensor of energy that is activated by an increase in the AMP/ATP ratio ^[105,115]	AMPK regulates metabolism, decreases energy expenditure, mediates cell cycle checkpoints, inhibits pro-survival growth pathways and modulates mitotic progression ^[115]
Bcl-2/Bcl-XL	Members of the Bcl-2 family of proteins that inhibit macroautophagy (by binding beclin 1) and pro-apoptotic BH3-only proteins ^[105]	Antiapoptotic proteins that inhibit pro-apoptotic BH3-only proteins (BNIP3, Bad, Bik, Noxa, Puma and BimEL) ^[105]
Survivin	Interacts with beclin-1 ^[103]	Member of the Inhibitor of Apoptosis (IAP) family of proteins that inhibits caspases. It is also involved in chromosome segregation during cell division ^[116]
PINK1	(PTEN-induced kinase 1/PARK6), mitochondrial protein that spans the outer mitochondrial membrane upon mitochondrial depolarization, recruiting Parkin to facilitate mitophagy ^[105]	Mitochondrial protein (mutated in some forms of Parkinson disease) that is processed in a membrane potential-dependent manner to maintain mitochondrial function ^[105]

VMP1	(vacuole membrane protein 1), localizes to the plasma membrane of the ER. Interacts with beclin 1 and is required for autophagy ^[105]	Required for protein secretion and Golgi organization, regulates cell proliferation, anchorage-independent growth and secretory membrane transport. It is a component of initial cell-cell contacts and tight junctions ^[117]
DAPK	Death associated protein kinase, it phosphorylates beclin 1 to activate autophagy by causing dissociation from Bcl-2 ^[105]	Regulates cell death, inhibits cell motility and adhesion, promotes membrane blebbing and stress fiber formation ^[118]
Bif-1	Bax-interacting factor 1)/endophilin B1, protein that interacts with beclin 1 <i>via</i> UVRAG and is required for macroautophagy ^[105] . It has membrane curvature-inducing activity, indicating that it may play a role in biogenesis of isolation membranes ^[119] .	Involved in mitochondrial fission and coat protein complex I (COPI)-vesicle formation ^[119] . Regulates receptor degradation and cytokinesis when present in a class III PI3K subcomplex containing Vps15, Vps34, beclin 1 and UVRAG ^[120]
UVRAG	UV irradiation resistance-associated gene, component of the class III PI3K complex that activates autophagy. It disrupts beclin 1 dimers and forms a heterodimer that activates autophagy. It binds Bif-1 to activate class III PI3K and competes with Atg14L for binding to beclin 1, directing class III PI3K to function in the maturation step of autophagy ^[105]	Regulates receptor degradation and cytokinesis when present in a class III PI3K subcomplex containing Vps15, Vps34, beclin 1 and Bif-1 ^[120] . Regulates coat protein complex I (COPI)-vesicle tethering in the ER ^[121]
Ambra1	Activating molecule in beclin 1-regulated autophagy, binds beclin 1 and positively regulates autophagy ^[105] . Regulates ULK1 stability and kinase activity and is phosphorylated and inactivated by mTOR, inhibiting its action on ULK1 ^[122] . Also, changes in its subcellular localization are important during autophagosome formation ^[123]	
HMGB1	High mobility group box 1, a chromatin-associated nuclear protein that translocates to the cytoplasm in response to stress. Binds to beclin 1, displacing Bcl-2 and promoting autophagy. Autophagy also promotes the release of HMGB1 from the nucleus and the cell which further induces autophagy ^[105]	In the nucleus it is a DNA chaperone, sustains nucleosome dynamics and chromosome stability, modulates gene transcription, recombination and participates in DNA repair and telomere maintenance. It regulates mitochondrial function and when secreted regulates inflammation, immunity, migration, proliferation, metabolism and apoptosis ^[124]
NAF-1	Nutrient-deprivation autophagy factor-1, integral membrane component of the IP3 receptor complex. It binds Bcl-2 at the ER and is required for Bcl-2 to bind beclin 1, resulting in the inhibition of autophagy ^[105]	Required for Bcl-2 dependent regulation of the IP3 channel at the ER and regulates Ca ²⁺ homeostasis ^[125]

tant point of regulation for autophagy induction. Beclin 1 is one of the subunits of the complex and its incorporation, which is essential for its kinase activity, is regulated by its association with other proteins, such as Bcl-2, Survivin, PINK1 or VMP1 (Figure 2). The phosphorylation of beclin 1 by death-associated protein kinase (DAPK) or phosphorylation of Bcl-2 by c-Jun N-terminal kinase (JNK) triggers the dissociation of beclin 1 from Bcl-2 in response to various stimuli, thereby inducing autophagy. AMPK can also stimulate autophagy in response to glucose starvation by phosphorylating beclin 1 on a residue that promotes its incorporation into the PI3K complex^[1,17]. Recent evidence also suggests that the Atg proteins are substrates for transcriptional regulation as well as post-translational modifications such as phosphorylation, ubiquitination and acetylation^[18]. The activity of the class III PI3K complex can be manipulated by pharmacological activators (such as BH3 mimetics) and inhibitors (such as spautin). Inhibitors of lysosomal enzymes (such as cystatin B) and lysosomotropic agents that increase the lysosomal pH (such as chloroquine, hydroxychloroquine and Bafilomycin A) are also used to block the degradative activity of autolysosomes and block autophagy at the degradation step (Figure 1)^[2,17].

AUTOPHAGY AND CANCER

Alterations in the autophagic pathway have been observed in several disorders, including metabolic diseases, myopathy, neurodegenerative disorders, infectious and

inflammatory diseases, autoimmune disorders, aging and cancer^[2,19]. Cancer was one of the first diseases genetically linked to an impairment of autophagy with the proposal that *beclin 1* functioned as a haploinsufficient tumor suppressor. *Beclin 1* was found to be monoallelically deleted in a high percentage of ovarian, breast and prostate cancers and its heterozygous disruption in mice results in increased spontaneous malignancies including lung cancers, liver cancers, lymphomas and mammary precancerous lesions^[20-22]. More recently, studies have established a relationship between beclin 1 expression and cancer prognosis since low levels of beclin 1 are associated with a worse prognosis in gastric, colorectal, pancreatic, oesophageal and breast cancers as well as in chondrosarcoma, whereas high levels of its expression are associated with improved survival in high-grade gliomas, hepatocellular carcinomas and B cell lymphomas^[2]. Nevertheless, deletions of *beclin 1* have recently been found mostly associated with *BRCA1* in breast and ovarian human tumors (suggesting that *BRCA1* loss is the driver mutation and that *beclin 1* is lost because of its proximity to it). Furthermore, in a recent comprehensive study no evidence was found for *beclin 1* mutations or loss in other cancers, casting doubt on whether it is a real tumor suppressor in human cancers^[23].

Other ATG proteins have been shown to be involved in the suppression of tumorigenesis. For instance, *Atg 4C* knockout mice do not develop more spontaneous tumors than their wild-type littermates but they are more prone to develop chemically-induced fibrosarcomas^[24] and *LC3* is localized to 16q24.1, a locus frequently deleted in liver,

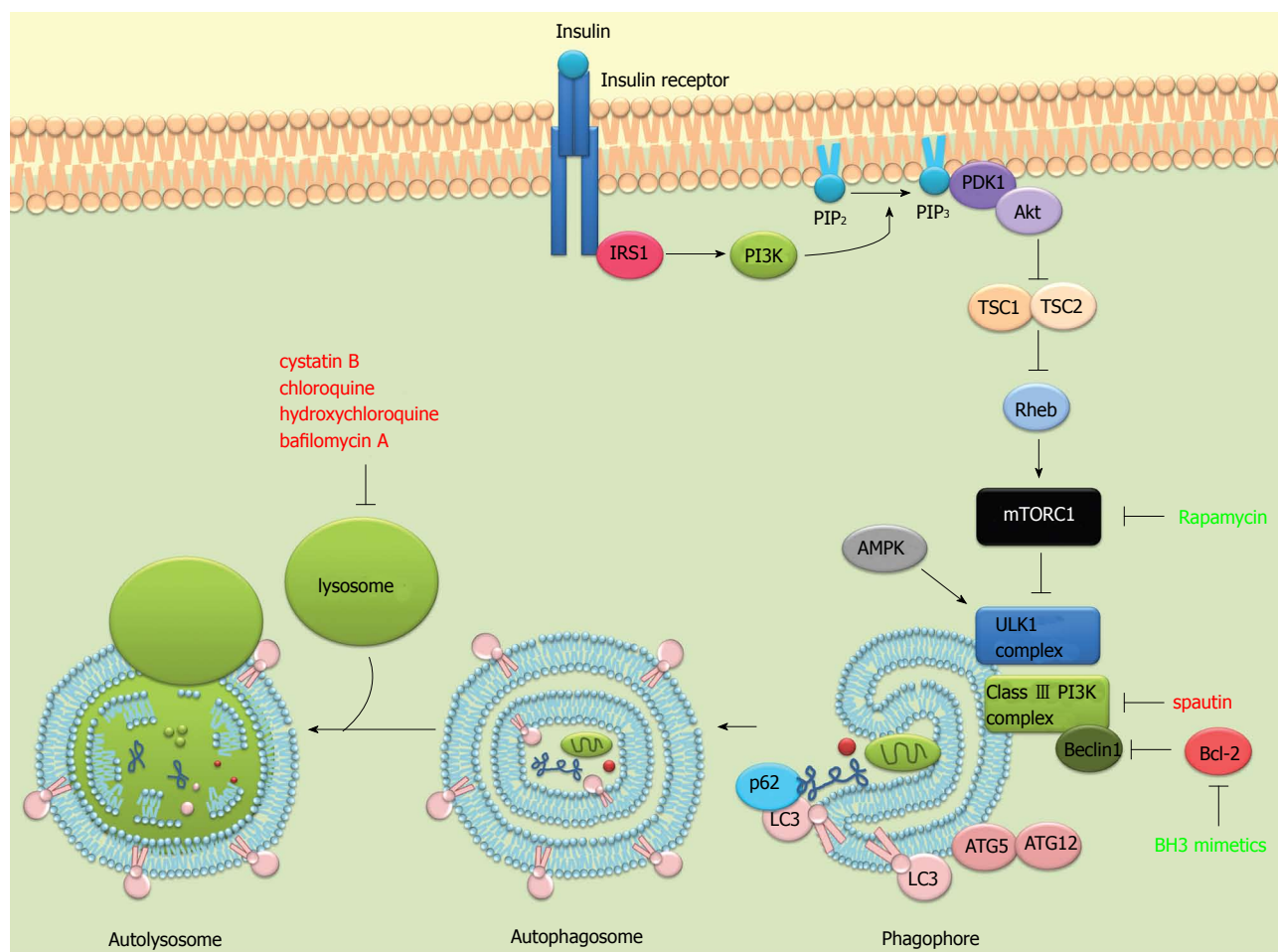


Figure 1 The autophagic process and some of its major regulators. The phosphatidylinositol 3-kinase (PI3K) pathway is triggered by the binding of insulin or growth factors to the insulin receptor, activating PI3K. Activated PI3K converts PIP₂ to PIP₃ which then recruits PDK1 and Akt to the plasma membrane. Activated Akt then phosphorylates and inactivates TSC 1/2, leading to the activation of Rheb and mammalian target of rapamycin complex 1 (mTORC1). Under nutrient-rich conditions, mTORC1 suppresses autophagy by phosphorylating and inhibiting the ULK1 complex. Under starvation conditions or rapamycin treatment, mTOR dissociates from the ULK1 complex and autophagy gets activated. ULK1 is also directly phosphorylated and activated by the AMP-activated protein kinase (AMPK) in response to energy restriction. The autophagic process involves the degradation of cytosolic proteins and organelles in the lysosomes via autophagosomal delivery. Two complexes regulate the formation of the phagophore, the ULK1 complex and the beclin 1-VPS34 (class III PI3K) complex. The elongation of the autophagosomal membrane is mediated by two ubiquitin-like protein conjugation systems: ATG12-ATG5 and ATG8/LC3. LC3 can additionally recruit adaptor proteins such as p62 to autophagosomes mediating selective autophagy of cellular structures, protein aggregates and microorganisms. LC3II (LC3 bound to phosphatidylethanolamine) is recruited to both the inner and outer autophagosomal membrane. Autophagosomes fuse with lysosomes to form autolysosomes where they are degraded together with their luminal content. Pharmacological modulators of autophagy are labeled according to their effect on autophagy. Red: Inhibits autophagy; Green: Induces autophagy.

breast, prostate and ovarian cancers^[25]. Also, p62 has been found to be upregulated in human tumors^[26], *Bif-1* (a protein part of the beclin 1/class III PI3K complex that enhances its activity) knockout mice have a higher incidence of tumors than wild-type mice, especially lymphomas^[27], and *UVRAG* (a beclin 1 binding protein also part of the class III PI3K complex) is localized to the 11q13 human chromosomal region which is frequently implicated in the development of malignancies, including breast and colon cancers^[28].

The previous observations suggest a tumor suppressive role of autophagy. However, mosaic deletion of *Atg5* in mice resulted in benign tumor development only in the liver^[29]. When taken together, these studies do suggest that autophagy has tumor suppressor functions particularly in the liver and that its inhibition predisposes to the development of cancer induced by additional

agents or mutations in other tissues. However, it is important to note that since all the autophagy regulators also affect other things too, it is difficult to be sure that any effects seen when a particular autophagy regulator is inactivated are due to autophagy as opposed to other functions. Consistent with this, the more pronounced effects observed with heterozygous disruption of *beclin 1* when compared with other *ATG* genes might be caused by effects on autophagy together with other autophagy-independent mechanisms. The different phenotypes observed in the *Atg5* or *Atg7* knockout mice (which are viable but die after birth presumably due to nutrient and energy depletion) when compared to *beclin 1* knockout mice (which are embryonic lethal) indicates that beclin 1 has important functions independent of its role on the autophagic pathway^[30]. Indeed, beclin 1 can bind anti-apoptotic proteins of the Bcl-2 family (such as Bcl-2 and

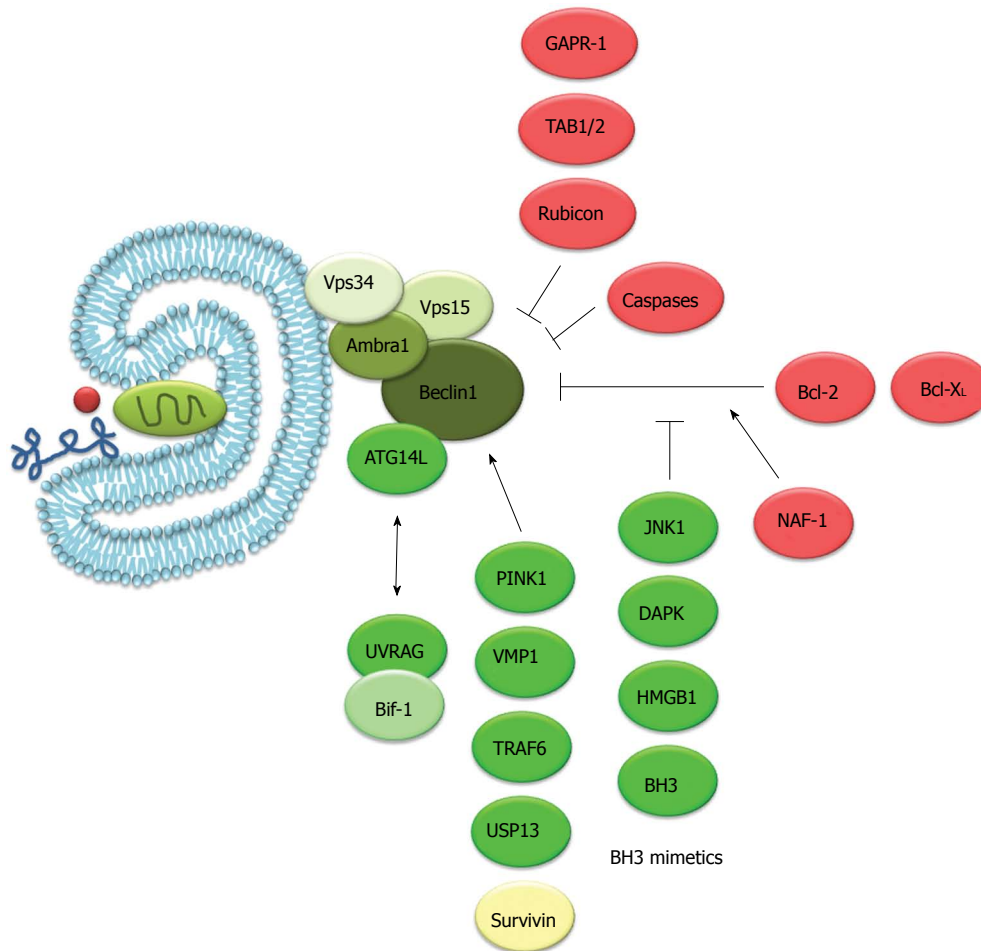


Figure 2 Beclin 1, its protein interactions and its role in autophagy. Beclin 1 has been found to form part of different class III phosphatidylinositol 3-kinase (PI3K) complexes. Each complex consists of beclin 1, Vps34, Vps15 and Ambra1. ATG14L activates the complex and induces the formation of autophagosomes. UVRAG and ATG14L are present in mutually exclusive class III PI3K complexes and UVRAG and Bif-1 have been shown to activate the complex. UVRAG has also been shown to function in autophagosome maturation and endocytic trafficking possibly independent from its interaction with beclin 1. Rubicon has also been shown to bind beclin 1 and can inhibit autophagosome formation and maturation. TAB1/2, two upstream activators of the TAK1-IKK signaling pathway interact with beclin 1 and their dissociation seems to be necessary for autophagy induction. GPR-1 can also bind beclin 1 and inhibit autophagy probably through beclin 1 tethering in the Golgi apparatus. Bcl-2/Bcl-XL can bind beclin 1 and inhibit autophagy. JNK1 phosphorylates Bcl-2 while DAPK phosphorylates beclin 1 and disrupt their interaction. Additionally, BH3-only proteins (tBid, Bad, BNIP3) or BH3 mimetics (ABT737) bind Bcl-2 and release beclin 1. Beclin 1 has also been found to be a substrate of caspases-3, 7 and 8 during apoptosis, in which cleavage of beclin 1 suppresses autophagy. Other beclin 1 interacting proteins that induce autophagy are PINK1 and VMP1. TRAF6 and USP13 have been shown to regulate beclin 1 ubiquitination. Survivin, an anti-apoptotic protein can also bind beclin 1 and regulate TRAIL induced apoptosis. NAF-1, a component of the IP3 receptor complex contributes to the interaction of Bcl-2 with beclin 1 at the ER^[32,99-104]. Proteins and drugs shown are color-coded according to their effect on autophagy. Red: Inhibits autophagy; Green: Induces autophagy; Yellow: Unknown.

Bcl-XL^[31]) and is also known to bind USP13 and regulate p53 levels (Figure 2)^[32]; these other functions could contribute to tumor suppression effects.

The mechanisms by which autophagy itself can decrease tumor formation have been suggested to involve degradation of damaged mitochondria that could otherwise induce oxidative stress, DNA damage and genomic instability (Figure 3). This situation of chronic tissue damage can also provoke an inflammatory response that can further promote tumor growth through cytokine production, and autophagy inhibition has been shown to increase cytokine production in some cases^[6,33,34]. Also, autophagy suppression leads to up-regulation of p62 which activates a NRF2 mediated antioxidant survival response that could lead to tumor promotion^[4].

Once a tumor is formed, tumor cells are exposed to

many and varied stresses including hypoxia, starvation and lack of growth factors, all known to be autophagy inducers. Increasing evidence in the literature suggests that cancer cells robustly activate autophagy to survive such stresses as they are encountered during tumor progression and metastasis. For example, autophagy is known to be most prominent in hypoxic tumor regions, where it sustains survival of tumor cells under metabolic stress^[3,4] and autophagy is also needed for survival during detachment from the extracellular matrix so that tumor cells can avoid anoikis^[35].

Many cancer cells have high levels of basal autophagy even in fed conditions. This is in contrast to normal cells in which autophagy normally occurs at low levels and is only up-regulated in response to stresses like starvation^[4]. In this regard, it has been suggested that transformation

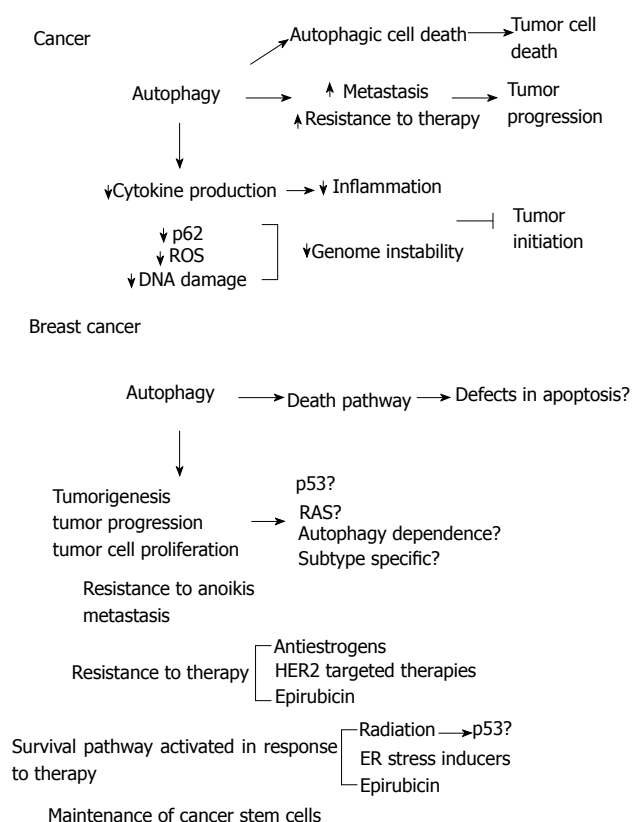


Figure 3 Autophagy in cancer and breast cancer. Different roles have been described for autophagy in cancer. Autophagy can limit tumor initiation by decreasing inflammation and genome instability. Once a tumor is established, autophagy can induce tumor progression by facilitating metastasis and resistance to therapy. On the other hand, it has also been proposed that autophagy could function as a cell death process that could be induced during therapy. In breast cancer, although some evidence suggests that autophagic cell death occurs in apoptosis defective cells, most of the evidence in the literature suggests a tumor promoting role for autophagy. Autophagy has been shown to promote tumorigenesis, tumor proliferation and progression. This could depend on the p53 or RAS mutation status of the cells. Also, if breast cancer is an autophagy-dependent disease remains to be determined as well as if this dependence is subtype specific. Autophagy has also been involved in resistance to anoikis, facilitation of metastasis, resistance to therapy and maintenance of breast cancer stem cells. Moreover, when cancer cells are made resistant to the therapies shown in the figure, they show increased autophagy and inhibition of autophagy reverts this resistance. Therapies that are known to induce autophagy in breast cancer are also shown. With these treatments, autophagy inhibition has been shown to increase cell death. Regarding radiation, results are controversial and the role of autophagy could depend on the p53 status of the cell.

mediated by certain oncogenes, like RAS, induces a metabolic switch in cancer cells that makes them addicted to autophagy under basal, but especially under starvation conditions and that autophagy inhibition would be particularly beneficial for the treatment of these tumors^[36,37]. This has been shown to be true in chloroquine-treated mouse xenografts models of pancreatic ductal adenocarcinoma (PDAC), in which activating Kras mutations are frequent^[38] and in a mouse model of Kras^{G12D} driven non-small-cell lung cancer (NSCLC). In the latter, autophagy inhibition diverted tumor progression from adenomas to oncocytomas, a more benign type of tumor, particularly in p53 deficient tumors^[33]. On the other

hand, in another model of NSCLC driven by Braf^{V600E}, the downstream effector of Kras, autophagy inhibition increased tumor burden. In this work, “autophagy dependence” became apparent only at later time points, when the tumors had been established. This study also shows a change in tumor type to more benign oncocytomas and a greater survival advantage than Kras^{G12D} driven tumors by autophagy inhibition^[39]. Similar to the previous study, autophagy inhibition by mosaic deletion of *Atg5* or *Atg7* in the pancreas induced increased formation of pre-cancerous lesions in a Kras^{G12D} mouse model of pancreatic ductal adenocarcinoma, but these lesions did not progress to carcinoma. Interestingly, this only occurred in a p53 wt background^[40] suggesting that a single oncogenic event such as Ras mutation may only cause dependence on autophagy in some circumstances and that the full spectrum of tumor mutations may ultimately determine just how autophagy-dependent a tumor will be. Further support for this idea comes from other studies where, in contrast to Ras-driven autophagy-dependence, it has been shown that autophagy restrains the proliferative potential of Ras transformed cells so that autophagy inhibition dramatically increased clonogenic growth^[41]. Moreover, autophagy has also been shown to restrict proliferation of cells carrying activated PI3K^[42], suggesting that autophagy might have different, and even opposing effects, depending on the cellular context and the transformation event. In conclusion, the role of autophagy in cancer is complex and is both context and probably tissue dependent (Figure 3). Thus, although numerous studies have addressed this issue, it remains inconclusive in which tumors autophagy should be targeted. This has important practical implications because current clinical trials are using chloroquine or hydroxychloroquine with the purpose of inhibiting autophagy in a variety of cancers without selecting patients who would more likely benefit from this treatment. We believe that further studies are needed to identify the tumors where autophagy inhibition will be effective before autophagy can be successfully targeted in the context of cancer treatment.

BREAST CANCER

Estimates of the worldwide incidence and mortality of cancer ranked breast cancer as the most frequent cancer among women with an estimated 1.67 million new cases diagnosed in 2012 (25% of all cancers) with slightly more cases in less developed than in more developed regions. Breast cancer mortality rates have declined in part due to therapeutic advances and it ranked as the fifth cause of death from cancer overall in 2012. However, it was still the most frequent cause of cancer death in women in less developed regions and the second in more developed regions after lung cancer^[43], underscoring the need for better strategies in both prevention and therapy.

Breast cancer is a heterogeneous disease. Clinically, it is classified as hormone receptor positive, HER2 (ERBB2) positive and triple negative breast cancer. Hor-

hormone receptor positive breast cancers are the most numerous and diverse accounting for 60%-70% of breast cancers. They express the estrogen receptor (ER) and usually respond to endocrine therapy. HER2 positive breast cancers overexpress the HER2 receptor tyrosine kinase and respond to targeted therapies against this receptor such as trastuzumab (monoclonal antibody targeting HER2) or lapatinib (a dual-kinase inhibitor targeting both the epidermal growth factor receptor and the HER2 receptor)^[44,45]. This type of cancer accounts for 10%-15% of breast cancer patients. Finally, triple negative breast cancers (TNBC) do not express hormone receptors (ER or progesterone receptor, PR) or HER2. Although the metastatic potential in these cancers is similar to that of other breast cancer subtypes, they are associated with a shorter median time to relapse and death. They have an increased incidence in younger women, in patients with germline *BRC1* mutations or of African ancestry^[46]. Cytotoxic chemotherapy remains the mainstay of treatment of triple-negative disease since there is not a clear agent that targets a defining vulnerability in this disease^[47].

More recently, gene expression analysis has led to the definition of five molecular “intrinsic” subtypes of breast cancer (Luminal A, Luminal B, HER2-enriched and basal-like) and a Normal Breast-like group which have differences in incidence, survival and response to treatment^[48]. Luminal cancers include the ER positive group and are subdivided in luminal A and luminal B subtypes. Luminal B tumors have a lower expression of ER or estrogen-regulated genes, low or no PR expression, higher tumor grade, higher expression of proliferation-related genes, and activation of growth factor receptor signaling, such as IGF-1R and PI3K/AKT/mTOR pathways when compared to Luminal A tumors. They are also considered to have a lower sensitivity to endocrine therapy and higher sensitivity to chemotherapy than luminal A tumors^[49]. The increased aggressiveness of luminal B tumors has been attributed to inactivation of the p53 pathway and hyper activation of *MYC* and *FOXM1*^[46]. Prospective trials are now evaluating the validity of prognostic gene signatures to distinguish between these two subtypes and identify those women (with luminal A cancers) who can be spared adjuvant chemotherapy^[49].

The majority of HER2+ tumors fall into the HER2 enriched subtype. About 30%-40% of these tumors are ER+, most are ER- and some of these tumors are clinically triple negative but still express the gene signature of HER2 activation. It remains unknown whether those patients with the HER2 enriched subtype but having triple negative cancers would also benefit from HER2 targeted therapies^[50].

Most basal-like tumors are triple negative and further molecular profiling has shown extensive heterogeneity of both TNBC and basal tumors. Molecularly, most, if not all of these cancers show a high frequency of *TP53* mutations or loss of the p53 pathway activity, loss of *RB1* and *BRC1*. They show increased signaling of the PI(3)K/AKT, *MYC* and HIF1/ARNT pathways and

hyper activation of *FOXM1*^[46]. The high prevalence of these mutations together makes them highly proliferative, aneuploid tumors with a very high apoptotic rate and geographic or central tumor necrosis^[48,50,51] with an inferior prognosis when compared to the luminal subtypes^[52,53]. The heterogeneity of TNBC is widely acknowledged. Another subtype belonging to this group, the claudin-low subtype, is characterized by the low expression of genes involved in cell-cell adhesion like claudins and E-cadherin, increased expression of immune response genes, high mesenchymal features, low epithelial differentiation and a stem cell-like phenotype^[48]. More recently, Lehmann *et al*^[53] defined six TNBC subtypes: basal-like 1 and 2, immunomodulatory, mesenchymal, mesenchymal stem-like and luminal androgen receptor, providing a broader classification for those TNBC tumors that do not fit in the basal-like group. In this work, the molecular signature of each subtype was used to predict which therapy should be used in cell lines belonging to each subtype with promising results, opening up the opportunity to use better molecular classification of tumors to improve the choice of treatments in the clinic^[53].

Some novel therapies in current clinical trials in TNBC include the use of fibroblast growth factor receptor (FGFR), vascular endothelial growth factor (VEGF), PI3K-AKT-mTOR pathway and PARP [poly (ADP-ribose) polymerase] inhibitors as well as the use of androgen receptor (AR) antagonists in AR positive breast cancers^[54]. Also, signaling pathways that are thought to be important for resistance to conventional breast cancer therapy are currently under investigation in clinical trials including: EGFR, HER2 and HER3 inhibitors, AMPK-activating compounds, restoration of the p53 signaling pathway by MDM2 inhibitors and inhibitors of the following proteins and signaling pathways: PI3K-AKT-mTOR, Notch signaling, CDK4/6, HDAC, HSP-90, BCR-Abl, VEGFR/PDGFR/Raf, VEGFR/PDGFR/c-kit, c-MET/VEGFR2, FGFR, Hedgehog signaling and MEK^[55].

AUTOPHAGY AND BREAST CANCER

The suggestion that beclin 1 is a tumor suppressor was particularly important for breast cancer since it was found to be deleted in 40%-75% of sporadic human breast and ovarian cancers. Beclin 1 expression is frequently low in human breast epithelial carcinoma cell lines and tissues and expressed at high levels in normal breast epithelia. However, as discussed previously, the *beclin 1* gene maps to 17q21, a region that is deleted in approximately 50% of sporadic breast carcinomas^[56], and *beclin 1* is next to *BRC1*, a tumor suppressor gene whose loss is a driver of some breast and ovarian cancers^[23]. In this regard, recent evidence suggests that although *beclin 1* is lost in breast and ovarian cancers, it is co-deleted with *BRC1*, consistent with *BRC1* loss being the primary driver mutation in these cancers and suggesting that *beclin 1* itself may not be a tumor suppressor in this context^[23]. However,

er, it would be interesting to explore if breast and ovarian cancers with loss of *BRCAl* and *beclin 1* have a different outcome than those that only lack *BRCAl*.

Even though *beclin 1* might not be a tumor suppressor gene in human tumors, overexpression of *beclin 1* in the MCF7 breast cancer cell line decreases proliferation, *in vitro* clonogenicity and tumorigenesis^[20], and the *beclin 1* loss that occurs in breast cancer could have important effects independent of autophagy through its interaction with Bcl-2. This relationship would be particularly important since Bcl-2 is itself overexpressed in 50%-70% of cancers, including breast cancer^[57]. An inverse correlation of *beclin 1* and Bcl-2 expression has been described in breast cancer tissue and Bcl-2 expression was correlated with histological grade, tubule formation, nuclear pleomorphism, mitotic count, estrogen receptor and distant metastasis^[58]. These findings suggest that the interaction between these two proteins might be particularly important for breast cancer tumorigenesis since loss, or low levels of *beclin 1* would increase free Bcl-2 and an antiapoptotic response. Moreover, it has also been shown that the growth-promoting activity of Bcl-2 correlates with its ability to bind *beclin 1* and inhibit autophagy rather than with its anti-apoptotic function, thus contributing to tumorigenesis^[59].

In contrast to the tumor suppressive role of autophagy discussed previously, autophagy has been shown to have a tumor promoting role in breast cancer. Two recent papers studied the role of autophagy in mammary tumorigenesis. In the first one, conditional knockout of the essential autophagy protein FIP200 in mammary epithelial cells in the MMTV-PyMT mouse model of breast cancer (which is known to induce mammary adenocarcinomas following PyMT-mediated activation of Ras, Src, and PI3K) reduced tumorigenesis, metastasis and increased survival. Gene expression profiling of mammary tumors in this study revealed increased expression of immune responsive genes in the autophagy deficient tumors, suggesting that FIP200 deletion might trigger enhanced anti-tumor immune response and contribute to the suppression of mammary tumorigenesis and progression^[6]. In the second study, the authors used mice deficient in *Palb2* in the mammary gland, which produced tumors with diverse histology but generally of high grade, invasive, with increased DNA double strand breaks, DNA damage and mutations in p53. In this work, autophagy inhibition by allelic loss of *beclin1* delayed tumor formation. The authors proposed that autophagy was being activated in response to DNA damage and oxidative stress and mediated survival of tumor cells in collaboration with p53, since allelic loss of *beclin1* did not have an effect in tumor formation when p53 was also deleted from the mammary gland^[60]. Both studies found a tumor promoting role of autophagy in oncogene-driven breast cancer models and thus suggest that autophagy addiction might be a potential therapeutic target in breast cancer (Figure 3).

As mentioned above, autophagy addiction has been

particularly linked to strong oncogenic insults like RAS transformation or alterations in the RAS pathway, which induce alterations in metabolic pathways to meet biosynthetic demands. Although RAS transformation is not a common event in breast cancer, other oncogene pathways activated in breast tumors, including HER2, Myc and activated PI3K, produce metabolic alterations similar to RAS transformation. Moreover, components of the PI3K and RAS-RAF-MEK pathway are amplified in basal-like cancers and autophagy addiction can be induced by RAS transformation in breast cancer cells^[3,37,46]. For instance, HRas^{V12} expressing MEFs were found to need autophagy for anchorage-independent growth and the MDAMB231 cell line (KRAS mutated) was found to need autophagy for proper proliferation in both anchorage-independent and in attached, nutrient rich conditions. In both cases, autophagy promoted survival through facilitation of glycolysis^[37]. In agreement with these observations, autophagy is necessary for lumen cell survival in breast cancer MCF10A cells expressing the oncogenic PI3KCA mutant in 3D acini. Interestingly, in this work autophagy inhibition promoted adhesion-independent proliferation in soft agar transformation assays in 3D culture but not in 2D conditions and was suggested to be mediated by an increase in p62 after autophagy inhibition^[42].

Autophagy is induced in non-transformed and oncogene-transformed breast cell lines following matrix detachment and protects them from anoikis or detachment-induced cell death^[35]. Also, chloroquine treatment, a pharmacological agent that blocks autophagy by accumulating in the lysosome and blocking the degradation of autophagosomes, decreased tumor growth, increased survival and decreased metastasis in the 4T1 model of breast cancer^[61]. Additionally, most breast cancer tumors will very likely have alterations in the autophagic pathway since mutations commonly present in breast cancers are known to be important regulators of the autophagic pathway. This is particularly the case of PI3K mutations, alterations in the PI3K-mTOR pathway, p53, EGFR and Bcl-2^[46,58].

AUTOPHAGY AND BREAST CANCER TREATMENT

Endocrine therapy is the mainstay in ER positive disease. While adjuvant endocrine therapy reduces breast cancer mortality, many ER+ tumors develop resistance and eventually recur. Although the mechanisms of endocrine therapy resistance are not well understood, autophagy has been suggested to be involved in them^[62]. In this regard, autophagy is induced in response to anti-estrogen therapy in the MCF7 breast cancer cell line and its inhibition sensitized to tamoxifen treatment in a tamoxifen-resistant cell line^[63]. It has also been shown that blocking autophagy sensitizes to restoration of antiestrogen sensitivity by Bcl-2 and BCL2L2 co-inhibition in a MCF7-derived anti-estrogen resistant cell line. Importantly, in

the former work, autophagy inhibition with 3-MA or beclin 1 knockdown, decreased necrotic cell death and induced apoptosis in the resistant cells after treatment with the antiestrogen ICI 182780 in response to Bcl-2/BCL2L2 co-inhibition^[64]. These observations suggest that autophagy is not only important for the development of anti-estrogen resistance but also for defining the type of cell death, which could also be important for a successful therapy.

Several important roles for autophagy have been described for HER2 positive breast cancers. Therapies targeting the HER2 receptor like trastuzumab (an antibody targeting HER2) or lapatinib (a small molecule tyrosine kinase inhibitor that targets both EGFR and HER2) are known to induce autophagy in both sensitive and resistant cells^[65,66]. Autophagy has also been implicated in the development of resistance to treatment since cells that have been made resistant to trastuzumab exhibit high levels of autophagy and its pharmacological or genetic inhibition sensitized to trastuzumab in cells that have acquired or inherent resistance^[66,67]. Also, a significant association has been found between loss of beclin 1 and HER2 amplification in breast cancers and this work found that loss of beclin 1 predicted response to trastuzumab alone or in combination with other drugs^[56] suggesting an important role for autophagy in this type of cancer. Additionally, a recent study found a direct interaction between both proteins. In this regard, beclin 1 overexpression was found to enhance HER2 phosphorylation and to decrease response to lapatinib whereas beclin 1 knockdown enhanced lapatinib-induced apoptosis^[65]. Also, ATG12 has been reported to be upregulated in trastuzumab resistant cell lines and its knockdown sensitized JIMT1 resistant cells to trastuzumab treatment both *in vitro* and *in vivo*^[68].

Although the previous evidence suggests that autophagy inhibition would be a promising therapeutic target in combination with HER2 targeted therapies, a recent study found conflicting results in NSCLC where a direct association between beclin 1 and EGFR was also found^[69]. Although these findings are in line with the previous observations on beclin 1 and HER2 receptor, in this work, active EGFR was found to bind and phosphorylate beclin 1 decreasing its association with VPS34 and autophagy in NSCLC cell lines. Tyrosine kinase inhibitor (TKI) treatment disrupted beclin 1 association with EGFR and restored autophagy in TKI sensitive cells. These results associated autophagy inhibition with an enhanced cancer cell survival in response to TKI inhibitors and resistance *in vitro*. Also, cells expressing a tyrosine phosphorylation mutant of beclin 1 (EGFR-phosphorylated sites), which decreased binding to VPS34 and autophagy, showed enhanced tumorigenesis and reduced response to erlotinib *in vivo*. The conclusions of this study were that autophagy should not be inhibited in combination with TKI therapy since they found that the tumors with the least autophagy and the greatest amount of cell death also were the most aggressive. It is unclear if these major differences are due to effects that are specific to lung cancer *vs* breast cancer

or if the interaction of beclin 1 with HER2 is different to the one with EGFR.

Chemotherapy in breast cancer often includes the use of anthracyclines, taxanes (docetaxel, paclitaxel), and DNA damaging agents like cyclophosphamide, fluorouracil or platinum-based compounds^[70,71]. Adjuvant systemic chemotherapy is used particularly in the management of TNBC, which lacks a targeted treatment, and is also given with or preceding trastuzumab for patients with HER2-positive, invasive breast cancer^[72]. Most of these agents are known to be good autophagy inducers in different types of cancer^[15]. In breast cancer cell lines, specifically in the MCF7 cell line, epirubicin was found to induce autophagy and a non-apoptotic form of cell death. In this work, autophagy inhibition increased drug toxicity by inducing apoptosis. Moreover, sensitivity to epirubicin was partially restored by autophagy inhibition in an MCF7 cell line that was made epirubicin-resistant^[73]. On the other hand, pharmacological inhibition of autophagy was found to enhance doxorubicin induced cardiotoxicity. Importantly, autophagy induction with rapamycin increased survival of doxorubicin-treated mice in the same work^[74]. Although the mechanisms by which rapamycin increased mice survival could have involved autophagy-independent mechanisms, this work brings up an important point about trying to manipulate autophagy systemically during cancer treatment, since it could have undesirable side-effects in other tissues even if it has the desired effects in tumor tissue and the overall effect could still be disadvantageous for treatment of a patient.

Microtubule stabilizing agents (taxanes) are used in cancer therapy because of their ability to inhibit mitosis. However, they are also known to have profound effects on autophagy. Microtubules support the assembly of pre-autophagosomal structures, direct their movement possibly to mediate formation of mature phagophores and also mediate trafficking of autophagosomes towards lysosomes. Microtubules also regulate two major complexes involved in the initiation of the autophagic response: mTORC1 and class III PI3K complex^[75]. Indeed, part of the cytotoxic effect of taxol has been shown to be dependent on its ability to block autophagosome transport and maturation, since treatment with 3MA or knockdown of ATG7 or VPS34 decreased cell death induced by paclitaxel in MCF7 and SKBR3 breast cancer cell lines. Importantly, this work also found increased expression of *ATG* genes in docetaxel sensitive primary breast tumor biopsy samples (when compared to resistant samples), probably indicating higher levels of autophagy and suggesting that levels of ATG proteins could be investigated as possible prognostic markers for clinical taxane effectiveness^[76]. Notably, other microtubule targeting drugs like vinblastine, a microtubule depolarizing drug, was found to stimulate formation of autophagosomes, decrease fusion with endosomes and disrupt autophagosome motility^[77]. So, despite having different effects on the autophagic pathway, drugs that disrupt microtubules certainly decrease autophagic flux. This is important

since it is often thought that autophagy is always induced in response to stress induced during chemotherapy, underscoring the need to understand the molecular mechanisms by which specific therapies affect the autophagic pathway.

Genotoxic stress as a result to ionizing radiation or chemotherapeutic drugs is known to increase p53 levels and induce cell cycle arrest, apoptosis, senescence or autophagy^[78]. In this regard, p53 has recently been shown to be an important regulator of autophagy with dual roles depending on its subcellular location. In the nucleus, p53 is pro-autophagic in a transcription dependent and independent manner and in the cytoplasm it can act as a repressor of autophagy^[8]. It is thus not surprising that DNA damaging agents and radiotherapy used for breast cancer treatment can induce autophagy in breast cancer cell lines^[79-81]. However, genetic inhibition of autophagy was not found to sensitize to cisplatin treatment and chloroquine treatment only mildly sensitized one mouse breast cancer cell line (67NR) but not another one (4T1)^[79]. Moreover, autophagy inhibition sensitized the MCF7^[81] but not the 4T1 cell line^[80] to radiation therapy. These different effects could be due to the p53 status (MCF-7 cells have wild type p53 and 4T1 cells are p53-null), possibly suggesting that autophagy inhibition together with DNA damaging agents should be used especially in those cancers with wild type p53 or with p53 mutants that maintain certain functions. So, despite numerous reports in the literature reporting a cytoprotective role of autophagy during DNA damaging agents in other types of cancer^[82], whether this is true for breast cancer and if this is dependent on the p53 status remains to be determined. This last statement is of particular importance since *p53* is frequently mutated in basal-like breast cancer, where the combination of *p53* mutations together with inferred pathway activity suggests that loss of p53 function occurs within most, if not all basal-like cancers^[46] and in which chemotherapy is most frequently used.

As mentioned above, triple negative breast cancers lack a molecular-directed therapy. However, recent studies have described molecular characteristics of the disease that are now targets of experimental agents, including poly (ADP-ribose) polymerase (PARP, for BRCA-associated tumors) inhibitors, angiogenesis blockers, EGFR inhibitors^[52,83] and endoplasmic reticulum stress inducers^[84]. With regards to endoplasmic reticulum stress, TNBC are known to be highly proliferative, aneuploid tumors^[48,50], conditions that are known to lead to proteotoxic stress in cancer cells and that could lead to the induction of autophagy. In this regard, autophagy inhibition seems to be a promising target since combination of autophagy inhibitors plus endoplasmic reticulum aggravators nelfinavir and celecoxib was synergistic in enhancing tumor cell killing particularly in TNBC cells^[84] and autophagy inhibition sensitized MCF7 cells to bortezomib treatment^[85]. Also, treatment with HDAC6 inhibitor panobinostat induced endoplasmic reticulum stress and autophagy in TNBC cells. Moreover,

chloroquine treatment synergized with panobinostat and induced cell death both *in vitro* and *in vivo*^[86].

Although most of the evidence described above suggests that autophagy should be inhibited in order to improve breast cancer therapy, some studies also suggest that autophagy could be involved in the cell's demise implying the opposite—that one should sometimes increase autophagy during breast cancer treatment. For example, Bcl-2 knockdown in MCF7 cells has been shown to induce autophagy and non-apoptotic cell death, which was decreased by knocking down ATG5, suggesting that autophagy could be involved in cell death in this model^[87]. These studies were performed in the MCF7 cell line, a widely used model for breast cancer and which is known to lack the executioner caspase-3 and thus have reduced susceptibility to apoptosis^[82]. Although this raises the caveat that pro-death effects of autophagy in this experimental model are due to its unusual deficiency in apoptosis, it is a relevant model for breast cancer since Bcl-2 is known to be overexpressed in breast cancers, resistance to apoptosis is thought to be an intrinsic property of many tumor cells and anti-apoptotic members of the Bcl-2 family have been found to be amplified in human cancers, including breast cancer^[88].

AUTOPHAGY AND BREAST CANCER STEM CELLS

According to the cancer stem cell hypothesis, tumorigenic cancer stem cells or tumor initiating cells (TICs) are those cells within a tumor that have self-renewal and tumorigenic capacities. They can “differentiate” into cancer cells with limited proliferative potential, creating a hierarchical organization within a tumor^[89]. Breast cancers are known to follow the cancer stem cell model, where tumors can be separated into tumorigenic (CD44⁺/CD24^{-low} or ALDH1⁺) and non-tumorigenic components, suggesting that only a minority of cells in a tumor can proliferate extensively and that some therapies that shrink tumors might not be curative because they fail to eliminate TICs. Breast cancer claudin-low tumors and cell lines have features of tumor-initiating cells since they express the same gene signature as the CD44⁺/CD24⁻ tumor fraction^[50]. Moreover, recent evidence suggests that TICs are also enriched in basal tumors and cell lines and their abundance has been associated with a worse overall survival^[90,91].

It is predicted that autophagy should be especially crucial for quality control mechanisms and maintenance of cellular homeostasis in stem cells due to their unique ability to self-renew and differentiate and relatively long life^[92]. Recent studies suggest that autophagy does in fact play a crucial role in the origin, maintenance and systemic distribution of TICs. The first paper linking autophagy to TICs described higher levels of LC3B and ATG5 in DCIS (ductal carcinoma *in situ*) derived tumorigenic spheroids when compared to epithelial cells in the same culture. Moreover, treatment of DCIS culture cells

with chloroquine suppressed xenografts tumor formation^[93]. The authors suggest that autophagy is activated in DCIS cells to persist and proliferate in the metabolically stressed intraductal space^[94]. Another recent paper reported that autophagy inhibition by ATG12, ATG8 knockdown or chloroquine treatment could decrease the number of CD44⁺/CD24⁻ cells in the MDAMB231 and JIMT1 breast cancer cell lines by increasing CD24 transcription^[95]. Finally, a recent study found increased autophagic flux in ALDH⁺ cells from mammospheres of the MCF7 cell line when compared to the bulk population of cells, increased beclin 1 expression in mammospheres when compared to adherent cells and decreased mammosphere size and formation by knockdown of beclin 1 as well as decreased tumor-forming ability in beclin 1 knockdown cells from mammosphere cultures of MCF7, SKBR3 and SK-3rd breast cancer cell lines^[96]. All these studies suggest that autophagy inhibition could preferentially target the TIC population in a tumor suggesting that combination of autophagy inhibition with other therapies would eliminate stem cells that survive the original treatment.

CONCLUSION

Research on autophagy and breast cancer treatment has dramatically increased in the past years. Despite being an area with many unanswered questions, recent discoveries in breast cancer biology and the autophagic pathway have increased our understanding on the implications and importance of trying to manipulate autophagy during breast cancer treatment. For example, it has been suggested that autophagy should be preferentially inhibited together with treatments that increase the tumor cell's dependency on it, like proteasomal inhibitors. Such combination treatment should probably be especially used to treat highly proliferative, aneuploid tumors with high proteotoxic stress, *e.g.*, basal-like tumors. On the other hand, autophagy inhibition could perhaps be avoided in combination with those therapies that themselves are known to negatively target the autophagic pathway, like microtubule-targeting agents. Therefore, autophagy manipulation will probably not be a generally applicable mechanism of sensitization to therapy in breast cancer and it will most likely depend on the type of breast cancer and on the treatment used. Moreover, evidence in the literature suggests that breast cancers (at least those with oncogenic events similar to the ones in the MMTV-PyMT or *Palb2*^{-/-} mouse models) display a certain degree of autophagy dependence and we recently found that many TNBC cell lines are particularly sensitive to autophagy inhibition when compared to luminal cells^[97], indicating that autophagy dependence in breast cancer may be subtype-dependent.

There are, however, some unresolved questions on autophagy and cancer that should be carefully addressed. For example, the fact that autophagy modulation can regulate the type of cell death in response to therapy is

an important thing to consider. In this regard, autophagy inhibition was shown to decrease necrosis and induce apoptosis in an MCF7 anti-estrogen resistant cell line in response to ICI 182780 treatment and Bcl-2/BCL2L2 co-inhibition^[64]. However, it has also been shown that although autophagy inhibition decreased survival in apoptosis deficient epithelial cells exposed to metabolic stress, it also changed the mode of cell death from apoptosis to necrosis, stimulating cytokine and chemokine production, macrophage infiltration and tumor growth *in vivo*^[34]. It has also been shown that autophagy induces ATP secretion during chemotherapy and that this is necessary for the establishment of a therapeutic immune response^[5]. Moreover, a recent study found that while autophagy inhibition sensitizes to radiotherapy both *in vitro* and *in vivo* in immune deficient mice, it increases tumor volume in an immune competent model *in vivo*. This work suggests that autophagy inhibition can decrease irradiation-induced release of ATP from tumor cells and impair an anti-tumoral immune response^[98]. This raises important questions about the immunogenicity of cell death and the possible final outcome in therapy when autophagy is inhibited-in a person, it may be better to directly kill slightly fewer tumor cells while allowing a robust anti-tumoral immune response than to obtain more direct tumor cell killing but losing the immune response. In breast cancer, autophagy has been shown to inhibit rather than stimulate the immune response (since autophagy inhibition was found to induce cytokine release) and autophagy deficient tumors had increased immune cell infiltration and decreased tumor growth and metastasis^[6]. The different effects might be related to the specific interplay of the immune system with cancer cells and to the type of therapy employed. Thus, if these effects are particular to the type of cancer (or the subtype of breast cancer) and the treatment used remains an important open question.

Finally, although most of the evidence in the literature suggests that autophagy should be inhibited in combination with breast cancer therapies, the fact that autophagy seems to be involved in cell death at least with some treatments should also be considered. If this effect is particular to a subtype of breast cancer, to specific mutations found in the tumor or to a certain treatment is something that needs to be studied further. Also, the fact that autophagy is involved in tumor suppression and that protective effects of autophagy have been described for diseases other than cancer (such as neurodegenerative, infectious diseases and ageing) raises concerns with regard to whether autophagy inhibition during cancer treatment could induce tumor formation in other tissues or promote other diseases in patients^[2]. Thus, a careful analysis of the full spectrum of effects of autophagy on cancer will be needed in order to successfully modulate it for therapeutic purposes. This may be a particular challenge for breast cancer therapy due to the high heterogeneity of this type of cancer but holds the opportunity for personalizing treatment protocols to maximize the benefit to breast cancer patients.

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Breast cancer-related lymphedema: Symptoms, diagnosis, risk reduction, and management

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Abstract

The global burden of breast cancer continues to increase largely because of the aging and growth of the world population. More than 1.38 million women worldwide were estimated to be diagnosed with breast cancer in 2008, accounting for 23% of all diagnosed cancers in women. Given that the 5-year survival rate for breast cancer is now 90%, experiencing breast cancer is ultimately about quality of life. Women treated for breast cancer are facing a life-time risk of developing lymphedema, a chronic condition that occurs in up to 40% of this population and negatively affects breast cancer survivors' quality of life. This review offers an insightful understanding of the condition by providing clinically relevant and evidence based knowledge regarding lymphedema symptoms, diagnosis, risk reduction, and management with the intent to inform health care professionals so that they might be better equipped to care for patients.

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Key words: Lymphedema; Breast cancer; Symptoms; Diagnosis; Risk reduction; Management

Core tip: Lymphedema is one of the most dreaded and

unfortunate outcomes of breast cancer treatment. Up to 40% of the women treated for breast cancer had lymphedema. Currently, there is no cure for this chronic condition. Even more distressing is that women who treated for breast cancer are facing a life-time risk of developing lymphedema. Lymphedema elicits daily stress and negative impact on breast cancer survivors' the quality of life. This paper offers an insightful understanding of the condition by providing clinically relevant and evidence based knowledge regarding lymphedema symptoms, diagnosis, risk reduction, and management with the intent to inform health care professionals so that they can be better equipped to care for patients.

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INTRODUCTION

The global burden of breast cancer continues to increase largely because of the aging and growth of the world population. More than 1.38 million women worldwide were estimated to be diagnosed with breast cancer in 2008, accounting for 23% of all diagnosed cancers in women^[1]. Given that the 5-year survival rate for breast cancer is now 90% and currently there are more than 2.9 million breast cancer survivors in the United States^[2], experiencing breast cancer is ultimately about quality of life. Women treated for breast cancer are facing a life-time risk of developing lymphedema, a chronic condition that occurs in up to 40% of this population^[3-6].

Breast cancer-related lymphedema results from obstruction or disruption of the lymphatic system associated with cancer treatment (removal of lymph nodes and radiotherapy); patient personal factors [obesity or

Table 1 Bivariate association between each symptom and lymphedema

Symptom	OR	95%CI	P-value
Arm swelling	561	76.04-71644.49	< 0.0001
Arm heaviness	17.46	8.22-39.25	< 0.0001
Arm firmness	10.33	5.04-22.16	< 0.0001
Increased arm temperature	9.07	2.98-29.94	0.000
Seroma formation	8.61	3.54-21.54	< 0.0001
Arm tightness	7.78	3.84-16.84	< 0.0001
Limited arm movement	5.86	2.94-11.93	< 0.0001
Tingling in affected arm	5.54	2.79-11.26	< 0.0001
Arm aching	5.14	2.60-10.46	< 0.0001
Limited fingers movement	4.56	1.92-10.66	0.0008
Limited elbow movement	4.39	1.53-12.21	0.0069
Limited wrist movement	4.23	1.58-10.99	0.0049
Limited shoulder movement	3.84	1.94-7.64	0.0001
Stiffness in the affected arm	3.55	1.75-7.16	0.0005
Burning in the affected arm	2.86	1.11-6.93	0.0299
Arm Redness	2.47	1.02-5.66	0.0450
Numbness in the affected arm	2.4	1.21-4.71	0.0124
Tenderness	2.07	1.06-4.03	0.0320
Pain in the affected arm	1.99	1.01-3.89	0.0463

Modified from the publication Ref.[23].

higher body mass index (BMI)] can increase the risk of lymphedema; and infections or trauma can trigger lymphedema^[4-6]. Lymphedema has elicited psychosocial problems that affect breast cancer survivors' daily lives^[7,8]. Significantly lower quality of life is observed in breast cancer survivors with lymphedema than in those without the condition^[9-12]. Management of lymphedema remains a major challenge for patients and health care professionals. Routine check-ups for lymphedema management, long-term physical therapy, management equipment (compression garments, bandages, special lotions), and repeated cellulitis, infections, and lymphangitis create financial and economic burdens not only to survivors but also to the health care system^[11]. Breast cancer survivors with lymphedema have significantly higher health care costs than those without it, they spend more days annually either hospitalized or visiting physicians' offices; they also have more days absent from work, which could adversely affect employment^[11,12]. Women treated for breast cancer often report being unaware that lymphedema was a possible outcome of cancer treatment and that health care professionals are not well informed and/or not helpful in guiding them on how to reduce the risk of lymphedema and manage this debilitating condition^[8,13]. The purpose of this paper is to offer an insightful understanding of the condition by providing clinically relevant and evidence based knowledge regarding lymphedema symptom, diagnosis, risk reduction, and management with the intent to inform health care professionals so that they might be better equipped to care for patients.

LYMPHEDEMA SYMPTOMS

Symptom assessment is essential since very often observable swelling and measurable volume changes are absent

during the initial development of lymphedema^[14-16]. Breast cancer survivors with lymphedema in the ipsilateral upper extremity report experiencing multiple symptoms, including swelling, heaviness, tightness, firmness, pain/aching/soreness, numbness, tingling, stiffness, limb fatigue, limb weakness, and impaired limb mobility of shoulder, arm, elbow, wrist, and fingers^[8,13-16]. These symptoms may be the earliest indicator of increasing interstitial pressure changes associated with lymphedema^[15,16]. As the fluid increases, the limb may become visibly swollen with an observable increase in limb size. Recent research shows that limb volume change has significantly increased as breast cancer survivors' reports of swelling, heaviness, tenderness, firmness, tightness, and aching have increased^[17]. Clinicians and researchers have long recognized that lymphedema symptoms may indicate an early stage of lymphedema in which changes cannot be detected by objective measures^[8,15]. The early stage of lymphedema may exist months or years before overt swelling occurs^[14-16].

Recent research demonstrates significant bivariate associations between each symptom and lymphedema^[16] (Table 1). A significant relationship exists between an increased number of symptoms and an increase in survivors' limb volume measured by infra-red perimeter^[17]. On average, breast cancer survivors reported 4.2 mean numbers of symptoms for survivors with < 5.0% limb volume change (LVC); 5.5 mean numbers of symptoms for 5.0%-9.9% LVC, 7.0 mean numbers of symptoms for 10.0-14.9% LVC, and 12.5 mean numbers of symptoms for \geq 15% LVC^[17]. A count of lymphedema symptoms is able to differentiate healthy adults from breast cancer survivors with lymphedema and those at risk for lymphedema^[16]. A diagnostic cutoff of three symptoms discriminated breast cancer survivors with lymphedema from healthy women with sensitivity of 94% and specificity of 97% [AUC (area under the curve) = 0.98]. A diagnostic cutoff of nine symptoms discriminated at-risk survivors and survivors with lymphedema with sensitivity of 64% and specificity of 80% (AUC = 0.72)^[16].

Since swelling is one of the key observable signs of lymphedema, objective measures are usually considered superior to symptom assessment or patient's perception of lymphedema. Perhaps, from the patient's perspective it is only the symptom experience and the perception of lymphedema that matter clinically because it is symptom experience and the perception of lymphedema that elicit tremendous distress and impair survivors' quality of life more than a measurement of inter-limb volume or girth size^[8,15]. In the absence of objective measurements capable of detecting early development of lymphedema, assessing symptoms may be a useful and cost-effective screening tool for detecting lymphedema.

DIAGNOSING BREAST CANCER-RELATED LYMPHEDEMA

Breast cancer-related lymphedema is a chronic syndrome

of abnormal swelling and multiple symptoms, resulting from abnormal accumulation of protein-rich lymph fluid in the interstitial tissue spaces due to an imbalance between lymph fluid production and transport^[13,14]. Because swelling is the cardinal sign of lymphedema, traditionally, lymphedema has been clinically diagnosed by health care professionals' observations of swelling and has often arbitrarily been defined in research as a 2 centimeters increase in limb girth, a 200-mL or more increase in limb volume, or a 5% or greater limb volume change^[17-19]. Inconsistency in the criteria defining lymphedema and the use of different measures has presented tremendous difficulty in diagnosing lymphedema. Breast cancer-related lymphedema can also occur in the shoulder, breast, and thoracic regions, unfortunately, no epidemiological studies have explored the incidence of lymphedema in the shoulder, breast, and thoracic regions due to lack of instruments to quantify swelling in these difficult-to-measure areas. Quantification of lymphedema by measuring limb size or girth or limb volume has been a major objective measure in research and clinical practice for diagnosing lymphedema using sequential circumference limb measurement, water displacement, and infra-red Perometry^[16]. Bioelectrical impedance is emerging as a possible alternative^[20-23]. Emerging assessment tool such as sonagraph needs more research to determine its reliability, sensitivity, and specificity.

Sequential circumferential arm measurements

Measuring limb size or girth or limb volume has been the most widely used diagnostic method in research. A flexible non-stretch tape measure for circumferences is usually used to assure consistent tension over soft tissue, muscle, and bony prominences^[19]. Measurements are done on both affected and non-affected limbs at the hand proximal to the metacarpals, wrist, and then every 4 or 10 centimeters from the wrist to axilla. The most common criterion for diagnosis has been a finding of ≥ 2 centimeters or ≥ 200 mL difference in limb volume as compared to the non-affected limb or 5% or 10% volume difference in the affected limb^[19].

Water displacement

Water displacement is seldom used in clinical settings because of spillover and hygienic concerns. Patients submerge the affected arm in a container filled with water and the overflow of water is caught in another container and weighed or measured. This method does not provide data about localization of the edema or shape of the extremity^[19,23]. The method is contraindicated in patients with open skin lesions. Patients may find it difficult to hold the position for the time needed for the tank overflow to drain^[19,23].

Infrared perometry

The infrared perometer is an optoelectronic device that works similarly to computer-assisted tomography, but makes use of light instead of X-rays^[19,23]. The volume and

shape of the limb can be measured and volume changes can be calculated. Perometry and circumference are reliable measurement of limb volume change over time in individuals undergoing breast cancer treatment^[19].

Bioelectrical impedance analysis

Bioelectrical impedance analysis (BIA) measures impedance and resistance of the extracellular fluid using a single frequency below 30 kHz^[20,21]. The device uses the impedance ratio values between the unaffected and affected limb to calculate a *Lymphedema Index*, termed as L-Dex ratio. A recent published study has demonstrated that the L-Dex ratio with a cutoff point of $> +7.1$ can discriminate between at-risk breast cancer survivors and those with lymphedema with 80% sensitivity and 90% specificity (AUC = 0.86)^[20]. In comparison, using the industrial recommended cutoff point of L-Dex $> +10$ can only identify 66% of true lymphedema cases among at-risk breast cancer survivors, that is, miss 34% of true lymphedema cases [AUC = 0.81 sensitivity = 0.66 (95%CI: 0.51-0.79)]. Since early treatment usually leads to better clinical outcomes, it is important to have higher sensitivity to avoid missing large number of true lymphedema cases. Since there are still about 20% of true lymphedema cases are missed by BIA with a cutoff point of $> +7.1$, it is critical for health care professionals to incorporate other assessment methods, including self-report, clinical observation, or perometry, to ensure the accurate detection of lymphedema^[20]. The BIA technique currently is not appropriate in assessing bilateral limb lymphedema.

LYMPHEDEMA RISK REDUCTION

Over 50% of breast cancer survivors were found to be exceptionally worried about their risk of developing lymphedema^[6]. Multiple factors may be associated with this fear, including symptom experience, type of cancer surgery, education level, earlier experiences, or the way that health care professionals educate and counsel survivors about risk reducing practices.

While lymphedema incidence has been reported less frequently in women who underwent sentinel lymph node biopsy only (SLNB), lymphedema has by no means become a minor or disappearing problem. A large number of women each year still face the life-time risk of developing this progressive and debilitating condition even with the most conservative estimates suggesting that 3% of women with sentinel lymph node biopsies and 20% of those who have axillary dissections develop lymphedema at 12 mo following breast cancer surgery^[6]. It is essential to note that surgical removal of lymph nodes and radiation remains the optimal choice for treating breast cancer with positive cancerous lymph nodes. As a result, current surgical approaches for diagnosis of and treatment for breast cancer continue to make patients with invasive cancer susceptible to the risk of lymphedema.

Risk factors that are directly related to breast cancer treatment may be mostly unavoidable for patients treated

for breast cancer, including breast surgery (lumpectomy and mastectomy), removal of lymph nodes (sentinel lymph node biopsy and axillary lymph node dissection), radiotherapy, or chemotherapy^[4-6]. There are also known risk factors that are not directly related to breast cancer treatment. These risk factors may actually be modified, such as obesity, weight gain after diagnosis, minor upper extremity infections, injury or trauma to the affected limb, or overuse of the limb^[4-6].

For decades, patient education has emphasized on precautionary lifestyle to avoid the modifiable risk factors. Breast cancer survivors are cautioned to avoid such activities as repetitive activity, lifting weighted objects, needle punctures, blood draw, as well as to use of compression garments for air travel^[24]. A recent systematic review evaluated the scientific evidence for current recommended risk reduction recommendations. The review concluded that some commonly practiced precautionary lifestyle recommendations were proved to be not true or “fiction”, such as avoid air travel/wear compression garment for air travel, avoid pressure, avoid extremes of temperature/apply sunscreen/avoid sun burn, avoid vigorous exercise; while precautionary recommendation of avoiding needle sticks/injection needs more research evidence. Only maintaining normal weight is an evidence based recommendation^[24]. Thus, to date, the insufficiency of high quality evidence is lacking to support these practices to reduce the risk of developing lymphedema and effective management of lymphedema.

Inflammation-infection and higher body mass index (BMI) are the main predictors of limb volume change and lymphedema besides treatment-related risk^[3-6]. Women who had previous inflammation-infection in the breast, chest, or arm were 3.8 times more likely to develop lymphedema^[5]; weight gain and obesity (BMI > 30 kg/m²) increases lymphedema risk: survivors with each increase of 1 kg/m² in their BMI were 1.11 times more at risk for developing lymphedema^[5,25].

Patient education focusing on risk reduction strategies is promising for lymphedema risk reduction. A recent study of 136 breast cancer survivors demonstrated patients who received lymphedema information reported significantly fewer symptoms and more frequent practice of risk reduction behaviors than those who did not^[26]. After controlling for confounding factors of treatment-related risk factors, patient education remains an important predictor of lymphedema outcome. While rigid prevention measures may promote fears and frustration, one essential risk reduction behavior under patient control is maintaining optimal body weight, because excess body weight is associated with decreased lymphatic function^[27-29].

Preventing infection and trauma that may trigger the onset of lymphedema is vital for lymphedema risk reduction^[5,27,28]. Infection is a significant risk factor and is the most frequently occurring complication of lymphedema^[5]. Risk increases with breaches in skin integrity. Daily skin care that maintain skin moisture and integrity

may be promising to preventing infection and trauma in the affected limb^[29,30]. Fluid accumulation can cause skin dryness and irritation, increasing the risk of cellulitis and skin infection. Water-based and low pH moisturizers are recommended to discourage infection^[30,31].

In the past, breast cancer survivors were cautioned to restrict physical exercises as a way to reduce their risk for lymphedema. A growing body of evidence suggests that exercises, including whole body exercises (walking, running), weight training, resistance training, do not necessarily increase lymphedema risk^[31,32]. Breast cancer survivors should be encouraged to perform all postoperative exercises, resume normal activities as tolerated, and be as fit as possible, while monitoring their affected limbs^[31]. In addition to a broad range of benefits, from weight control, physical fitness, positive emotion, and quality of life, physical exercise can promote lymph fluid drainage through large muscle movement. Survivors should be instructed to perform physical exercise according to the general exercise guidelines^[31,32] (Table 2).

To facilitate effective lymphedema risk reduction, health care professionals can assist patients by presenting or reinforcing risk reduction information. Emphasis on self-protection rather than rigid rules fosters patient empowerment^[26]. An empowered patient assumes responsibility for reminding health care professionals to avoid use of the affected arm rather than expecting health care professionals to remember to do so.

MANAGEMENT OF BREAST CANCER-RELATED LYMPHEDEMA

Once breast cancer-related lymphedema is established, there is no cure. Management of lymphedema focuses on swelling reduction and symptom alleviation while minimizing exacerbations of swelling. Treatments include pharmacological therapy, surgery, complete decongestive physiotherapy (CDT), mechanical pneumatic pumps, and infection prevention and treatment^[29-44]. Emerging treatment such as low-dose laser needs more research to determine its efficacy.

Pharmacological management of lymphedema uses benzopyrones, flavonoids, diuretics, hyaluronidase, pantothenic acid, and selenium^[35]. Poor quality of existing trials on pharmacological agents makes it impossible to draw conclusions about the effectiveness of pharmacological approach for lymphedema among breast cancer survivors^[35].

Surgical treatment for lymphedema includes microsurgical lymphovenous or lympholymphatic anastomoses, debulking, and liposuction^[34]. Surgical procedures aiming at enhancing lymphatic function by removing excess fluid or tissue in the affected area have been shown to be only marginally effective^[34]. Surgery does not cure lymphedema, use of compression is necessary after surgery^[34]. Potential complications may occur with surgical management, such as recurrence of swelling, poor wound healing, and infection; thus surgical treatment should only be

Table 2 General exercise guidelines for breast cancer survivors

	Suitability	
	Survivors at-risk for Lymphedema	Survivors with Lymphedema
(1) Initiate exercise at lower intensity gradually increasing intensity as tolerated, monitoring the affected limb for signs and symptoms of lymphedema	Yes	Yes
(2) Walking, swimming, cycling and low impact aerobics are recommended.	Yes	Yes
(3) Modify physical exercise to reduce the risk of trauma and injury. Exercise to the extent that the affected body part is not sore or fatigued	Yes	Yes
(4) Flexibility exercises should be performed to maintain range of movement	Yes	Yes
(5) Appropriate warming up and cooling down should be implemented as part of exercise regime	Yes	Yes
(6) Compression garments should be worn during exercise	Not established	Yes

considered when other treatments fail, and with careful consideration of the benefits to risks ratio.

Chronic lymphedema leads to formation of excess subcutaneous adipose tissue secondary to slow or absent lymph flow^[36]. Liposuction can help to remove excess fat tissue^[36-38]. Liposuction increases skin capillary blood flow without further damaging already compromised lymph transport capacity in breast cancer survivors with lymphedema^[36-38]. Patients are able to maintain limb size reduction with the use of compression garments after liposuction. Liposuction does not correct inadequate lymph drainage and is contradictory when pitting edema is present.

Complete decongestive therapy (CDT) is the standard care for lymphedema in the United States, but it is time-consuming, expensive, and requires lifelong maintenance. This approach includes manual lymph drainage, multi-layer, short-stretch compression bandaging, gentle exercise, meticulous skin care, education in lymphedema self-management, and elastic compression garments^[39-41]. In the treatment phase, patients generally receive 2-h treatments 5 d a week for 3 to 8 wk. Once treatment phase is completed, the patient continues self-management phase at home with skin care and exercise, self-massage, and use of a compressive sleeve and glove during the day and/or arm bandaging at night^[40,41]. Studies have shown long-term volume reduction as high as 50%-63% in up to 79% mean volume reduction of patients who are 100% adherent^[39,40]. Lifelong adherence to prescribed treatment regimen is required to prevent progression of disease. Adherence to the prescribed management routine can be difficult because even the most customized garments or sleeves sometimes are uncomfortable, unsightly, and laborious to put on^[39-41]. A constellation of complex factors (*e.g.*, physical, financial, aesthetic, time) can influence survivors' adherence with management routines. From the patient's perspective, the complete decongestive therapy itself is a constant reminder of cancer experience that prevents her from living a normal life^[8].

Mechanical pneumatic pumps use electricity to inflate a single-chamber or multi-chamber sleeve that produces external limb compression. A decreased tissue capillary filtration rate facilitates tissue fluid reduction and, consequently, limb volume decrease^[42]. Lymph formation decreases, but lymph transport is not improved. Pneumatic

pumps can reduce swelling, but concern exists regarding the way in which the rapid displacement of fluid in the other areas of the body. The use of pumps does not eliminate the need for compression garments and may not provide more benefit than garments alone^[42]. Using pumps may cause complications, including lymphatic congestion and injury proximal to the pump sleeve, and increased swelling adjacent to the pump cuff in up to 18% of patients^[42].

Infection prevention and treatment is another important aspect of lymphedema management. Infection is the most common lymphedema complication^[43]. Lymph stasis, decreased local immune response, tissue congestion, and accumulated proteins and other debris foster infection^[43]. Patients and health care professionals should be vigilant about any signs and symptoms of infection, such as fever, malaise, lethargy, and nausea. Prompt oral antibiotics are the first line of treatment for acute infection to prevent the need for intravenous therapy and hospitalization^[29-30,44]. Preventive antibiotics have been highly effective for patients who experience repeated serious infections or inflammatory episodes^[43,44]. Skin care optimizes the condition of the skin and prevents cellulitis and infection^[43,44]. Lymphedema can cause skin dryness and irritation, increasing the risk of cellulitis and skin infection. Water-based and low pH moisturizers are recommended to discourage infection^[31].

CONCLUSION

Lymphedema is one of the most important factors that elicit daily stress in breast cancer survivors since there is no cure for this condition^[6-7]. In addition, breast cancer survivors face a life-long risk of developing lymphedema since there is no defined period of time after cancer treatment when the risk no longer exists^[3]. To reduce the risk of lymphedema and maintain optimal lymphedema management, patient self-care is ultimately necessary to promote lymph drainage and prevent inflammation-infection. Optimal self-care typically includes adherence to risk reduction behaviors, optimal weight management, use of compression garments, exercises, healthy lifestyle practices, and seeking assistance for lymphedema-related problems. Health care professionals should focus on empowering patients with skills and knowledge that helps

patients to reduce lymphedema risk and achieve optimal management and risk reduction. Empowering patients for optimal self-care is a great impetus to long-term success of lymphedema risk reduction and management.

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WJCO 5th Anniversary Special Issues (2): Breast cancer**Mechanisms and therapeutic advances in the management of endocrine-resistant breast cancer**

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Abstract

The estrogen receptor (ER) pathway plays a critical role in breast cancer development and progression. Endocrine therapy targeting estrogen action is the most important systemic therapy for ER positive breast cancer. However its efficacy is limited by intrinsic and acquired resistance. Mechanisms responsible for endocrine resistance include deregulation of the ER pathway itself, including loss of ER expression, post-translational modification of ER, deregulation of ER co-activators; increased receptor tyrosine kinase signaling leading to activation of various intracellular pathways involved in signal transduction, proliferation and cell survival, including growth factor receptor tyrosine kinases human epidermal growth factor receptor-2, epidermal growth factor receptor, PI3K/AKT/mammalian target of rapamycin (mTOR), Mitogen activated kinase (MAPK)/ERK, fibroblast growth factor receptor, insulin-like growth factor-1 receptor; alterations in cell cycle and apoptotic machinery; Epigenetic modification

including dysregulation of DNA methylation, histone modification, and nucleosome remodeling; and altered expression of specific microRNAs. Functional genomics has helped us identify a catalog of genetic and epigenetic alterations that may be exploited as potential therapeutic targets and biomarkers of response. New treatment combinations targeting ER and such oncogenic signaling pathways which block the crosstalk between these pathways have been proven effective in preclinical models. Results of recent clinical studies suggest that subsets of patients benefit from the combination of inhibitor targeting certain oncogenic signaling pathway with endocrine therapy. Especially, inhibition of the mTOR signaling pathway, a key component implicated in mediating multiple signaling cascades, offers a promising approach to restore sensitivity to endocrine therapy in breast cancer. We systematically reviewed important publications cited in PubMed, recent abstracts from ASCO annual meetings and San Antonio Breast Cancer Symposium, and relevant trials registered at ClinicalTrials.gov. We present the molecular mechanisms contributing to endocrine resistance, in particular focusing on the biological rationale for the clinical development of novel targeted agents in endocrine resistant breast cancer. We summarize clinical trials utilizing novel strategies to overcome therapeutic resistance, highlighting the need to better identify the appropriate patients whose diseases are most likely to benefit from these specific strategies.

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Key words: Endocrine therapy; Endocrine resistance; Breast cancer; Therapeutic advances; Targeted therapy

Core tip: Endocrine therapy is the important systemic therapy for hormone receptor positive breast cancer. However, treatment resistance is common. Multiple mechanisms responsible for endocrine resistance have been identified over the past decade. New treatment

combinations targeting estrogen receptor and growth factor receptor signaling which block the crosstalk between these pathways are effective in preclinical models and clinical studies. In this review, we summarize the complex genomic and epigenetic regulatory pathways involved in endocrine resistance, in particular focusing on the clinical trials utilizing novel strategies to overcome therapeutic resistance.

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INTRODUCTION

Estrogen receptor (ER) is expressed in about 75% of human breast cancers which is the one of the leading cause of death for women globally. The estrogen-bound ER functions through ligand-activated transcriptional regulation (genomic actions) and by acting as a component of signaling cascades outside of the nucleus (non-genomic actions)^[1-4]. Clinical observations and laboratory studies suggest ER signaling pathway is the major driver in promoting proliferation, survival and invasion of ER-positive breast cancer cells^[5]. Endocrine therapy is the mainstay of treatment for patients with ER-positive breast cancer, especially those with metastatic disease. Endocrine therapies include treatments which target ER by blocking receptor binding with an antagonist or by depriving the tumor of estrogen. The three broad groups of currently approved anti-estrogen therapies are selective estrogen receptor modulators (SERMs) such as tamoxifen, raloxifene and toremifene, which block activity of ER; selective estrogen receptor down regulators (SERDs) such as fulvestrant, which induce destabilization and degradation of ER; and aromatase inhibitors (AIs), including steroidal/irreversible (anastrozole and letrozole) and nonsteroidal/reversible (exemestane) inhibitors, which decrease estrogen production in peripheral tissues and within the tumors through inhibition of the enzyme aromatase^[5-11]. Endocrine therapy as the first targeted therapy in cancer treatment has successfully improved outcome of millions of breast cancer patients in the past 30 years^[5,12].

There is evidence that some breast tumors are more resistant to endocrine therapy than others, despite expressing ER. This is supported by stratification of ER positive tumors into luminal A and luminal B subtypes based on molecular profiling studies over the last decade. The luminal B subtype is more aggressive and less endocrine sensitive, while the luminal A subtype is more indolent and endocrine responsive^[13-15]. Recently The Cancer Genome Atlas (TCGA) data reinforces that luminal B cancers represent a unique subtype of breast cancer, with a distinctive biology from that of luminal A cancers. Multigene tests performed on the primary breast

tumor are increasingly utilized in clinical practice to assist in adjuvant therapy decision making and to distinguish which patients might benefit most from a combination of endocrine therapy plus chemotherapy, rather than endocrine therapy alone. For example, the 21-gene (OncotypeDx) and 70-gene (MammaPrint) assays can classify ER positive tumors according to their aggressiveness, risk of recurrence, and likelihood of benefitting from adjuvant endocrine or chemotherapy. PAM50 is a 50 gene expression assay to separate breast tumor samples into known intrinsic molecular subtypes (basal-like, HER-2 enriched, luminal A and luminal B) and correlate with risk of relapse. The progesterone receptor (PR) is expressed in half of patients with ER+ breast tumors^[16]. Clinical studies have shown that ER+/PR+ tumors are more responsive to endocrine therapy than ER+/PR- tumors^[17]. Furthermore, down-regulation of PR correlates with high growth factor activity, indicating that loss of PR in ER positive breast tumors could serve as a predictor of endocrine therapy outcome^[16,17]. However, no biomarkers that predict resistance to endocrine therapy with certainty are available currently. Therefore most patients with ER positive breast cancers are treated with endocrine therapy, in adjuvant and/or metastatic setting. Tamoxifen is the treatment of choice in premenopausal patients. And aromatase inhibitors (*e.g.*, letrozole and anastrozole) have become the treatment of choice as first-line therapy in postmenopausal patients. On disease progression, second-line treatment options include other classes of AIs (steroidal or nonsteroidal) and the ER antagonists, fulvestrant and tamoxifen^[18]. But the effectiveness of endocrine therapy is limited by high rates of *de novo* or intrinsic resistance (existing before any treatment is given) and acquired resistance during treatment (resistance that develops during a given therapy after an initial period of response). One third of patients will have recurrent disease within 15 years after being treated with tamoxifen for 5 years^[11]. About 50% of patients with metastatic disease do not respond to initial endocrine treatment^[8]. Inevitably the vast majority of patients with ER-positive advanced breast cancer will become refractory to endocrine therapy.

A plethora of mechanisms have been proposed to explain resistance to endocrine therapy, including deregulation of various components of the ER pathway itself^[11,14,19], activation of escape pathways that provide tumors with alternative cell proliferative and survival stimuli^[20-24], alterations in cell cycle and apoptotic machinery^[3,25], modulation in epigenetics and microRNA profile^[1,4,6,26]. In this review, we summarize the key mechanisms that have been implicated in the development of endocrine resistance in breast cancer. We give an overview of the completed and ongoing clinical trials with novel agents targeting these alternative mechanisms, with the goal to overcome endocrine resistance in breast cancer.

LITERATURE SEARCH

PubMed was searched for articles in English published between January, 2000 to February, 2014 using the terms

“breast cancer”, “endocrine resistance”, as well as the individual terms of the molecular components under molecular mechanism listed in this Review. Reference lists from key articles were searched for additional material. Abstracts from the ASCO annual meetings and the San Antonio Breast Cancer Symposium were considered (2010-2012). ClinicalTrials.gov was searched for relevant trials. Articles were identified on the basis of the authors’ knowledge of the advances in endocrine resistant breast cancer research.

MOLECULAR MECHANISM OF ENDOCRINE RESISTANCE

De novo resistance in breast cancer is characterized by loss of ER (the ER α isoform) expression and ER gene mutations such as deletion and point mutation. Patients carrying inactive alleles of cytochrome P4502D6 (CYP2D6) deficiency cannot convert tamoxifen to its active metabolite, endoxifen, therefore are resistant to tamoxifen^[27]. By contrast, multiple mechanisms have been detected to account for the acquired resistance to endocrine therapies. Although it is beyond the focus of this review to summarize all of the known mechanism of endocrine resistance in breast cancer, we can focus on the molecular changes in some of the key pathways involved and their clinical implications (Figure 1).

DEREGULATION OF CLASSIC ESTROGEN SIGNALING

The classic function of ER is its nuclear function, also known as genomic activity, to regulate the expression of genes important for normal and cancer cell proliferation and survival^[3]. The nuclear estrogen receptors (ER α and ER β) have similar structure, consisting of a central DNA-binding domain flanked by two autonomous transcriptional activation domains. In classic estrogen signaling, ligand-bound ER activates gene expression-either through direct binding of dimeric ER to specific DNA response elements in complexes including co-activators, or function as a coregulator through protein-protein interactions with other transcription factors, such as activation protein 1 (Ap1), specificity protein 1 (Sp1) and nuclear factor (NF- κ B) to facilitate binding to serum response elements and activation of transcription^[28-30].

Mechanisms of endocrine resistance include the loss of ER α expression which occurs in 15%-20% of resistant breast cancers, ER α mutations which present in < 1% of ER-positive tumors, the expression of ER splicing variants, specifically the truncated variant ER α 36, and estrogen related receptors (ERR)^[11,14,31-33]. Deregulation of ER co-regulators has been implicated in endocrine resistance as well. For example, increased Ap1 and NF- κ B transcriptional activity are associated with endocrine resistance. Overexpression of nuclear

receptor co-activator 3 (nCOA3, also known as AIB1 or SRC3), detected in two-thirds of all breast cancers, has been implicated in clinical and experimental tamoxifen resistance^[3,21,34].

Post-translational modifications (phosphorylation, methylation and ubiquitination) of ER and its co-regulators are regulated to influence ER activity, interactions with other proteins including cytoplasmic signaling molecules^[21,35,36]. Aberrant regulations at this post-translational level contribute to endocrine resistance as well^[3].

ACTIVATION OF GROWTH FACTOR RECEPTOR PATHWAYS

The ER can also be activated by ligand independent fashion, as a consequence of signaling events downstream of membrane receptor tyrosine kinases (RTKs). RTKs are the intracellular portions of a class of growth factor receptors including HER2 (ERBB2), epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor (IGFR). The bidirectional crosstalk between the RTK signaling and ER pathways has been implicated in the development of resistance to endocrine therapy in preclinical studies. Many clinical trials have begun to test several attractive strategies, such as manipulation of growth factor signaling networks and the use of tyrosine kinase and multikinase inhibitors that may delay or even overcome the resistance of breast cancers to endocrine therapy.

HER2 pathway

HER2 (Human epidermal growth factor receptor 2/ERBB2) is a member of the HER receptor tyrosine kinase family, which plays an important role in promoting cell proliferation and malignant growth in breast cancer. Over-expression of HER2 occurs in approximately 30% of metastatic breast cancers (MBC) and is associated with aggressive disease course and poor outcome with reduced disease-free and overall survival rates. Both pre-clinical and clinical evidence suggested that HER2 over-expression confers resistance to anti-estrogen agents in ER positive tumors^[10]. Activation of the Her2 pathway, even without HER overexpression, confers tamoxifen resistance in ER positive cancer cells^[37]. Preclinical studies demonstrated that tamoxifen resistant cells have the ability to switch between HER2 and the ER pathway for cell growth and survival. Upregulation of HER2 signaling occurs in some tumors with disease progression during endocrine therapy. Recent studies show that HER2 gene expression is repressed by the PAX2-ER-tamoxifen complex in sensitive breast cancer cell lines; while in tamoxifen resistant cell lines, the ER coactivator AIB-1/SRC-3 competes with PAX2 for binding, leading to increased HER2 transcription^[38]. In addition, HER2 activation decreases ER level and increase ER phosphorylation, even in the absence of estrogen^[38-40]. HER2 signaling alters ER mediated transcription through disrupting the interac-

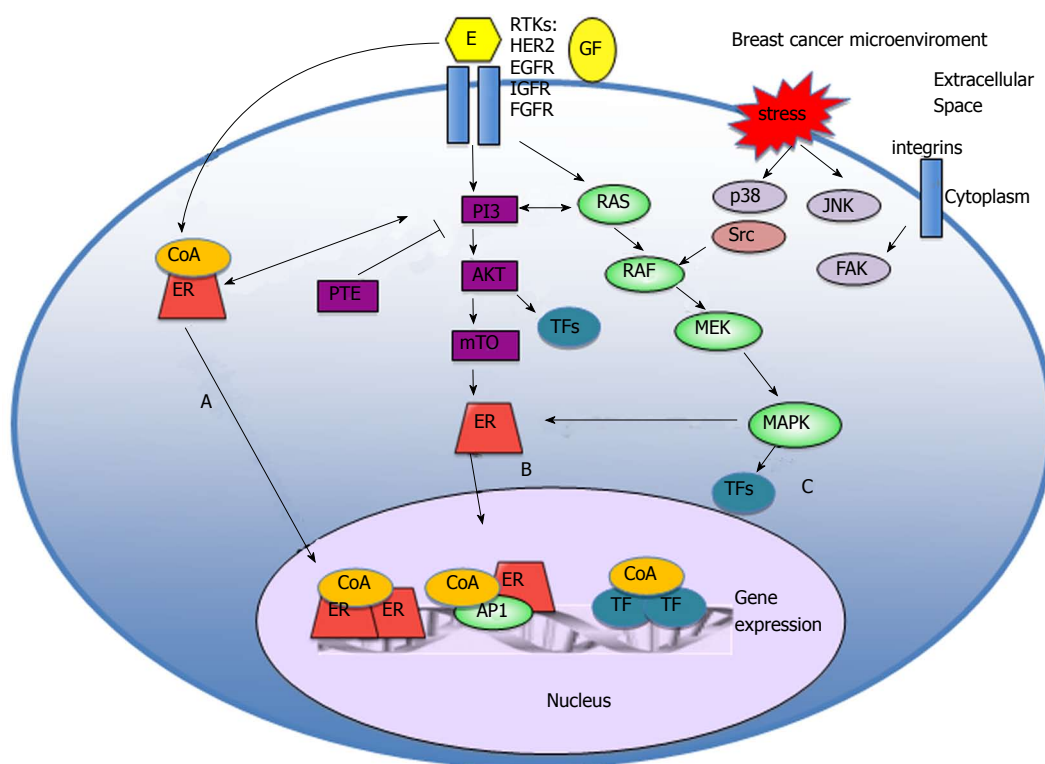


Figure 1 Estrogen receptor action at molecular level. A: Ligand dependent activation: in classic estrogen signaling, ligand-bound ER activates gene expression—either through direct binding of dimeric ER to specific DNA response elements in complexes including co-activators, or function as a coregulator through protein - protein interactions with other transcription factors to facilitate binding to serum response elements and activation of transcription; B: Ligand independent activation: the ER can also be activated by ligand independent fashion, as a consequence of signaling events downstream of membrane receptor tyrosine kinases (RTKs); C: Non-genomic mechanisms: signaling can be mediated through non-genomic mechanisms by ER that is localized at the cell membrane or in the cytoplasm. ER: Estrogen receptor; mTOR: Mammalian target of rapamycin; FGFR: Fibroblast growth factor receptor; IGF-1R: Insulin-like growth factor-1 receptor; EGFR: Epidermal growth factor receptor.

tion between ER and its coregulators (corepressors and coactivators). HER2 also activates downstream signaling pathways, such as the phosphoinositide 3-kinase (PI3K)/AKT pathway and mitogen activated kinase (MAPK) pathway, as discussed later^[3,15,19].

The interdependence of ER and HER2 pathways is highlighted by examples in which treatment with AIs or downregulation of ER with fulvestrant has inhibited the growth of HER2-positive tumors that had progressed with trastuzumab or lapatinib. In addition, HER2 inhibition with trastuzumab or lapatinib restores or upregulates ER levels or transcriptional activity in breast cancer cells^[24,41]. These data provide rationale for combined inhibition of ER and HER2 pathway, and clinical studies have demonstrated the benefit of targeting both the ER and HER2 in ER positive/HER2 positive breast cancer. In the phase III TAnDEM (Trastuzumab in Dual HER2 positive ER positive Metastatic Breast Cancer) trial, 207 postmenopausal women with HER2 positive ER positive MBC were randomized to anastrozole alone or anastrozole plus trastuzumab. The combination arm was clearly associated with a longer progression free survival (PFS) (4.8 mo *vs* 2.4 mo, $P = 0.0016$) and a higher clinical benefit rate (CBR) (42.7% *vs* 20.3%)^[42]. Similarly, in the randomized, double-blind phase III study of letrozole with or without lapatinib in MBC, PFS and clinical benefit

were superior in the combination arm compared with the AI-alone arm in 219 patients with ER positive/HER2 positive MBC^[43]. Both trials suggest that both HER2 and ER should be simultaneously targeted for maximal therapeutic efficacy.

EGFR pathway

Among the four HER family members (HER1-4), HER1 is better known as epidermal growth factor receptor (EGFR). Binding of EGF-related growth factors leads to receptor homo and/or heterodimerization (with HER2) and activation of downstream signaling cascades including PI3K/AKT and MARK pathways. In breast cancer, overexpression of EGFR and subsequently increased activity of MAPK and PI3K/AKT signaling pathways confer estrogen independency, resistance to endocrine therapy and poorer prognosis^[44-46]. For example, activation of ErbB3, EGFR and Erk is shown to be essential for growth of human breast cancer cell lines with acquired resistance to fulvestrant^[47]. In preclinical study, Gefitinib, a small molecule inhibitor of EGFR, effectively inhibited EGFR-HER2 heterodimerization, phosphorylation and downstream signaling in the tamoxifen resistant MCF-7 cell line^[48,49].

Lapatinib is a dual tyrosine kinase inhibitor blocking EGFR and HER2. In cell models of HER2 positive

breast cancer with acquired endocrine resistance, lapatinib restores hormone sensitivity^[50]. Johnston *et al.*^[51] reports in the randomized, double-blind phase III study, 1286 postmenopausal women with ER positive MBC, were randomized to receive letrozole with or without lapatinib. The benefit of combination therapy was observed in the ER positive, HER2 positive, but not in the ER positive, HER2 negative group. Letrozole plus lapatinib significantly increased PFS *vs* letrozole-placebo (8.2 mo *vs* 3.0 mo, HR = 0.71; 95%CI: 0.53-0.96; $P = 0.019$) in HER2 positive population^[46,51]. There was also a trend toward a prolonged PFS for the combination observed in patients who experienced relapse less than 6 mo since prior adjuvant tamoxifen discontinuation. These data suggest that there is benefit with the addition of an EGFR/HER2-targeted therapy to an AI in patients who experience relapse early during prior tamoxifen therapy which is consistent with preclinical models where EGFR activity is enhanced in association with endocrine resistance^[51].

Several selected EGFR inhibitors are being investigated as monotherapy or in combination with endocrine therapy in an attempt to overcome or prevent endocrine resistance. However, clinical trials that target EGFR in ER positive breast cancer have yielded mixed results. In the randomized placebo controlled phase II trial of tamoxifen with or without gefitinib, 290 patients were stratified into an endocrine naïve group who had not received endocrine therapy within one year prior to enrollment, and another group who had developed recurrence during or after AI therapy. PFS was not significantly prolonged in the endocrine naïve group (8.8 mo *vs* 10.9 mo, $P = 0.31$) or the group who had AI^[52]. Another small randomized placebo controlled phase II trial enrolled a total of 93 ER + metastatic breast cancer patients with or without prior endocrine therapy. In this study, combination of anastrozole with gefitinib showed a statistically significant increases in PFS compared to anastrozole plus placebo (14.7 mo *vs* 8.4 mo, HR = 0.55; 95%CI: 0.32-0.94). Similarly, subset analysis of PFS for patients who had received prior endocrine treatment compared with those who were endocrine therapy naïve showed a more pronounced benefit for patients that had not previously received endocrine therapy^[47]. These trials have suggested targeting EGFR could delay resistance to endocrine therapy in endocrine naïve patients.

Strategy of combined targeting the ER and EGFR was assessed in the neoadjuvant setting as well. Polychronis and colleague conducted the double-blind, placebo -controlled Phase II trial^[53]. 56 patients with ER and EGFR expressing breast cancer were randomized to receive gefitinib and placebo, or gefitinib plus anastrozole, for 4-6 wk prior to surgery. The combination arm showed a significant reduction in Ki67, which is the primary end point, than the monotherapy arm (5.6% difference, $P = 0.0054$). In contrast, Smith *et al.*^[54] reported a separate randomized phase II trial of neoadjuvant anastrozole alone or with gefitinib, in which 206 postmenopausal women with early stage ER positive breast

cancer were randomized to receive 16 wk of anastrozole monotherapy, 16 wk of anastrozole with 14 wk of gefitinib (preceded by two weeks of placebo) or 16 wk of gefitinib before surgery. There was no difference in proliferation index as measured by Ki67 for either gefitinib regimen when compared to anastrozole alone. Moreover, there was no difference in overall objective response (48% *vs* 61%, $P = 0.08$). The authors concluded that addition of gefitinib/EGFR inhibitor to neoadjuvant anastrozole did not improve clinical or biologic effect^[54]. The selection of EGFR overexpressing breast cancer cases in Polychronis *et al.*'s study might account for the difference in these trial results. One could postulate that the ideal setting for testing combination of endocrine therapy and EGFR inhibitors is in the patients with acquired resistance since it is associated with adaptive upregulation of growth factor receptor signaling. Further biomarker studies in patients who had prior endocrine therapy are clearly warranted to identify a phenotype that may predict relapse and subsequent benefit from combined endocrine therapy and EGFR inhibitors.

Mitogen activated kinase pathway

The mitogen activated kinase pathway (MAPK) pathway is stimulated by the RAF serine/threonine kinase, and signals to additional downstream cytoplasmic serine-threonine kinases that ultimately activate MAP kinases such as, ERKs, c-jun N-terminal kinases, and p38MAPKs with resultant downstream phosphorylation of transcription factors. As discussed earlier, the MAPK pathway is important in mediating HER2-and EGFR-induced endocrine resistance. In addition, studies show that ERK and p38 phosphorylate AIB1 and ER coactivators^[3,8]. Clinical trials targeting the MAPK pathway directly using MAPK inhibitors in combination with endocrine therapy are ongoing. Results on the randomized phase II trial, fulvestrant with or without AZD6244 (selumetinib, a MAPK Inhibitor) in advanced stage breast cancer progressing after aromatase inhibitor are awaited (NCT01160718).

The PI3K-AKT- mammalian target of rapamycin pathway

The PI3K-AKT (a serine/threonine kinase) pathway plays a central role in cell survival, proliferation and angiogenesis and is frequently deregulated in cancer^[45]. Phosphatidylinositol 3-kinase (PI3K) consists of a regulatory subunit (p85) and a catalytic subunit (p110). PI3K is activated by growth factor RTKs and G-protein-coupled receptors (GPCRs). PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to produce phosphatidylinositol 3,4,5- trisphosphate (PIP3). In turn, PIP3 recruits several adaptor proteins such as phosphatidylinositol-dependent kinase 1 (PDK1) and AKT (a serine/threonine kinase), which when activated, drive cell proliferation and survival. Through dephosphorylation of PIP3 and PIP2 respectively, PTEN and INPP4B provide negative regulation of this pathway. AKT activates the mammalian target of rapamycin (mTOR) -containing complex 1 (mTORC1), which regulates protein synthe-

sis^[25]. Activating mutations or genetic amplification of PI3K catalytic subunit, amplification of downstream targets such as *Akt*, amplification of upstream receptors such as *erbB2/HER2* and loss of negative regulators such as PTEN have all been described in breast cancer^[55-57]. The Cancer Genome Atlas (TCGA) analysis confirms the high mutation frequency of *PIK3CA* in luminal/ER-positive breast cancer. *PIK3CA* somatic mutation is present in approximately 32% of luminal B subgroup, 49% of luminal A, 42% of HER2-enriched, and only 7% of basal-like breast cancer. Within the same pathway, PTEN mutation/loss and *INPP4B* loss were observed in more luminal B (24%, 16% each) than luminal A subtype (13% and 9% respectively)^[14,58]. The PI3K-AKT pathway is widely viewed as an important therapeutic target and PI3K pathway inhibitors are being studied in clinical trials.

Preclinical studies have associated PI3K pathway activation with *de novo* and acquired resistance to endocrine therapy. Increased phosphorylation of mTOR substrates and AKT is observed in estradiol deprived breast cancer cell lines. Oncogene overexpression that activate PI3K/AKT signaling (*e.g.*, HER2, type 1 insulin-like growth factor receptor (IGF1R), activated mutant AKT1) and RNAi-mediated knockdown of PTEN lead to resistance to tamoxifen, fulvestrant, and estrogen deprivation in ER-positive breast cancer cells. Studies using long-term estrogen-deprived (LTED) ER-positive breast cancer cell lines have shown that endocrine resistance develops concomitantly with amplification of PI3K/AKT/mTOR signaling^[59]. Similar changes have been observed with chronic exposure of MCF-7 cells and xenografts to fulvestrant^[23].

Moreover, inhibition of PI3K has reversed antiestrogen resistance in experimental models. For example, treatment with the PI3K/mTOR inhibitor BEZ235 or the mTOR inhibitor everolimus prevents the growth of LTED cell lines in the absence of estrogen^[60]. Everolimus in combination with tamoxifen had an additive anti-tumor effect in breast cancer cells *in vitro*^[61]. In another study, the combination of temsirolimus with an ER antagonist synergistically inhibited the growth of breast cancer cells *in vitro* and growth in a xenograft model of breast cancer (mTOR)^[62]. In a separate study, high levels of AKT activity conferred resistance to letrozole and fulvestrant through alteration of the cell cycle and apoptotic response in an *in vitro* breast cancer cell model^[63]. Treatment with everolimus plus either letrozole or fulvestrant restored responsiveness in the resistant cells and results in synergistic inhibition of the proliferation and induction of apoptosis^[60].

These preclinical studies indicated the promise of drugs targeting PI3K network (PI3K, AKT, mTOR) in ER positive breast cancer resistant to endocrine therapy. Table 1 summarizes the randomized trials in which inhibitors of PI3K pathway have been combined with endocrine therapy. Neoadjuvant treatment with letrozole and the mTOR inhibitor everolimus more effectively

reduced tumor cell proliferation and improved clinical response compared with letrozole alone in patients with early-stage ER-positive breast cancer^[64]. Two studies (BOLERO-2 and TAMRAD trials) have demonstrated superior benefit of mTOR inhibition in combination with endocrine therapy in advanced resistant ER positive breast cancers. In the phase III randomized BOLERO-2 trial, 724 patients with ER positive metastatic breast cancer (MBC) who had recurrence or progression while receiving previous therapy with a nonsteroidal aromatase inhibitor (either letrozole or anastrozole) were randomly assigned to everolimus and exemestane *vs* exemestane and placebo. The median PFS was significantly longer in the combination arm (10.6 mo *vs* 4.1 mo, HR 0.36; 95%CI: 0.27-0.47; $P < 0.001$, according to central assessment)^[65]. The combination of exemestane and everolimus has been approved for ER positive advanced breast cancer in United States and Europe based on the magnitude of these positive results. TAMRAD is a randomized phase II trial of tamoxifen with or without everolimus in patients with aromatase inhibitor (AI)-resistant metastatic breast cancer. Patients in the combination arm showed an improved clinical benefit rate (61% *vs* 42%), time to progression (8.6 mo *vs* 4.5 mo), and overall survival compared with patients receiving tamoxifen alone. Notably patients with acquired endocrine resistance (relapse > 6 mo after AI treatment) derived the greatest benefit from the combination compared with those with primary resistance (relapse during adjuvant AI or within 6 mo of AI treatment in the metastatic setting) with an improvement in the median PFS of 12.4 mo *vs* 1.5 mo, respectively^[66].

In contrast, Wolff *et al*^[67] examined letrozole with or without temsirolimus as first line therapy for patients with ER positive MBC who had no prior endocrine therapy for advanced disease in a randomized phase III trial. The study was terminated early due to lack of efficacy in the combination arm. Differences in results between the temsirolimus trial and the everolimus trials are likely attributable to different dosing schedules and pharmacokinetics, as well as different patient populations. It is possible that by selecting the more resistant cases, the TAMRAD and BOLERO-2 trials were enhanced with breast cancers that are likely to be driven by PI3K-mTOR signaling. Studies to identify predictive biomarker that could be used to select patients who would likely benefit from the combined mTOR and ER targeting approach are needed. In addition to mTOR inhibitors, drugs targeting other components of the PI3K pathway are in clinical development. Furthermore, isozyme-specific PI3K inhibitors have been developed in the hope of increasing therapeutic benefit while decreasing toxicity. Pan-PI3K inhibitors BKM120 and XL-147, dual PI3K/mTOR inhibitors BEZ235 and XL-765, and AKT inhibitor MK2206 have entered phase I, or phase I / II trials in combination of endocrine therapy.

Hedgehog signaling

The hedgehog (Hh) signaling pathway is highly conserved

Table 1 Clinical trials of targeted agents in endocrine resistant breast cancer

Agent	Class	Type of study	Study design	Patient population	Status/Results	Ref.
Targeting receptor tyrosine kinases signaling pathway						
PI3K/AKT/mTOR						
Everolimus	mTOR inhibitor	Phase III randomized	Exemestane +/- everolimus	ER+/HER2- LABC/MBC pts failed previous therapy with a nonsteroidal AI	PFS: 10.6 vs 4.1 mo, HR 0.36; $P < 0.001$, favoring combination arm	[76]
Everolimus	mTOR inhibitor	Phase II randomized	Tamoxifen +/- everolimus	ER+/HER2- MBC pts after previous therapy with AI	CBR: 61% vs 42%; TTP: 8.5 vs 4.5 mo, $P = 0.008$, favoring combination arm	[77]
Temsirolimus	mTOR inhibitor	Phase III randomized	Letrozole +/- temsirolimus	First line therapy for patients with ER positive MBC	No difference in CBR, terminated early	[78]
Everolimus	mTOR inhibitor	Phase II randomized	Letrozole +/- everolimus	Neoadjuvant therapy in ER + breast cancer	RR (by U/S): 58% vs 47%; $P = 0.035$, favoring combination arm	[75]
Sirolimus	mTOR inhibitor	Phase I / II	Tamoxifen +/- sirolimus	Pts with ER+ MBC	$N = 400$, TAM + SIR: 193; TAM alone: 207, ORR: TAM + SIR 40%; TAM alone 4%; Time to progression: TAM + SIR: 11 mo TAM alone: 3 mo	Bhattacharyya <i>et al</i> Eur.J.Cancer 47, Abstract 16LBA (2011)
Bkm120	Pan-PI3K inhibitor	Phase III randomized	Fulvestrant + BMK120	ER+/HER2- LABC/MBC Postmenopausal pts, AI Treated, Progressed on or After mtor Inhibitor		NCT01633060
Bkm120	Pan-PI3K inhibitor	Phase I b	Fulvestrant + BMK120	Postmenopausal pts with ER+ MBC	Ongoing, to determine the maximum tolerated dose of BKM120	NCT01339442
Bez235	Dual PI3K-mTOR inhibitor	Phase I b	Letrozole + BEZ235	Postmenopausal pts with ER+ MBC		NCT01248494
BMK120 or BEZ235	Pan-PI3K inhibitor	Phase I b	Letrozole + BMK120 or BEZ235	Postmenopausal pts with ER+ MBC		NCT01248494
XL147 or XL765	Pan-PI3K inhibitors/ dual PI3K/mTOR inhibitor	Phase I / II	Letrozole + XL147 or XL765	ER+/HER2- MBC pts refractory to a previous AI therapy		NCT01082068
GDC-0941 or GDC-0980	dual PI3K/ mTOR inhibitor	Phase II randomized	Fulvestrant + GDC-0941 or GDC-0980	Part I : ER+/HER2- postmenopausal LABC/ MBC refractory to AI; part II : criteria plus pik3 camutation		NCT01437566
Gdc-0032	PI3K inhibitor	Phase I / II	GDC-0032 + fulvestrant	ER+/HER2- LABC/MBC Postmenopausal pts		NCT01296555
Byl719	PI3K- α inhibitor	Phase I	BYL719 + letrozole or exemestane	ER+/HER2- LABC/MBC pts		NCT01870505
Mk2206	AKT inhibitor	Phase I	Endocrine therapy + MK2206	Postmenopausal pts with ER+ MBC		NCT01344031
Mk2206	AKT inhibitor	Phase II	MK2206 monotherapy	LABC/LRBC/MBC with pik3ca mutation or AKT mutation or PTEN loss		NCT01277757
Azd5363	AKT inhibitor	Phase I / II	Paclitaxel +/- AZD5363	Parta: all MBC, partb: ER+ MBC, stratified by PIK3CA mutation		NCT01625286
Igf-1r Amg 479	IGF1R mAB	Phase II randomized	Addition of AMG 479 to either exemestane or fulvestrant	MBC or LABC pts who had progressed on prior endocrine therapy	No statistically significant difference in PFS (PFS: 3.9 vs 5.7 mo, favoring placebo arm, $P = 0.44$), OS or CBT between two arms	[87]
Bms-754807	dual IGF-1R/ insulin receptor kinase inhibitor	Phase II randomized	BMS-754807 +/- letrozole	MBC or LA BC pts who had progressed on prior nonsteroidal AI		NCT01225172
Dalotuzumab (MK-0646)	IGF1R mAB	Phase I / II	MK-0646 and fulvestrant and dasatinib	ER+/HER2- MBC pts without prior therapy in metastatic setting		NCT00903006

Cixutumumab	IGF1R mAB	Phase I / II	Cixutumumab and temsirolimus	MBC or LA BC pts progressed on one to two chemotherapy		NCT00699491
Ridaforolimus (mk-8669) with dalotuzumab (mk-0646)	mTOR inhibitor and IGF-1R mAB		Ridaforolimus and dalotuzumab <i>vs</i> standard care	Er + bc		NCT01234857
Fgf Dovitinib (TKI258)	TKI inhibits FGFR1-3, VEGFR and PDGFR	Phase II Phase I / II	Dovitinib monotherapy, stratified by FGF amplification	4 groups of MBC pts: (group 1: FGFR1+, HR+), (group 2: FGFR1+, HR-) (group 3: FGFR1-, HR+), (group 4: FGFR1-, HR-)	Dovitinib has activity in breast cancers with amplified FGF pathway	[94]
Dovitinib (TKI258)	TKI inhibits FGFR1-3, VEGFR and PDGFR		Dovitinib(TKI258) + AI	ER+/HER2- postmenopausal MBC resistant to AI with fgfr1 amplification status confirmed		NCT01484041
Dovitinib (TKI258)	TKI inhibits FGFR1-3, VEGFR and PDGFR	Phase II randomized	Fulvestrant +/- Dovitinib, stratified by FGF	Postmenopausal pts with HER2-/HR+ LA BC or MBC progressing within 12 mos of completion of adjuvant endocrine therapy or after \leq 1 prior endocrine therapy in the advanced setting		NCT01528345
Azd4547		Phase II	amplification	HER2-MBC with fgfr1 amplification		NCT01795768
Azd4547		Phase II	Fulvestrant +/- AZD4547	ER+ postmenopausal LABC or MBC with fgfr1 polysomy or gene amplification resistant to endocrine treatment (Adjuvant or First-line Metastatic)		NCT01202591
Targeting cell cycle regulators Pd 0332991	CDK4/6 inhibitor	Phase I / II randomized	Letrozole +/- PD 0332991	First line therapy for postmenopausal pts with ER+/HER2- MBC		[99]
Pd-0332991 (palbociclib)	CDK4/6 inhibitor	Phase III randomized	Letrozole +/- PD 0332991	First line therapy for postmenopausal pts with ER+/HER2- MBC		NCT01740427
Lee011		Phase I b/ II	LEE011 + exemestane +/-everolimus	Postmenopausal pts with ER+/HER2- LABC/MBC		NCT01857193
Epigenetic therapy Vorinostat	HDAC inhibitor	Phase II	Vorinostat + tamoxifen	ER+ MBC progressed on previous endocrine therapy	N = 43; 34 evaluable, 7 (21%) PR; 4 (29%) SD; ORR 19%, CBR 40%	[105]
Entinostat	HDAC inhibitor	phase II randomized	Exmestane +/- entinostat	MBC or LA BC pts who had progressed on prior nonsteroidal AI	N = 130; PFS: 4.3 <i>vs</i> 2.3 mo (HR 0.73, 95%CI: 0.50 to 1.07; P = 0.06); OS: 28.1 <i>vs</i> 19.8 mo (HR 0.59, CI, 0.36 to 0.97; P = .036), favoring combination	[106]
Panobinostat	HDAC inhibitor	Phase I / II	Panobinostat + letrozole	MBC, triple negative phase II portion		NCT01105312
Vorinostat	HDAC inhibitor	phase II	Vorinostat + AI	ER + MBC pts who previously derived benefit from AI		NCT01153672
Vorinostat		phase II	Vorinostat/placebo + nab-paclitaxel + carboplatin (n = 62)	Primary operable breast cancer, triple-negative or high grade ER-positive, HER2-negative	Ongoing	NCT00616967

MBC: Metastatic BC; LABC: Locally advanced BC; mAB: monoclonal antibody; ORR: Objective response rate; CBR: Clinical benefit rate response or stable disease >24 wk; PR: Partial response; SD: Stable disease; TKI: Tyrosine Kinase Inhibitor; PI3K/AKT/mTOR: PI3K-AKT- mammalian target of rapamycin (mTOR) pathway; IGF1R: Insulin-like growth factor-1 receptor pathway; FGF: Fibroblast growth factor. signaling.

and plays a critical role in embryonic development. The Hh pathway has been increasingly recognized as playing

a crucial role in carcinogenesis in the last decade. Three mammalian Hh ligands have been identified in humans, as denoted by the prefixes Sonic, Indian, and Desert (SHH, IHH, and DHH). They activate the Hh signaling pathway by binding to the cell surface receptor Patched (PTCH), which otherwise represses the activity of the transmembrane receptor like protein Smoothened (SMO). Release of SMO from PTCH-mediated repression subsequently leads to the modulation of GLI (glioma-associated oncogene homolog) transcription factors. There are three mammalian GLI proteins, GLI1, GLI2 and GLI3. GLI1 is a transcriptional activator; GLI2 can either activate or repress gene expression; GLI3 acts as a transcriptional repressor. Aberrant activation of the Hh pathway has been reported in several malignancies including breast cancer^[68,69].

Traditionally, four major mechanisms have been proposed account for aberrant activation of the Hh pathway: (1) Hh ligand-independent mechanism - Loss of function mutations in PTCH or gain of function mutations in SMO lead to constitutive activation of this pathway; (2) Autocrine signaling- tumor cells produce Hh ligand to activate the Hh signaling; (3) Paracrine signaling - Hh ligand produced by tumor cell stimulates stromal and endothelial cells that produce growth factors supporting tumor growth and survival; and (4) Reverse paracrine signaling-Hh ligand produced by stromal cells support tumor growth and survival. Upon the pathway activation, the GLI transcription factors activate or inhibit transcription by binding to their responsive genes and interacting with the transcriptional complex. A ligand-dependent autocrine model of activating the Hh signaling has been described in breast cancer^[69,70].

We recently show noncanonical Hh signaling as an alternative growth promoting mechanism that is activated in tamoxifen-resistant breast tumors. Importantly PI3K/AKT pathway plays a critical role in regulating Hh signaling by protecting key components of this pathway from proteasomal degradation. We showed that activation of Hh signaling correlated inversely with disease-free and overall survival in a cohort of 315 patients with breast cancer with poor disease outcome. Furthermore, we observed that among ER positive, node-positive patients, Hh activation in the primary tumors was an independent prognostic factor for worse disease-free survival. Add treatment of tamoxifen-resistant xenografts with anti-Hh compound GDC-0449 blocked tumor growth in mice. These promising preclinical results describe a signaling event linking PI3K/AKT pathway with Hh signaling that promotes endocrine resistance^[71]. Targeting Hh pathway alone or in combination with PI3K/AKT pathway could therefore be a novel therapeutic option in treating endocrine resistant breast cancer. We are currently planning a phase I / II clinical trial using GDC-0449 (vismodegib), an oral compound approved for the management advanced basal cell carcinomas in patients with ER positive MBC that are resistant to endocrine therapy. Interestingly, Hh signalling has been shown to condition the bone mi-

croenvironment for osteolytic metastasis of breast cancer^[45], therefore Hedgehog inhibitors are candidate drugs for the treatment of patients with bone metastases which is the most common site of metastasis in ER positive breast cancer.

Insulin-like growth factor-1 receptor pathway

Studies have shown that ligand activation of Insulin-like growth factor-1 receptor (IGF-1R) and its downstream pathways stimulate tumor growth by inhibition of apoptosis and promotion of transformation, metastasis and angiogenesis^[72]. IGF-1R is expressed in 90% to 95% of breast cancer and is often co-expressed with ER^[73]. The crosstalk between IGF-1R and ER pathway is critical for the development of IGF-1R -mediated endocrine resistance in breast cancer. For example, estrogen activates IGF1R pathway through genomic and nongenomic mechanism. IGF-1R plays a direct role in ER phosphorylation. In addition, activation of IGF-1R signaling is associated with loss of PR expression, which itself is associated with high proliferative ER positive breast cancer^[74]. IGF1R overexpression also renders resistance to tamoxifen and fulvestrant through activation of MAPK and PI3K pathway.

Multiple agents interrupting the IGF-1 signaling pathway are developed and tested in clinical trials. AMG 479, a humanized monoclonal antibody antagonist of IGF1R, is tested with exemestane or fulvestrant in postmenopausal women with ER positive locally advanced or metastatic breast cancer who had disease progression on prior endocrine therapy in a randomized phase II trial. No statistically significant difference in PFS (PFS: 3.9 mo *vs* 5.7 mo, favoring placebo arm, $P = 0.44$), OS or CBT between two arms in this study^[75]. Ongoing trials with IGF-1R inhibitors are listed in Table 1. Correlative studies of these trials will be critical to determine whether there is a benefit adding IGF-1R inhibition to anti-estrogen therapy in patient cases with aggressive features, such as increased proliferation.

Fibroblast growth factor signaling

Fibroblast growth factor receptor (FGFR) signaling system includes at least 18 FGF ligands and four transmembrane tyrosine kinase FGF receptors, and it is involved in cancer cell proliferation, migration, angiogenesis, and survival^[76]. Multiple studies indicate that deregulated FGFRs can function as driving oncogenes stimulating tumorigenesis in a variety of human malignancies in addition to its role as an escape mechanism of anti-VEGF (vascular endothelial growth factor) therapies^[76,77]. A variety of FGFR pathway alterations have been identified in cancer and include activating mutations; chromosomal translocations resulting in expression of FGFR-fusion proteins with constitutive FGFR kinase activity; aberrant splicing of *FGFR* and isoform switching which substantially alter ligand specificity; gene amplifications or receptor overexpression through post-transcriptional regulation. Subsequently, aberrant activation of downstream path-

ways results in mitogenic and antiapoptotic responses in cells^[78,79].

FGFR family members are frequently overexpressed in breast cancer^[28]. *FGFR1* is the most commonly amplified genes following *erb2/HER2* in breast cancer, present in about in 8%-15% of all breast cancer^[14,76]. Large series have shown that *FGFR1* amplification is associated with high proliferation as assessed by Ki-67 immunostaining, drives resistance to endocrine therapy and is an independent predictive factor of poor prognosis^[22].

Preclinical models of breast cancer cells with amplification of *FGFR1* or *FGFR2* have demonstrated sensitivity to inhibition of FGFR^[80]. Several antibodies and small molecule inhibitors of FGFR are currently in early-phase clinical trials. Dovitinib (TKI258) is a first generation oral tyrosine kinase inhibitor (TKI) which inhibits FGFR1-3, VEGFR and platelet-derived growth factor receptor (PDGFR). Dovitinib inhibits proliferation in *FGFR1*- and *FGFR2*- amplified, but not *FGFR*-normal, breast cancer cell lines. Dovitinib monotherapy was evaluated in the phase II trial selecting patients on the basis of hormone receptor (HR) status and *FGFR1* amplification status. The mean reduction in target lesions was 21.1% in patients with FGF pathway-amplified breast cancer based on qPCR assay, compared with a 12.0% increase in target lesions in patients who did not present with FGF pathway-amplified breast cancer. Therefore, preliminary results suggest Dovitinib has antitumor activity in advanced breast cancer with FGF pathway alterations and warrants further investigation^[81].

CELL CYCLE SIGNALING AND APOPTOSIS

Experimental model data and clinical correlations indicate anti-estrogen treatment leads to a G₁ phase-specific cell cycle arrest and reduction in growth rate. Several molecular consequences that result in apoptosis have been documented. Aberrant regulation of positive and negative regulators of the cell cycle has been shown to interrupt and inhibit the antiproliferative effects of endocrine therapy, leading to treatment resistance^[3]. For example, overexpression of the positive regulators MYC, cyclins E1 and D1 cause endocrine resistance either by activating cyclin-dependent kinases critical for G₁ phase or by relieving the inhibitory effects of the negative cell cycle regulators p21 and p27^[3,74]. Importantly, expression and activity of these negative cell cycle regulators are down-regulated by multiple growth factor receptors and their downstream signaling pathways by modulating specific transcription factors, microRNAs, or by interfering protein phosphorylation. Moreover, increased expression of anti-apoptotic molecules such as BCL-2 and BCL-XL and decreased expression of pro-apoptotic molecules such as BAK, BIK and caspase 9 lead to endocrine resistance as well^[82]. Of note, activation of growth factor receptor signaling *via* the PI3K/AKT pathway is critical modulators of many apoptotic/survival molecules^[83]. Cyclin D1 is a

well-studied ER target gene that is required for estrogen-induced cell proliferation. Cyclin D1 binds to and activates cell cycle-dependent protein kinases four and six (CDK4/6) essential for mediating RB-induced cell cycle progression at the G₁/S checkpoint^[53,74]. Cyclin D1 amplification and overexpression was a common oncogenic event in breast cancer and preferentially occurred within luminal tumors, and more specifically within luminal B subtype. In the Cancer Genome Atlas (TCGA) network studies, Cyclin D1 is amplified in 58% of luminal B breast cancers with CDK4 gain in 25% of this subtype. In comparison, only 29% of luminal A tumors has Cyclin D1 amplification with 14% has CDK4 gain^[14]. Furthermore, Wang *et al*^[84] report that the alternatively spliced message, cyclin D1b, is aberrantly regulated in response to therapeutic challenge and promotes resistance to estrogen antagonists. Recently, Thangavel *et al*^[85] noted that a unique gene signature indicative of RB protein loss of function could identify luminal B breast cancers most likely to be resistant to endocrine therapies. Therefore targeting cyclin D1 and its downstream mediators of ER action CDK4/6 may provide a viable strategy to treat endocrine resistant breast cancers.

A phase I / II clinical trial testing the efficacy of letrozole with or without PD-0332991 (an oral CDK4/6 inhibitor) was conducted as first-line treatment of ER-positive advanced breast cancer (NCT00721409). This trial excluded patients who have previously been treated for advanced breast cancer. Thus the patient population is not determined to be endocrine resistant. The preliminary results were very impressive and showed significant prolongation of median PFS with the combination when compared to letrozole alone (26.2 mo *vs* 7.5 mo; HR = 0.32, 95%CI: 0.19-0.56, *P* < 0.001)^[86]. The result of the randomized, multicenter, double-blind phase III study of palbociclib (PD-0332991), plus letrozole *vs* placebo plus letrozole for postmenopausal women with ER positive, HER2 negative MBC who have not received any prior systemic treatment for advanced disease is awaited (NCT01740427)^[87]. Trials using other CDK inhibitors (Novartis) are also underway.

EPIGENETICS AND ENDOCRINE RESISTANCE

Epigenetics is defined as reversible changes in gene expression without change in the DNA sequence. DNA methylation is mediated by the action of DNA methyltransferases (DNMTs). DNMTs directly interact with histone deacetylases (HDACs) and the methyl-CpG-binding domain (MBD) family of proteins at the promoter regions to form a repressive transcription complex. DNA methylation, histone modification, and nucleosome remodeling are the major epigenetic changes that are dysregulated in breast cancer. Several genes involved in proliferation, anti-apoptosis, invasion, and metastasis have been shown to undergo epigenetic changes in breast cancer^[88,89].

There is increasing evidence that epigenetic modification plays a potential role in the development of endocrine resistance in breast cancer. The epigenetic regulation of ER is mediated through the recruitment of multi-molecular complexes containing HDAC1, DNMT1 and other co-repressors to the promoter region. Methylation of the gene encoding ER- α is one of the mechanisms of loss of ER expression in ER negative breast cancer cell. The epigenetic silencing of ER target genes is crucial to the development of ER independent growth and endocrine treatment resistance. A number of preclinical studies have shown that epigenetic therapy can impact expression of ER. For example, inhibition of DNMTs in ER negative breast cancer cells leads to induction of ER expression^[90,91]. HDAC inhibitors can restore ER expression, either alone *via* chromatin remodeling or in combination with DNMT inhibitors^[89]. The TCGA study highlights the finding that breast cancer molecular subtypes harbor specific patterns of epigenetic hardwiring and further demonstrates luminal B is a distinct subtype from luminal A not only based on the mRNA-based assay but also at the methylation and protein levels^[14]. Five DNA methylation groups were identified from 802 patient samples. Interestingly, the hypermethylated group 3 was significantly related to Luminal B subtype. Comparison between DNA methylation status and mRNA expression profile of group 3 with other groups led to identification of over 4000 differentially methylated genes and almost 2000 differentially expressed genes^[14]. Collectively, these data provide basis for the biological rationale for combining endocrine therapy with epigenetic-targeted therapies.

A phase II study of vorinostat, a HDAC inhibitor, in combination with tamoxifen was conducted in MBC patients who had progressed on previous lines of hormone therapy^[92]. The overall response rate was 19% and CBR was 40% (defined as Complete Response, Partial Response or Stable Disease of > 6 mo in duration) in 43 patients treated. The results from the randomized double blind phase II study of exemestane with or without entinostat, a benzamide HDAC inhibitor, are promising for reversal of AI endocrine therapy resistance. 130 postmenopausal women with locally recurrent or metastatic ER-positive breast cancer progressing on treatment with a nonsteroidal AI were enrolled. In this study, PFS was 4.3 mo *vs* 2.3 mo (HR 0.73, 95%CI: 0.50-1.07, *P* = 0.055) and OS was 28.1 mo *vs* 19.8 mo (HR = 0.59, 95%CI: 0.36-0.97) for the group receiving combination therapy *vs*. exemestane alone^[93]. Trials combining letrozole and panobinostat, vorinostat and AI therapy in metastatic breast cancer, vorinostat and tamoxifen in early stage breast cancer, are ongoing. Based on the higher frequency of methylation observed in Luminal B tumors, it is possible that luminal B breast cancers may represent a better target for epigenetic therapy than other subtypes.

MICRO RNA

Micro RNA (miRNAs) is a class of small noncoding,

single-stranded, highly conserved RNAs (19-25 nucleotides) involved in essentially all aspects of physiological and pathological cellular processes, such as development, proliferation, differentiation and apoptosis. MiRNA can either cleave mature mRNA molecules or inhibit their translation through base-pairing within the 3'-UTR of protein coding genes. Research over the past decade has demonstrated that about one third of human genes appear to be targeted by miRNAs and each miRNA is thought to regulate multiple genes. Interestingly, specific miRNA signatures have been associated with different molecular subtypes of breast cancer. In the Cancer Genome Atlas network analysis, 7 breast cancer subtypes were identified on the basis of MiRNAs expression and correlated with molecular subgroups^[14]. We have explored the potential role of specific miRNAs in endocrine resistance, especially resistance to tamoxifen, in breast cancer. Studies from our and other groups showed miR-221, miR-222 and miR-181b are up-regulated, whereas miR-21, miR-342 and miRNA-489 are downregulated in the tamoxifen-resistant cells. Multiple mechanisms of these miRNAs in conferring resistance to tamoxifen have been published. Mir-221 and -222 target the cell cycle inhibitor, p27/Kip1 through posttranslational modification and sequestration of p27 protein, or through miRNA-mediated suppression. Mir-221 and -222 overexpression is known to suppress ER α expression at protein level which leads to tamoxifen resistance in ER positive breast cancer^[19]. We recently reported that TIMP3, a tissue metalloproteinase inhibitor, is down-regulated by miR-221, -222 and -181b. We showed miRNA-mediated regulation of TIMP3 level and inhibition of metalloproteases contribute to tamoxifen resistance in cell culture models, mouse xenograft models, as well as in primary breast tumors. Direct injection of antago miRNA-221/222 to tamoxifen resistant xenografts in mice caused decrease in miRNA-221/222 level and restoration sensitivity to tamoxifen^[94]. Other groups subsequently reported up-regulation of miR-221 and -222 is implicated in resistance to fulvestrant as well^[32].

Investigation during the last decade demonstrate emerging regulatory role of miRNAs in endocrine resistant breast cancer. Future studies evaluating miRNAs as prognostic and predictive markers, as well as novel therapeutic targets to overcome resistance are warranted.

CONCLUSION

Recent progress in the field of endocrine therapy has produced a significant number of active compounds. Patients with ER-positive advanced breast cancer are treated with different endocrine agents serially at tumor progression, often resulting in long periods of disease control with no significant toxicity. Inevitably, however, vast majority of patients will become refractory to endocrine therapy. Therefore resistance to endocrine therapy continues to be a subject of great importance. In this review, we have summarized the complex genomic and epigen-

etic regulatory pathways involved in endocrine resistance. A combination of ER-targeted and HER2- targeted therapies is our current standard-of-care therapy in ER positive, HER2 positive breast cancer. Early results from clinical trials suggest that subsets of patients may benefit from a combination of inhibitor targeting certain growth factor pathway with endocrine therapy. The combination of exemestane and mTOR inhibitor everolimus has been approved for ER positive advanced breast cancer in USA and Europe based on the magnitude of positive results in two randomized phase III trials. The use of epigenetic therapy or miRNA/antimiRNA-based therapy with existing endocrine therapy in breast cancer is a topic of active interest.

Many challenges still remain as we try to identify the subsets of patients most likely to benefit from these novel targeted agents. Efforts should be directed at defining biological markers that could predict the efficacy of a specific agent. The use of genome-wide approaches in detecting gene alterations that drive resistance to endocrine therapy will hopefully promote personalized cancer medicine in management of endocrine resistance breast cancer. Clearly, future clinical trials with prospective patient selection based on predictive biomarkers are needed.

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Diagnosis and surgical management of breast cancer metastatic to the spine

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Abstract

Breast cancer is the most common malignancy and the second leading cause of death in Western women. Breast cancer most commonly metastasizes to the bone and has a particular affinity with the spine, accounting for 2/3 of osseous metastases discovered. With significant improvements in cancer therapies, the number of patients at risk for symptomatic spinal metastases is likely to increase. Patients may suffer from intractable pain and neurological dysfunction, negatively influencing their quality of life. Timely diagnosis of patients is crucial and has been aided by several breakthrough advances in imaging techniques which aid in detection, staging, and follow-up of bone metastases. Breast metastases are usually responsive to hormonal therapy and pharmacologic interventions, but skeletal metastases often require surgical intervention. The treatments are palliative but goals include the preserving or restoring neurologic function, ensuring spinal stability, and relieving pain. Advances in surgical techniques and instrumentation have allowed more effective decompression

and stabilization of the spine, and with the support of recent evidence the trend has shifted towards using more advanced surgical options in appropriately selected patients. In this review, the clinical presentation, diagnosis, patient selection, and surgical management of breast cancer metastatic to the spine are discussed.

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Key words: Breast cancer; Spine; Metastasis; Surgery; Outcomes; Decompression

Core tip: Breast cancer most commonly metastasizes to the bone and has a particular affinity for the spine. The treatment for symptomatic spinal metastases remains palliative and is not intended to prolong survival. Surgical advances in the last few decades have allowed improved spinal cord decompression and tumor resection. With the support of recent literature, the trend has shifted towards using more advanced surgical options in appropriately selected patients. Goals of treatment include restoration of and preservation of neurological function, maintaining spinal stability, and pain relief in an effort to achieve a better quality of life.

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INTRODUCTION

Breast cancer is the most commonly diagnosed malignancy and is the second leading cause of cancer-related death in the western world^[1]. The incidence of breast cancer has continued to rise in the last several decades. In

the United States alone, there will be an estimated 230000 new cases of invasive breast cancer diagnosed in 2013^[2]. Fortunately, the prognosis of breast cancer has improved following advances in pharmacologic and surgical techniques in controlling regional disease. As a consequence, the prevalence of patients with a history of breast cancer is increasing as the rate of survival improves, making breast cancer in many aspects a chronic condition. According to recent statistics from the American Cancer Society, over 2.9 million United States women with a history of breast cancer are alive, highlighting the large and increasing population at risk for long-term complications of breast cancer^[3].

While medical advances have prolonged survival for patients with breast cancer, metastatic progression involving distal sites such as bone, lung, liver, and brain remains common^[4]. The bone is the most common site of metastasis, with osseous metastases developing in 8% of all patients with breast cancer and 69% of patients with advanced disease^[5]. Consequences of bony metastases include pathologic fracture, spinal cord compression, anemia, and hypercalcemia^[6]. Breast cancer has a particular affinity for the spine, accounting for approximately two-thirds of the osseous metastases discovered^[7]. Of these lesions to the spine about one-third become symptomatic, causing intractable pain, neurological deficits, mechanical instability, and ultimately disability and a severe deterioration in quality of life^[8,9]. Breast cancer metastases constitute the most common cause of symptomatic spine metastases, accounting for 9%-40% of reported clinical series of spinal epidural metastases in the literature^[10].

The management of patients with symptomatic spinal metastases from breast cancer is often complex and requires a multidisciplinary approach^[11]. The optimum treatment algorithm has not been definitively defined and varies per patient, with available options including pharmacologic management, radiotherapy, and surgery. Early management of metastatic spinal tumors traditionally emphasized treatment with radiotherapy over surgical decompression. Still, surgery continues to play a critical role in the treatment of metastatic spinal tumors. Advances in surgical techniques and instrumentation have allowed more effective decompression and stabilization of the spine, and with the support of recent evidence the trend has shifted towards using more advanced surgical options in appropriately selected patients^[11]. However, while management strategies are continually evolving and physicians now have the capability to treat more aggressively, all therapies for spinal metastases unfortunately remain palliative. This review will detail the presentation, diagnosis, and surgical management options for patients with symptomatic breast cancer metastatic to the spine.

TUMOR CHARACTERISTICS

Multiple malignancies such as breast, prostate, kidney, and lung show a remarkable affinity to metastasize to bone^[4]. Metastatic lesions can spread to the bone *via*

several mechanisms, but the method of dissemination most likely responsible for breast cancer involves hematogenous seeding *via* venous routes^[12]. Spread may be accomplished through the Batson plexus, a network of veins that connects the vertebral veins with other beds of venous drainage. This importantly includes the azygos vein, which receives blood draining from the breast *via* the intercostal veins. The venous plexus of Batson lacks valves to control the flow of blood, so changes in pressure within the body can lead to variable flow through the plexus, allowing retrograde or antegrade seeding of tumor cells^[13]. The exact mechanism of metastatic seeding of the bone is unclear. There exists a prominent disparity between the abundance of circulating tumor cells and the relative rarity of metastatic seeding, suggesting a complex environmental barrier to metastasis^[14]. Nevertheless, when metastatic events occur in the spine they most commonly occur within the vertebral body with or without extension into the posterior elements^[15].

The majority of bone lesions caused by breast cancer is generally accepted to be osteolytic^[6]. In reality, breast cancer metastases can cause osteolytic, osteoblastic, or most commonly mixed osteoblastic and osteolytic lesions in the bone^[6]. Studies show that resident metastatic breast cancer cells secrete a multitude of osteolytic factors that directly and indirectly activate osteoclasts^[15]. Indirect stimulation is mediated by up-regulation of RANK-RANKL signaling, either by osteoblast-mediated osteoclastogenesis or *via* stimulation of host immune cells by factors such as PTHrP^[16]. In addition, there is evidence to suggest that bone breakdown releases previously deposited growth factors and cytokines within the matrix. This has a proliferative effect on the metastatic cells, thus creating a vicious cycle of bone resorption^[17].

Thus, the overall local reaction caused by metastatic breast cells is predominantly increased osteolysis. On the other hand, there is often an osteoblastic component due to three mechanisms. The first is tumor cells may influence other cells in its microenvironment such as stromal cells, which can differentiate into osteoblasts^[16]. Alternatively, metastatic cells may simply secrete factors which directly stimulate osteoblast proliferation and bone formation^[15]. Finally, there is commonly a local bone response to increased bone lysis as a natural response to injury^[6]. Thus the resultant lesion may be quite variable, with these factors on a molecular level dictating the degree of overall bone distortion by the infiltrating metastatic tumor.

CLINICAL MANIFESTATIONS

Pain is the most common symptom and is the presenting complaint in nearly 90% of patients with spinal metastases from breast cancer^[18]. Pain symptoms vary in intensity but may be vague and nonspecific, and patients with metastatic spinal cord compression have been found to have a delay in diagnosis of about 2 mo from first presenting to a physician to the time of diagnosis^[19]. As

the neurological status of the patient at time of diagnosis correlates strongly to the patient's prognosis, a diagnosis before the onset of neurological compromise is essential^[11,20]. Accordingly, any patient with a known history of malignancy who presents with new-onset back or neck pain should be promptly and thoroughly evaluated with a high suspicion for metastatic disease involving the spine. Common degenerative disorders less commonly affect the thoracic spine than the cervical or lumbar spine, hence pain in the thoracic spine warrants a high clinical suspicion for metastatic disease^[11]. Likewise, patients with persistent nonmechanical pain should have a low threshold for evaluation of a neoplastic etiology^[21].

Eliciting the type of spinal pain is important as one may receive clues to the etiology, location, and severity of the tumor infiltration. The pain may be biological, radicular, or mechanical pain. Biological, or local, pain is commonly described as a persistent deep "aching" unrelated to activity that is worse at night^[22]. The mechanism is thought to be caused from local periosteal stretching from either tumor growth or tumor-induced inflammatory process^[11]. Percussion over the spinous process may elicit local tenderness^[13]. This type of pain usually responds to corticosteroids, anti-inflammatory medications, and tumoricidal treatments^[22]. Radicular pain results from tumor infiltration causing compression or irritation of individual nerve roots. This pain classically presents in a dermatomal distribution as a band-like, burning, or shooting pain. Effective treatments for radicular pain include surgical nerve root decompression, conventional or stereotactic radiotherapy, or even neuroleptic medications such as gabapentin and pregabalin^[22]. Finally, mechanical back pain results from spinal instability caused by the predominantly osteolytic metastatic breast lesion. This pain is aggravated with movement, activity, or activities that require axial loading of the spine, such as sitting or standing^[13]. Mechanical pain is important to recognize as patients generally cannot find relief with pharmacologic or radiation therapy, and often require bracing or surgical stabilization^[13].

Neurologic dysfunction is a feared consequence and is the second most common presenting symptom of metastatic cancer to the spine^[22]. Symptoms are caused by direct tumor growth or pathological fracture that leads to compression of the spinal cord, individual nerve roots, or the cauda equina. First, epidural spinal cord compression may lead to varying degrees of motor weakness, gait instability, autonomic dysfunction, and diminished sensation below the level of injury^[22]. Bladder dysfunction is the most common autonomic finding and commonly correlates well with the degree of motor impairment^[11]. Manifestations of myelopathy may be noted on detailed physical exam, such as hyperreflexia, clonus, or positive Hoffman or Babinski signs. Second, compression of individual nerve roots can cause radicular pain, as mentioned above, as well as weakness or paresthesia in the associated muscle groups^[22]. Thirdly, compression of the cauda equina may manifest as the characteristic constellation of

symptoms including back pain, saddle anesthesia, bladder and bowel dysfunction, and lower extremity weakness. Importantly, patients who present with motor weakness may have a variable rate of neurologic deterioration, but in the absence of intervention usually progress to complete paralysis in the absence of any treatment^[11].

IMAGING MODALITIES

The available imaging modalities to evaluate for suspected breast cancer metastatic to the spine include plain radiographs, skeletal scintigraphy (SS) (bone scan), computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography CT (PET-CT), and single photon emission CT (SPECT). These modalities carry varying sensitivities, degrees of information, and costs in evaluating for spinal metastases. Given the ability of metastatic breast cancer to present as osteoblastic, osteolytic, or mixed lesions in the bone, each imaging technique may play a valuable role in the evaluation of the at-risk patient depending on the clinical situation.

Plain radiographs are commonly the initial imaging study ordered in evaluating a patient presenting with back pain or neurological symptoms. Given its low cost and widespread availability, plain radiographs are a useful screening test to assess for lytic or sclerotic metastatic lesions, large masses, and pathological fractures. This imaging modality may detect the rare osteoblastic breast metastasis, but this would be more fruitful in a more osteoblastic lesion such as prostate cancer. Regarding osteolytic lesions, which is the predominant radiographic presentation of metastatic breast cancer, bone metastases only become visible on plain radiographs after 30%-50% of bone mineral loss has occurred^[23]. Thus, plain radiographs are fairly insensitive and does not provide a definitive diagnosis.

Skeletal scintigraphy (bone scan) is highly sensitive in the detection of osseous metastases, provides images of the entire skeleton, and has been suggested as the first imaging study in asymptomatic patients^[24]. This technique detects regions of remodeling within the skeletal system. Osteolytic lesions are accompanied by secondary formation of bone, which allows osteolytic bone metastases to be detected with skeletal scintigraphy several months before they appear on plain radiographs^[6]. However, remodeling may also be a result of inflammation, infection, or fractures^[22]. Thus, SS has limited specificity and findings need to be correlated by further imaging studies.

CT has become the preferred imaging modality for evaluation of the osseous structures of the spine. The ability of CT scanners to distinguish among materials of different densities allows it to show superior skeletal detail, including bone marrow^[25]. Using the bone window setting, CT can be useful in determining the extent of tumor extension, osteoblastic vs osteolytic lesions, and assessing spinal stability^[22]. CT is particularly better than plain radiographs and SS for evaluation of lesions in the spine, as these studies do not visualize the spine in suf-

ficient anatomic detail^[25]. CT also plays an essential role in preoperative planning and postoperative monitoring. CT myelography is a useful tool in imaging patients with prior spinal instrumentation, as the instrumentation artifact makes visualization with MRI difficult^[22].

MRI is the gold-standard diagnostic modality in the imaging of metastatic spinal tumors^[13]. This modality has superior sensitivity compared to standard radiographs, CT, and bone scans due to its superior resolution of soft-tissue structures of the spine. MRI is able to define important preoperative parameters such as the extent of epidural extension, degree of spinal cord compression, surrounding edema, and spinal root impingement. Further, evaluation of neighboring structures such as the ligaments, paraspinal muscles, and joints are able to be evaluated. Gadolinium contrast provides additional definition of soft-tissue infiltration and information regarding its vascularity^[26]. T1 and T2 weighted studies and fat suppression studies are frequently ordered.

PET is a nuclear medicine technique that detects cellular metabolism of a glucose analog, most commonly fluorodeoxyglucose^[24]. PET scans have limited resolutions, but fusion with CT (PET-CT) allows more precise localization of radiotracer uptake. While this modality is more sensitive and specific than SS, its role is controversial due to its increased radiation dose and high cost^[27]. Currently it is only recommended if plain radiography, CT, SS, and MRI do not provide adequate information for diagnosis or treatment planning^[24].

SPECT is similar to SS but acquires images in a cross-sectional fashion instead of a planar fashion^[24]. SPECT shows a greater sensitivity and specificity for detecting spinal metastases^[28]. Additionally, SPECT is able to differentiate tumors from inflammatory and infectious lesions, unlike SS^[22]. Modern scanners now allow SPECT, diagnostic-quality CT, and fused SPECT-CT images to be done within 1 h on the same machine, which further improves the diagnostic accuracy of the modality^[29].

Metastatic involvement of the spine can lead to emergent situations, such as spinal cord compression or pathologic fracture. In these patients, diagnosis in a timely manner is critical as the fragility of the spinal cord and nerve roots mandates urgent intervention. Failure to do so may result in vascular damage and spinal cord “stroke”, leading to irreversible neurological defect. Plain radiographs may be an initial study of choice to evaluate for compression fractures and overall spinal stability. An emergent MRI, which carries excellent soft-tissue contrast, is indicated in order to evaluate for the extent of tumor progression or retropulsed fracture fragments. A T1-weighted, fat saturated sequence after IV gadolinium contrast is optimum for imaging of spinal metastases as intramedullary and osseous lesions are best seen with this sequence^[24]. CT (using bone windows) can be helpful for preoperative assessment of extent of bone destruction and mechanical instability.

MANAGEMENT

The management of patients with metastatic breast can-

cer to the spine is complex and frequently requires a multidisciplinary approach, involving numerous medical specialties (oncology, radiation oncology, pain management, rehabilitation medicine), surgery subspecialties (neurosurgery, orthopedics, surgical oncology), as well as radiologists and interventional radiologists. Advances in the past few decades have improved the treatment of both systemic disease as well as localized tumor burden to the spine. Cytotoxic agents remain the mainstay of treatment of patients with breast cancer. Hormone therapies such as selective estrogen receptor modulators and aromatase inhibitors have been shown to be effective against breast cancer, and human epidermal growth factor receptor 2 (HER2) targeting agents have also been effective in treating metastatic patients^[30,31]. Moreover, bisphosphonates given with vitamin D and calcium, which inhibit tumor-related osteoclast activity, and corticosteroids, which may have oncolytic effects on breast cancer and decrease peritumor edema, give physicians an ever-growing array of tools to combat this disease. However, the treatment for symptomatic spinal metastases remains palliative and is not intended to prolong survival. The goals of treatment include restoration of and preservation of neurological function, maintaining spinal stability, and pain relief in an effort to achieve a better quality of life.

Treatment modality and tumor evaluation

A proposed algorithm for the treatment of patients with symptomatic spinal metastasis from breast cancer is shown in Figure 1. Treatment decision-making can be further aided by considering neurologic, oncologic, mechanical, and systemic parameters (NOMS)^[32,33]. Neurologic evaluation incorporates the degree of epidural tumor extension, as the presence of neurological deficits usually correlate with high-grade tumor extension. The Weinstein-Boriani-Biagini staging system was developed for primary spinal tumors in order to describe tumor involvement of vertebral body and adjacent tissues^[13]. Briefly, the vertebral body in the axial plane is divided into 12 sectors and 5 tissue layers, which allows a reliable inter-physician description of tumor involvement. More recently, the Spine Oncology Study Group (SOSG) validated a 6-point scale devised specifically for metastatic spinal tumors^[34]. Metastatic tumors are graded on a 3 point scale depending on tumor infiltration, with grade 0 representing no epidural extension and grade 3 representing spinal cord compression without CSF fluid around the cord. Grade 2 and 3 tumors are highly likely to result in neurologic deficits and generally require surgical decompression.

The oncologic parameter takes into account specific features of the primary tumor histology. When compared to other metastatic spinal tumors, patients with breast cancer have a relatively long life expectancy^[10,35]. Patients with breast cancer are also at higher risk for vertebral compression fractures because of age, osteoporosis, and the osteolytic nature of the tumor^[36]. Patients with breast cancer are at increased risk of losing ambulation as compared with patients with other primary histologies^[37,38].

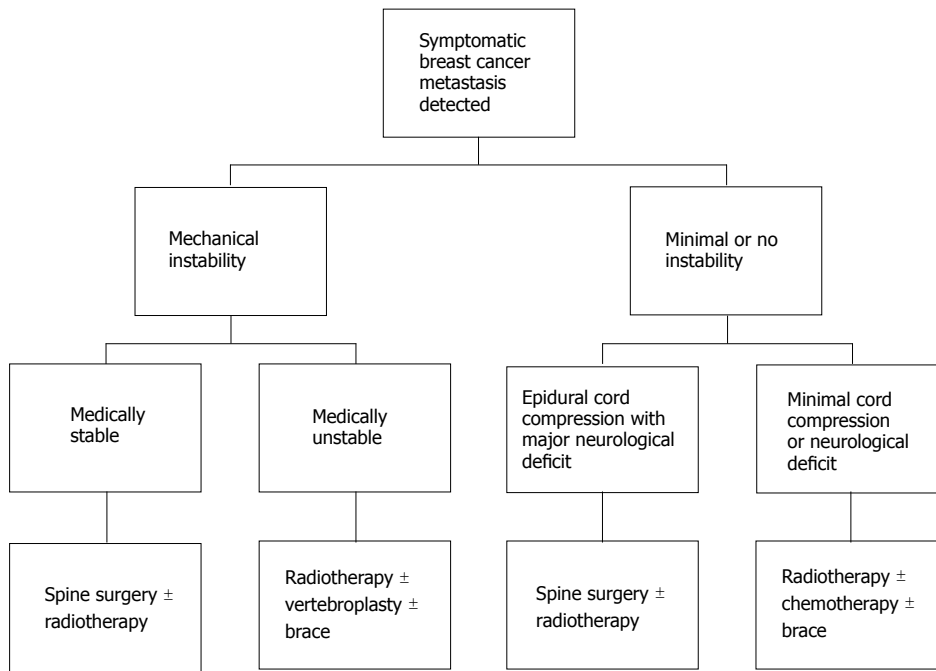


Figure 1 Proposed algorithm for treatment of patients with symptomatic spinal metastases from breast cancer.

For these reasons, patients with symptomatic spinal metastases from breast cancer may especially benefit from aggressive surgical intervention, in an attempt to achieve long-lasting symptom relief. Further, radiation response varies by tumor type and breast cancer metastases are generally more radiosensitive than other types of solid tumors^[22,35]. Thus, radiation therapy can be useful treatment consideration in this patient cohort, in addition to or independent of surgery.

Spinal instability in the setting of metastatic disease is caused by tumor invasion and distortion of the vertebral body and posterior elements. This results in movement-related pain, symptomatic or progressive deformity, or neural compromise under physiologic loads. The SOSG recently devised the first classification system to aid in predicting spine stability of neoplastic lesions, called the Spine Instability Neoplastic Score (SINS)^[39]. Briefly, the scoring system is a 6-point scale that takes into account location, pain, bone lesion type (lytic, blastic, or mixed), spinal alignment, extent of vertebral body collapse, and posterior element involvement. The SINS classification displays near-perfect intraobserver reliability and can be used to guide treatment decision-making, as unstable pathologies are likely to require surgical stabilization given that systemic or radiotherapy cannot restore spinal stability^[40].

Surgical management and patient selection

Patients with spinal metastases may be candidates for a wide range of surgical interventions, ranging from limited posterior decompression to radical tumor excision and reconstruction. Surgical advances in the last few decades have allowed improved and more aggressive spinal cord decompression and tumor resection with ac-

ceptably low morbidity. Although surgery may be palliative from an oncologic perspective, patients may benefit significantly given the appropriate surgical indications. In 2005, Patchell *et al.*^[41] reported the results from the first prospective and randomized controlled trial of direct decompressive surgery plus radiation compared to radiation therapy only. The patients in the surgical arm of the study demonstrated significantly superior postoperative functional improvement (ambulation, neurologic function, muscle strength, continence) and decreased analgesic requirements. Patients with spinal metastases from multiple primary lesions were enrolled, with breast cancer accounting for 11% of the patients in both arms of the study. However, postoperative results were not stratified by primary tumor type. Possible mechanisms underlying improvements following surgical resection and stabilization may include direct alleviation of neural compression by tumor-related spinal pathology, and reduction of mechanical pain caused by tumor-induced instability^[10].

The exact indications for surgery in patients with metastatic breast cancer to the spine are controversial, and evidence-based guidelines are not available due to the paucity of literature on this topic^[10]. Currently, it is generally agreed that aggressive surgical resection may be appropriate for patients with at least 3 mo expected survival who present with progressive neurological deficit, vertebral column instability, tumors that progress despite maximal radiotherapy, and medically intractable pain^[13,42,43]. The majority of the literature has studied metastatic spinal cord tumors as one large cohort, and there has been a recent effort to study histologic-specific spinal metastases. Surgical patients with various types of primary cancer have very different clinical characteristics, which can dictate a patient's surgical approach, prognosis,

and treatment options.

In 2007, Shehadi *et al*^[10] reported the largest retrospective cohort of breast cancer patients treated with aggressive resection of metastatic spinal disease. Eighty-seven patients were treated with aggressive decompression and instrumentation. Patients generally did well neurologically, with 53% of patients who presented with neurologic deficits improving and 85% of all patients maintaining or improving their neurologic function at 1 year. Further, postoperative pain levels were significantly reduced from a preoperative visual analog scale (VAS) of 6 to a median VAS of 2. In 2011, Tancioni *et al*^[35] reported on 23 breast cancer patients treated with more conservative decompressive surgery followed by radiotherapy. This retrospective study remarkably resulted in 96% complete remission of pain and 100% recovery of neurological defect with no major morbidity, which lasted until death or progression of disease at another site. Similarly, Chung *et al*^[44] reported on 15 breast cancer patients who underwent aggressive spinal cord decompression, with 56% of patients who presented with neurologic deficits improving and all patients maintaining or improving their neurological status after surgery.

Several scoring systems have been proposed to determine which patients with metastatic spinal disease would benefit most from decompressive surgery^[45]. Tokuhashi *et al*^[46] was the first to propose a scoring system designed to estimate patient prognosis, which guides excisional surgery (> 6 mo survival) *vs* nonoperative treatment or limited intralesional curettage (< 6 mo survival). Tomita *et al*^[47] also proposed a prognostic scoring system, recommending limited palliative decompression or supportive care *vs* total en bloc spondylectomy for long-term control. Both the Tomita and Tokuhashi scoring systems take into account the histology of the primary tumor as an important prognostic value, with breast cancer carrying the most favorable prognosis. However, both scores are based on a wide variety of tumor histopathologies with a limited number of breast cancer patients, multiple primary cancers are compiled into the same prognostic group, and medically intractable pain is not an indication for surgery. Therefore, these scores may not be entirely applicable for patients with metastatic breast cancer. Recent histopathologic-specific studies have reported potential prognostic variables, with estrogen receptor positivity, location of tumor, presence of other metastasis, and adjuvant radiotherapy potentially being important variables for patient risk stratification^[20,44,45]. However no same prognostic variable has been consistently reported and the studies are all retrospective with small sample sizes.

Surgical techniques

The surgical approach to metastatic tumors of the spine depend on the tumor location, extent of infiltration, and type of reconstruction needed. The approach can be anterior, posterior, lateral or a combination of the above depending on the tumor location. Lesions may also involve multiple levels and require multiple-level decompression

and excisions if the osteolytic lesions are extensive^[21]. Because the vertebral body is most commonly involved in metastatic disease, an anterior approach often represents the most direct route to the tumor^[22]. In the craniocervical region, access may be achieved with a transoral or transmandibular approach although these are associated with significant morbidity and are rarely used in the setting of metastatic disease. Recently, transnasal and transcervical approaches have been developed for improved access. The upper thoracic region (T1-T4) is difficult to access anteriorly due to obstruction by the great vessels and mediastinal organs^[48] and a transpedicular posterior or posterolateral approach is generally preferred^[13]. However, when necessary a manubriotomy, sternotomy, or trap-door approach may be used^[22].

The remaining thoracic region (T5-T11) may be accessed ventrally *via* a thoracotomy and L2-L5 may be accessed through a retroperitoneal approach^[49]. Care should be taken to screen patients who may have had previous radiation or surgery to the neck, thorax, or abdomen, as tissue planes may be disrupted and complicate ventral access. On the other hand, most spine surgeons have a greater familiarity with posterior approaches and thus this represents the most commonly used route for decompression and stabilization. Fortunately, T3-T12 nerve roots may generally be sacrificed without significant morbidity^[22]. Posterior stabilization involve multilevel pedicle instrumentation using titanium polyaxial screw-rod systems, which are indicated for resections at high-stress areas and multilevel vertebrectomies^[13,22]. Anterior reconstruction following a vertebrectomy is achieved using titanium or polyetheretherketone (PEEK) cages or with polymethyl-methacrylate (PMMA) cement and anterolateral plating. Newer surgical techniques such as minimally invasive surgery have shown efficacy in achieving neurological improvement and alleviating pain, while decreasing blood loss, operative time, and complication rates^[50].

Vertebroplasty and kyphoplasty

Less invasive operative techniques such as percutaneous vertebroplasty and kyphoplasty are cement formulations that can provide additional reinforcement to the vertebral body. Vertebroplasty, which involves PMMA injection into the vertebral body under fluoroscopic or CT guidance, stabilizes the anterior column and prevents further compression fractures. Kyphoplasty is a similar procedure in which an inflatable balloon is first inserted in order to provide a void to inject cement under low pressure, which restores vertebral body height and corrects kyphotic deformity^[22,51]. The analgesic properties are thought to be primarily secondary to mechanical stabilization, with potential contributions from thermoablation of nociceptive nerve endings and cytotoxic antitumor effects^[52]. Preliminary retrospective reports have demonstrated effective pain relief for metastatic breast cancer to the spine. For example, in 2008 Lee *et al*^[53] reported the results of vertebroplasty for patients with solitary metastases, 8 of whom had breast cancer. Following treatment, all breast

cancer patients experienced immediate pain improvement and reduction in analgesic requirement. Treatment strategies have also been proposed that combine percutaneous techniques with surgical resection as well as stereotactic radiosurgery^[54].

CONCLUSION

The prevalence of patients with a history of breast cancer is increasing as the rate of survival improves, highlighting the large population of patients at risk for symptomatic spinal metastases. The management of patients with metastatic breast cancer to the spine is often complex and requires a multidisciplinary approach. Precise diagnosis with history, physical, and imaging are imperative to initiate the appropriate treatment in a timely manner. The treatment for symptomatic spinal metastases remains palliative and is not intended to prolong survival. Surgical advances in the last few decades have allowed improved spinal cord decompression and tumor resection and continue to evolve. The goals of treatment include restoration of and preservation of neurological function, maintaining spinal stability, and pain relief in an effort to achieve a better quality of life. Further research should focus on pathology-specific whenever possible, given its implications for treatment selection and prognosis.

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WJCO 5th Anniversary Special Issues (2): Breast cancer

Weight gain following breast cancer diagnosis: Implication and proposed mechanisms

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pharmacologic agents. Although breast cancer prognosis does not appear to be significantly impacted, weight gain has negative consequences on quality of life and overall health. Future studies should explore change in body composition, metabolism and insulin resistance. Avoiding weight gain in breast cancer survivors following initial diagnosis and treatment should be encouraged.

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Key words: Breast cancer; Weight gain; Exercise; Survivorship; Insulin resistance

Core tip: Weight gain occurs in the majority of women following breast cancer treatment, especially those who are younger, closer to ideal body weight and who have been treated with chemotherapy. Although weight gain may be modest, changes are consistent with sarcopenic obesity. Women are unlikely to return to pre-diagnosis weight. Although the degree of weight gain does not appear to significantly alter prognosis, associated changes in metabolism and inactivity are of concern. Interventions should be promoted to avoid weight gain.

Abstract

Weight gain occurs in the majority of women following breast cancer treatment. An overview of studies describing weight gain amongst women treated with early to modern chemotherapy regimens is included. Populations at higher risk include women who are younger, closer to ideal body weight and who have been treated with chemotherapy. Weight gain ranges between 1 to 5 kg, and may be associated with change in body composition with gain in fat mass and loss in lean body mass. Women are unlikely to return to pre-diagnosis weight. Possible mechanisms including inactivity and metabolic changes are explored. Potential interventions are reviewed including exercise, dietary changes and

Makari-Judson G, Braun B, Jerry DJ, Mertens WC. Weight gain following breast cancer diagnosis: Implication and proposed mechanisms. *World J Clin Oncol* 2014; 5(3): 272-282 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i3/272.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i3.272>

INTRODUCTION

Weight gain following a diagnosis of breast cancer has been reported consistently in women treated for breast cancer, but was an unexpected finding when first described in women undergoing adjuvant chemotherapy by

Dixon and colleagues in 1978^[1]. After subsequent reports confirmed this observation, weight gain was included as a known side effect of adjuvant chemotherapy^[2]. Despite this, most women appear to be inadequately informed about this possibility, as demonstrated by one study reporting concern about treatment-associated weight gain in only 27% of survivors prior to therapy^[3].

WHY IS POST-TREATMENT WEIGHT GAIN OF CONCERN?

Obesity is a global issue associated with increased risk of developing post-menopausal breast cancer^[4] and a worse prognosis at the time of diagnosis^[5]. However, the effect of weight gain following a diagnosis of breast cancer is less well understood. While weight gain following diagnosis does not necessarily lead to obesity or its consequences, any impact on breast cancer prognosis and overall health, patient self-image, or quality of life (QoL) would be an undesirable outcome. These outcomes may be interrelated: weight gain can influence other medical conditions such as diabetes, heart disease, hypertension and hypercholesterolemia that may impact overall survival. For example, Erickson *et al*^[6] found associations between weight change and the incidence of developing diabetes in breast cancer patients in the Women's Healthy Eating and Living (WHEL) study. Perhaps most concerning, patients appear rarely to return to pre diagnosis weight^[7,8] which might be coupled with already well-described body image concerns (in 70% of women under age 50 in one study^[9]). Weight gain and receipt of mastectomy with or without reconstruction are two significant factors impacting body image and quality of life; weight gain following reconstruction can result in asymmetry in the reconstructed breast (autologous tissue) or in the contralateral breast in women who had implant surgery^[10,11]. McInnes *et al*^[12], in an early descriptive study of 50 women receiving chemotherapy, found weight gain of more than 2.5 kg in 62.5% and proposed a relationship between weight gain and QoL, while Ganz and associates identified the influence of weight problems after modern chemotherapy on QoL^[13]. Patients unaware of the possibility of weight gain exhibited distress about this unanticipated outcome^[14]; consequently post-treatment related weight gain is a serious source of concern and needs to be better understood.

DOES IT OCCUR, HOW MUCH, AND IN WHOM?

Weight gain following treatment for early stage breast cancer is a global concern with the phenomena described by investigators spanning the continents. Table 1 provides an overview of studies describing weight gain following a diagnosis of early stage breast cancer selected based on a focus on factors impacting weight gain after diagnosis rather than obesity at diagnosis and includes reports from

a diverse selection of nations. Studies focusing on prognosis related to weight gain are discussed later in the text.

Weight gain after chemotherapy was reported in France, a country that has the lowest rates of obesity in Europe^[15]. Obesity is less prevalent in Asian countries also, yet here too weight gain has been reported following diagnosis^[16,17]. In developing countries, such as Malaysia, 63% of women reported weight gain with a mean weight change of 2.73 kg^[18]. In the United States, weight gain is seen in different ethnic groups. In an exploratory assessment of weight change in a group of 37 African American breast cancer survivors, 47% reported weight gain after treatment^[19]. Vance and colleagues, in their 2011 review, noted that 50%-96% of breast cancer survivors reported weight gain with gains in the range of 2.5-6.2 kg^[20].

Early reports: Pre-anthracycline era

Initial reports describing weight gain in the 1990s described changes of up to 8-10 kg^[21]. The chemotherapy regimens used at the time included longer duration treatments of non-anthracycline containing regimens such as cyclophosphamide, methotrexate, and fluorouracil (CMF) and often use of up to one year of prednisone prescribed as an anticancer agent. (More recent steroid use has been limited to dexamethasone employed to avoid chemotherapy-induced emesis.)

Levine *et al*^[22] followed 32 women over a two-year period and reported in 1991 that 63% gained weight with an average gain at two years was of 6 kg. Subsequently Goodwin and colleagues, studying 535 patients treated between 1989 and 1996, determined that adjuvant chemotherapy treatment and onset of menopause were factors predicting weight gain one year after diagnosis^[23]. Most women in this study were treated with mostly non-anthracycline based chemotherapy, however some received anthracycline-containing cyclophosphamide, epirubicin, and fluorouracil (CEF) delivered for the same duration as CMF. Overall, the total group gained a mean of 1.6 kg at one year-84% of patients had gained weight-with no accompanying alteration in waist-hip ratio (WHR); however those receiving any type of chemotherapy gained 2.5 kg (95%CI: 1.8-3.2 kg).

More recent reports: Modern chemotherapy regimens

In more recent reports, covering a period of time during which adjuvant chemotherapy regimens incorporated anthracyclines and/or taxanes, weight gain is reported but generally to a lesser degree than earlier studies. In a prospective study from Turkey, Basaran *et al*^[24] reviewed weight change in 176 women receiving adjuvant chemotherapy between 2003 and 2007, 98% of whom received an anthracycline-based regimen, with or without a taxane; 72% had gained weight (median weight change: 3 kg) at end of year one. Age, menopausal status and comorbidities impacted the degree amount of weight gain.

Makari-Judson and associates conducted a retrospective review of 185 patients with early stage breast

Table 1 Overview of studies describing weight gain in women following a diagnosis of breast cancer

Ref.	Patient numbers	Average weight gain	Study type	Comment
Levine <i>et al</i> ^[22] , 1991	32	6.03 kg at 2 yr	Prospective	63% of women receiving chemotherapy gained weight
Goodwin <i>et al</i> ^[23] , 1999	535	1.6 kg at 1 yr	Prospective	Weight gain associated with chemotherapy and onset of menopause
Demark-Wahnefried <i>et al</i> ^[31] , 2001	53	2.5 kg at 1 yr with chemo 1 kg local rx at 1 yr 2.1 kg at 1 yr with chemo	Prospective	Study group premenopausal only Weight gain associated with chemotherapy Change in body composition (sarcopenic obesity)
Makari-Judson <i>et al</i> ^[8] , 2007	185	2.2 kg at 1 yr	Retrospective	Weight gain associated with chemotherapy Younger women closer to ideal BMI gained most Most received anthracycline chemotherapy
Saquiab <i>et al</i> ^[25] , 2007	3088	Measured as relative weight gain > 5% on 65% of chemotherapy recipients	Prospective	Weight gain associated with chemotherapy Both anthracycline and non- anthracycline treatments similar
Heideman <i>et al</i> ^[26] , 2009	271	2 kg at one year	Retrospective	Younger, closer to ideal BMI more likely to gain Weight gain associated with chemotherapy and hormone therapy
Gu <i>et al</i> ^[16] , 2010	5014	2 kg at 18 mo	Prospective	Stage 0-III Weight gain associated with chemotherapy and younger, lower BMI, premenopausal women
Tredan <i>et al</i> ^[15] , 2010	272	3.9 kg at 1 yr	Prospective	All participants received chemotherapy Anthracycline 41%, anthracycline and taxane 58% Gained weight despite dietary counseling
Basaran <i>et al</i> ^[24] , 2011	176	3 kg at 1 yr	Prospective	Weight gain associated with chemotherapy Anthracycline/taxane chemotherapy
Chen <i>et al</i> ^[17] , 2011	4561	1.7 kg dx to 18 mo	Retrospective	Stage 0-IV, patient reported weights, Association of weight gain with younger age, premenopausal, and higher stage.
Nissen <i>et al</i> ^[27] , 2011	49	1.95 kg at 1 yr	Prospective	Obese more likely to lose weight All participants received chemotherapy
Yaw <i>et al</i> ^[18] , 2011	368	2.73 kg reported at study entry (treatment completion)	Retrospective	Weight gain associated with being closer to ideal BMI Overweight/obese women lost weight Patient reported weight gain one year prior to diagnosis and at study entry

BMI: Body mass index.

cancer and evaluated weight change at diagnosis, 1, 2 and 3 years^[8]. Ninety percent of this patient population received an anthracycline-containing regimen [most commonly doxorubicin and cyclophosphamide (AC) for four cycles]. Weight gain at 2 years was greater than one year, plateauing at year 3. Recursive partitioning analysis associated weight gain at year 1 with younger age, closer to ideal BMI and adjuvant chemotherapy. In fact, older and overweight patients receiving chemotherapy tended to lose weight, although these patients constituted a smaller subgroup. Length of chemotherapy, specific chemotherapy regimens, and hormonal therapy were not found to be significant predictors of weight gain. Of patients who had gained weight at year 1, only one in five had returned to baseline by year 3.

Similar results emerged from the WHEL study, which assessed the association between chemotherapy, and tamoxifen and weight gain in 2972 participants^[25]. Relative weight gain rather than an absolute number was reported because the authors considered it less likely to be confounded by initial body weight; a clinically meaningful gain was defined as a greater than 5% gain from baseline to year 1. Chemotherapy was significantly associated with weight gain while tamoxifen was not. Anthracycline *vs* non-anthracycline, or shorter duration of therapy (with

AC) compared with longer [six cycles of cyclophosphamide, doxorubicin and fluorouracil (CAF)] were not significant variables. Weight gain peaked at year 2, and then plateaued. After six years of follow up, only 10% returned to pre diagnosis weight.

Weight gain ranging from 1.95 kg to 4.5 kg has been described in the first year after chemotherapy. In a Dutch study of 271 women, the average gain at one year was 2 kg^[26]. Women who received both chemotherapy and hormonal treatments gained 4.5 kg at one year compared to 2 kg at five years. Nissen and co-investigators followed prospectively 49 chemotherapy-treated women ages 40-54 and found a mean gain of 1.95 kg accompanied by increased body fat; patients who were closer to ideal BMI at diagnosis experienced the greatest weight gains^[27]. In a prospective, observational study of 272 chemotherapy-treated women from France, weight change was reported at 6 and 12 mo post therapy^[15]. Approximately one third of the study population reported that they had experienced weight gain of unspecified amount in the year prior to diagnosis. At one year after diagnosis, 60% of women had gained weight (mean 3.9 kg) despite dietary counseling.

Gu *et al*^[16] reported findings from the Shanghai Breast Cancer Survival Study (SBCSS) of 5014 women with

stage 0-III breast cancer diagnosed between 2002 and 2006 and followed at 6, 18 and 36 mo after diagnosis and determined mean weight changes of 1, 2, and 1 kg respectively. Thirty-seven percent of survivors gained greater than 5% of their baseline body weight at 18 mo, a percentage similar to western studies despite the comparatively low obesity incidence in China. Younger age, premenopausal status, lower BMI at diagnosis, receipt of chemotherapy or radiation were significantly associated variables. In this study, women with co-morbidities and advanced stage were more likely to lose weight at 36 mo. In another report from SBCSS, Chen *et al*^[17] described 4561 women with stage 0-IV breast cancer, measuring weight, height, and waist and hip circumference at study entry and again at 18 mo. Compared to patient reports from one year prior to diagnosis, there was a gain reported in 61% with a mean gain of 1.7 kg at 18 mo post diagnosis. Thirty-seven percent gained more than 5% of body weight, however 27% lost weight. An association with chemotherapy was found in univariate but not multivariate analyses however, 91% of this cohort received chemotherapy; multivariate analysis identified socio-demographics and lifestyle factors as significant. The inclusion of Stage IV patients in this study is more difficult to interpret since these women may have more comorbidities and poorer performance status.

Anti-estrogen therapy effect

Hormonal treatments, such as tamoxifen and aromatase inhibitors, are less frequently associated with significant weight gain. Tamoxifen use in the P1 prevention trial did not lead to a significant weight gain when compared to placebo^[28]; similarly, in the Women's Healthy Eating and Living (WHEL) study (see above), tamoxifen did not lead to significant changes in weight^[25]. Additionally, aromatase inhibitor usage did not lead to weight changes when compared to tamoxifen in the ATAC trial^[29].

Treatment with radiation does not appear to be an independent factor contributing to weight gain^[8]. Neither treatment with corticosteroids or adjuvant therapy with anti-estrogens appears to be strongly associated. Thus, when taking into consideration all breast cancer treatments, weight gain is most strongly correlated with use of cytotoxic therapies.

Menopause and age effect

Several studies describe an increased tendency toward weight gain in premenopausal women compared to postmenopausal women^[16,23,26]. However, inconsistencies in defining menopause in retrospective studies and the issue of chemotherapy-related amenorrhea complicate the interpretation of this effect on weight gain. Goodwin and colleagues identified the greatest weight gains (mean of 2.65 kg) in premenopausal women who experienced chemotherapy-associated amenorrhea (receiving either CMF or CEF, each delivering 6 mo of cyclophosphamide) and became postmenopausal^[23].

When weight gain is assessed following the use of

regimens less likely to cause chemotherapy-induced amenorrhea, younger age rather than menopausal status appears as a significant risk factor. Makari-Judson *et al*^[8], employing recursive partitioning analysis found women younger than age 59 gained the most weight with no independent effect of menopausal status. Irwin and colleagues determined associations between weight gain and both younger age and postmenopausal status; this was further refined as younger post-menopausal women gaining significantly more than older post menopausal women and women who developed treatment-associated menopause after diagnosis^[30]. Tredan and associates found no influence of menopausal status on weight gain^[15].

Pre-treatment weight and BMI effect

Women of normal weight or women with closer to ideal BMI were found to be more likely to gain weight after diagnosis in several studies^[8,16,27]. Women with higher BMI ($> 30 \text{ kg/m}^2$) are less likely to gain weight, and some studies have demonstrated that these women are more likely to lose weight^[8,27]. Nissen *et al*^[27] found women who were close to ideal BMI gained an average of 2 kg while overweight patients lost 1.4 kg, and those who were obese lost 1.9 kg. It is not clear why obese women may be more likely to lose weight following chemotherapy; although it is possible that this is related to co-morbidities, no studies have demonstrated decrease in energy intake however increased walking was identified as a factor favoring weight loss in the Nissen study.

IMPACT OF TREATMENT-ASSOCIATED WEIGHT GAIN

Body composition

Several authors have identified a change in body composition as well as weight gain. Nissen and colleagues conducted a longitudinal, randomized observational study of a physical activity intervention *vs* zoledronic acid and calcium^[27]. While the primary endpoints related to bone density, body composition measurements were made. Forty-nine women aged 40-55 were studied, all within 24 mo of their last menstrual period and all receiving chemotherapy. Chemotherapy included AC in 39%, and AC-T (doxorubicin and cyclophosphamide followed by paclitaxel for a total of 8 cycles of therapy) in 57%. There was no difference in weight gain between those receiving 4 compared with 8 cycles. Only normal ($< 25 \text{ kg/m}^2$) BMI at baseline was predictive of weight gain, with those having normal BMI gaining the most. Women of normal weight had an increase in fat mass in the torso and arms while younger subjects appeared to gain fat mass in arms. Weight gain in this study and in the Demark study is consistent with sarcopenic obesity, namely loss of lean body mass and gain in fat mass^[31].

Freedman and colleagues performed a prospective study of weight and body composition in 20 women with Stage I-III breast cancer receiving modern chemotherapy^[32]. Weight change and measurements of body

composition were compared with age and BMI-matched controls at the start of treatment, immediately after completion of chemotherapy and 6 mo later. Mean pre-treatment BMI was 24.1 kg/m^2 . Six months after completion of chemotherapy, a statistically significant mean weight gain of $1.09 \pm 2.46 \text{ kg}$ was seen from the immediate post chemotherapy weight; however, this was not statistically significant when compared to the experience of controls. Strikingly, fat mass increased while lean body mass decreased in the women who received chemotherapy but not in the healthy controls. Using computed tomography, an unfavorable change of decrease in the ratio of visceral adipose tissue to subcutaneous fat was detected following chemotherapy. Although this small study did not demonstrate significant weight gain, the deleterious change in body composition is consistent with findings from others and raises concerns about metabolic function.

Cheney and associates, also using computerized tomography to measure changes in weight and abdominal and visceral subcutaneous adipose tissue, assessed 34 women prior to treatment and 6-12 mo later^[33]. Although the findings were not significant in terms of weight change, there was in this study similar to the Freedman study, a significant loss in lean body mass and gain in fat mass.

A small study by Campbell *et al*^[34] followed 10 women undergoing adjuvant chemotherapy and measured resting energy expenditure using dual energy x-ray absorptiometry across cycles of treatment. Although participants did not gain weight, and there was no change in resting energy expenditure, there was an increase in total fat mass. This was felt to be consistent with a decrease in physical activity.

Irwin *et al*^[30] reported results from the Health, Eating, Activity, and Lifestyle (HEAL) study concerning 514 women with stage 0-III breast cancers, almost half of whom used tamoxifen but only 27% received chemotherapy. Women, post treatment, increased their weight by a mean of 1.7 kg and their body fat by 2.1%. Weight increases were greatest for patients who were younger, postmenopausal, had a higher stage of disease, and who reported a decrease in their physical activity. Higher stage of disease may correspond to receipt of chemotherapy, longer duration of treatment and potential longer duration of de-conditioning.

Breast cancer prognosis

Speculation regarding the impact of treatment-related weight gain on breast cancer outcomes has been stimulated in part by the findings of increased incidence and poorer outcomes in patients with greater pretreatment weight or BMI^[35]. While minimizing weight gain is important, clarification of its impact on survival and recurrence outcomes is of critical importance.

The influence of weight gain on prognosis is inconsistent across studies. Camoriano and colleagues examined 646 lymph node-positive breast cancer patients treated prospectively in two clinical trials with extended non-anthracycline chemotherapy, chemo-hormonal

therapy, or observation, and found that premenopausal women who gained more than the median weight gain (of 5.9 kg) experienced significantly higher risk of death that persisted after controlling for other factors (multivariate hazard ratio 1.62); a similar trend in postmenopausal women (median weight gain for observed patients 1.8 kg, and for treated subjects 3.6 kg) was not statistically significant^[36]. Kroenke and associates, reporting results from the Nurses' Health Study (NHS), noted that patients with greater BMI before breast cancer diagnosis had a greater risk of breast cancer recurrence and death if a never-smoker or if premenopausal^[37]. In addition, patients who were never-smokers who gained weight after treatment had a higher risk of breast cancer recurrence and mortality, and all-cause mortality, than did never-smokers who had maintained their pre-treatment weight; significant covariates in the analysis included nodal status and tumor size (the effect of weight gain was more pronounced in patients with small tumors and negative lymph nodes) and baseline BMI [the effect on survival was seen in normal weight but not overweight ($\text{BMI} \geq 25 \text{ kg/m}^2$) subjects].

In contrast, Caan *et al*^[38] evaluated a prospective cohort of women [1692 with complete data in the Life after Cancer Epidemiology (LACE) study] drawn from the Kaiser Permanente Northern California and Utah Cancer Registries. While similar findings as to patient characteristics associated with weight gain and pretreatment weight associations with mortality were found, no impact of treatment-associated weight gain with mortality was discovered.

A pooled analysis of the LACE, WHEL, NHS, and SBCSS studies was subsequently performed, incorporating 12915 patients with breast cancer diagnosed between 1990 and 2006^[39]. The analysis contained a mixture of patient-reported and measured outcomes. Weight gain after treatment tended to impact all-cause mortality in US but not Shanghai patients; overall there was no impact on breast cancer-specific mortality. However, overall mortality was increased in women who were normal weight who had gained 10% or more (mean 10.5 kg) of their pre-treatment weight when compared to overweight patients. The authors concluded that while evidence for poorer survival associated with weight gain was found, the relative weight gain was substantial. A higher mortality rate was found for patients who lost $\geq 10\%$ of their pretreatment weight, particularly if they were smokers and were leaner prior to treatment. Similarly, Bradshaw and colleagues described a cohort of 1033 women from Long Island, NY, diagnosed with breast cancer between 1996 and 1997 for which complete data were available^[40]. Self-reported weights were employed at age 20, and one year prior to diagnosis, one year after, and at time of follow up questionnaire completion. Mortality-all cause and breast cancer-specific-was increased for women reporting a loss of $> 5\%$ or a gain of $> 10\%$ compared to pretreatment weight.

While the effect of weight gain on prognosis is vari-

able among studies, it appears to be significant in a subset of women. Investigations that linked obesity with metabolic outcomes and prognosis provide lessons and potential mechanisms. Goodwin and associates found that mean fasting insulin levels were higher in women diagnosed with breast cancer and that this negatively impacted prognosis in this subset^[41]. Similarly, Borugian *et al.*^[42] determined that higher fasting insulin levels were associated with poorer prognosis.

Though the amount of weight gain observed as a consequence of chemotherapy regimens is modest on average, it may be sentinel of a more dramatic shift in body composition. Adipose tissue produces a variety of inflammatory cytokines, which may be directly responsible for altering risk^[43,44]. Some of these factors can act directly on the breast epithelium to stimulate the development and expansion of cancer cells. In particular, IL-6 was shown to expand substantially the population of cancer stem cells raising the prospect of recurrences with features of triple negative breast cancers (that is devoid of estrogen and progesterone receptors and HER2/*neu* overexpression) that have, stage for stage, poorer prognoses and fewer effective treatment options when compared to most other breast cancers. These effects may be magnified in the minority of women who suffer substantial weight gain.

PROPOSED MECHANISMS OF WEIGHT GAIN AND CHANGE IN BODY COMPOSITION

Weight gain is ultimately a result of energy surplus due to either increased intake or decreased energy expenditure. It is important to identify possible mechanisms of weight gain in order to advise women on how best to avoid it. While diagnosis- and treatment-related depression may alter eating patterns, most studies have demonstrated that caloric intake actually tends to decrease over the first year after breast cancer diagnosis, suggesting that weight gain is not likely a result of overeating^[45,46].

Decreased energy expenditure was demonstrated by Demark-Wahnefried *et al.*^[31] in a study of 36 women receiving chemotherapy and 17 receiving only local treatment. Body composition, resting energy expenditure, dietary intake recalls and physical activity were recorded. Mean weight gain in the local treatment group was 1 kg compared to 2.1 kg in the chemotherapy group. Reduced activity and with no change in caloric intake was identified in the women who gained weight, who exhibited a body composition pattern consistent with sarcopenic obesity (loss of muscle mass and increase in body fat) that has also been suggested by other investigators^[27,47].

Decreased exercise and inactivity appeared to be linked to a higher risk of weight gain in the HEAL study^[48]. The activity levels of 812 patients were measured after diagnosis; chemotherapy patients' exercise time dropped by 3.6 h/wk compared to 1.6 h/wk for

surgery only patients. This was especially evident in patients with BMI > 30 kg/m² that lost 2.8 h of exercise compared to those with BMI < 25 kg/m² that lost only 1.7 h/wk. Women reported being less active 4-12 mo after diagnosis with an average 2-h drop in activity. A 50% reduction in activity level was noted in those treated with chemotherapy, surgery and radiation, compared to a drop of 24% in those treated with surgery alone. Broderick found weight gain and high levels of sedentary behavior in a study of 24 women at completion of chemotherapy with no improvement one year later^[49]. Patients may need encouragement to exercise since chemotherapy treatment may foster a more sedentary lifestyle.

In the study by Demark and colleagues, all of the women treated with chemotherapy experienced treatment-associated amenorrhea, leading to speculation about the role of hormones and menopause in a change of metabolism^[31]. Body composition and waist-hip ratio may change with the onset of menopause; as estrogen levels decrease, body composition changes and fat moves from hip to waist (*i.e.*, "pear-shaped" to "apple-shaped"). Weight gain was associated with increase in estrogen and insulin levels^[30]. Additionally, abdominal fat correlates with cortisol levels.

The results from Makari-Judson and colleagues suggest that insulin resistance may be a contributing factor^[50]. A prospective, observational study of 100 women receiving adjuvant therapy for early stage breast cancer measured BMI, waist hip ratio (WHR), fasting glucose and insulin at diagnosis, 6, 12 and 24 mo. Weight gain was associated with higher baseline WHR and homeostatic model assessment index (HOMA-IR), a measure of insulin resistance. In particular, women receiving chemotherapy had evidence of a deleterious effect on measures of insulin resistance at 6 mo and experienced weight gain. At 12 mo, although these measures had returned to baseline, participants continued to gain weight. By 24 mo, no further weight gain occurred and measures of insulin resistance were similar to baseline.

Even in the absence of weight gain, the change in metabolic profile may have negative implications. Increased adiposity in the abdominal region can be associated with insulin resistance; insulin and adiponectin may influence tumor growth pathways^[51]. Guinan *et al.*^[52] described development of metabolic syndrome and insulin resistance in a population of 61 non-diabetic women at surgery and completion of chemotherapy and/or radiation (87% received chemotherapy and 84% radiation therapy). Although there was not a statistically significant gain in weight, percent body fat increased. There was a significant increase in fasting insulin and HOMA-IR which correlated with development of metabolic syndrome. Bell *et al.*^[53] demonstrated impaired glucose metabolism in a small study of eight patients early on during chemotherapy and raised concerns about this when compared to non-malignant matched controls.

Genetic polymorphisms associated with obesity include the FTO, fat mass and obesity-associated protein.

These genetic predictors of weight gain were described by Reddy and colleagues in a retrospective analysis of 459 breast cancer patients^[54]. Blood samples were tested for single nucleotide polymorphisms in FTO and adiponectin pathway polymorphisms. In the general population, these genetic factors may contribute to weight gain: FTO decreases satiety and activity levels while adiponectin regulates insulin-like growth factor. The model was enhanced by incorporating environmental interactions demographic and clinical factors predictive for weight gain. Although limited by the fact that some of the weights were self-reported, this model is intriguing for combining genetic predisposition to obesity with the strongest clinical correlates for weight gain, namely, age and baseline BMI.

Weight gain may be a harbinger of more fundamental metabolic changes induced by chemotherapy. In addition to the direct DNA damage in tumor cells that are a result of chemotherapy agents such as anthracyclines, these drugs also interfere with mitochondrial function and ATP production which can have diverse effects^[55]. Cardiomyocytes derive as much as 90% of ATP from mitochondrial oxidative phosphorylation, and thus, are extremely sensitive to the effects of anthracyclines. In animal models, exercise has been shown to mitigate cardiotoxicity^[56] in part alleviating the effects on mitochondrial function^[57]. While overt toxicity may not be evident, other tissues may suffer from impairments. Insulin resistance could result from disruptions in glucose homeostasis in the muscle and liver providing a basis to propose a role for exercise in limiting weight gain.

POTENTIAL INTERVENTIONS TO AVOID WEIGHT GAIN

By identifying predictors of weight gain and populations at higher risk, we can target interventions specifically to those populations. Krogh-Madsen demonstrated in a general population that women who exercised regularly and then stopped for as little as two weeks, experienced a 7% increase in abdominal fat^[58]. There is consistent evidence that physical activity declines after diagnosis and that the reduced energy expenditure is associated with weight and fat gain. In addition to less energy expended for physical activity, more inactivity (*e.g.*, sitting) may contribute to a relative energy surplus and increased adiposity, even in the absence of higher energy intake. Paradoxically, the reduced energy intake observed in some prior studies also lowers daily energy expenditure due to less diet-induced thermogenesis (that is, the energy costs of digesting, absorbing and storing ingested nutrients). Finally, amenorrhea conserves energy output and can contribute to overall energy surplus and weight gain.

The “dose” of physical activity required to offset the deleterious effects of diagnosis and treatment on body weight and adiposity is not well characterized in cancer patients but evidence from the general population suggests it is not trivial. Women in the National Weight Con-

trol Registry^[59], a database of over 6000 people (mainly women who have lost weight and kept it off for more than 6 mo) report close to an hour of physical activity a day; an Institute of Medicine report generally concurred with that recommendation^[60]. A well-controlled study by Jakicic *et al.*^[61] demonstrated that women who performed less than 240 min of exercise per week slowly regained lost weight over the course of a year. An important consideration to prescribing an effective “dose” of exercise is the degree of compensatory behavior in terms of greater inactivity (hopefully minor) and increased energy intake.

Nissen and colleagues found that exercise was a stronger predictor of weight change during chemotherapy than energy intake^[27]. Goodwin and associates found that weight gain could be prevented in a group of 61 breast cancer patients with BMI of 20-35 kg/m² (OR 1.73 for each additional 30 min of exercise per week) however, weight loss required a combination of caloric reduction and exercise^[47].

Data from the SBCSS, demonstrated in a population of mostly normal BMI women at diagnosis (only 32% had BMI > 25 kg/m²) who exercised regularly, (65% reported exercise at baseline and 74% at 18 mo after diagnosis), that exercise did not prevent weight gain in the short run^[17]. The inability to identify a protective effect in this study may have been a consequence of the small numbers of non-exercisers, shorter follow up or because the exercise considered included such low to moderate intensity activities as walking and tai chi.

Exercise may improve metabolic profile and body composition without weight loss. Ligibel and co-investigators studied overweight (BMI > 25 kg/m²) but non-diabetic, breast cancer patients who were at least 3 mo beyond the completion of treatment^[62]. After 16 wk, fasting insulin levels decreased by 28% and hip circumference decreased in the exercise group compared to those receiving usual care, but there was not a significant decrease in WHR and no significant weight loss.

Kim and colleagues performed a meta-analysis of aerobic exercise on body composition in breast cancer patients and included ten trials of exercise interventions in women during (8 trials) or at completion (2 trials) of adjuvant treatment^[63]. The study groups participated in median of three sessions per week, 30-40 min each of moderate exercise and were found to have decreased percent body fat, without significant change in weight or lean body mass. De Backer carried out a review of twelve studies of resistance or resistance plus aerobic training. The studies had different endpoints: one study demonstrated a decrease in WHR, but of those that measured weight, BMI, or fat mass, there was no significant change^[64].

Loprinzi, in a narrative review, described the influence of physical activity, not only on weight gain, but also on other side effects of adjuvant treatment including fatigue, depression, and QoL^[65]. Participation in regular exercise contributed to weight loss and enhanced QoL, mood and energy level. In this review it was suggested, based on the

analyses by Kim and De Backer, that in order to decrease percent body fat, aerobic exercise would be favored over resistance training^[63,64].

Interventions for weight management or loss over the past twenty years were summarized by Demark-Wahnefried^[66]. Twelve studies were included consisting of exercise and dietary counseling. The interventions were not specifically directed towards women who had gained weight after treatment. The recommendation for weight to be an important outcome for overall health, functional status and quality of life was supported even if prognostic impact is inconclusive.

Regardless of whether or not exercise leads to avoiding weight gain after treatment, exercise improves breast cancer prognosis. Holmes found that 3 to 5 h walking per week at average pace (2-2.9 mph for one hour) reduced risk of death by 6% at ten years^[67]. Exercise has additional benefits on mood and QoL independent of weight loss and should be encouraged following recovery from surgery even in the midst of adjuvant treatment. A number of ongoing prospective, intervention trials will help further elucidate the contribution of physical activity to breast cancer prognosis.

In summary, breast cancer patients with a more normal BMI appear more likely to gain weight after treatment compared to overweight and obese survivors. This is in contrast to midlife women in the general population, in whom greater BMI is associated with weight gain^[68]. The weight gain and increase in waist circumference experienced over time in the general population may be prevented with increased participation in regular physical activity; similarly, evidence suggests that exercise has a role in avoiding weight gain in women after a diagnosis of breast cancer.

Obesity at the time of diagnosis is consistently correlated with a poorer prognosis^[5]. This same group of women appears to be more likely to experience unintentional weight loss following chemotherapy. It is not clear if intentional weight loss after a diagnosis of breast cancer affects prognosis. Interventions in addition to exercise may be necessary for obese women to reduce their BMI.

Patients frequently ask about diet alterations as a way of improving their cancer prognosis, but data are scant to make firm, evidence-based recommendations. The Women's Intervention Nutrition Study (WINS) study found that a low fat diet was associated with a 24% reduction in risk of relapse. Patients who were estrogen receptor-negative seemed to benefit more; this subgroup experienced a 42% reduction in relapse^[69]. However, some of this improved prognosis might be attributable to concurrent weight loss.

Goodwin *et al.*^[70] studied the impact of metformin in a group of non-diabetic breast cancer patients with fasting insulin levels at least 45 pmol/L and BMI at diagnosis of at least 28 kg/m². After treatment with metformin for 6 mo, fasting insulin levels decreased by 22%, insulin sensitivity improved by 25% and weight was reduced by

1.9 kg. Ongoing prospective trials are testing this further. Thus, in overweight and obese women, strategies to avoid weight gain may differ from those needed to achieve a healthy weight and may include diet, exercise and pharmacologic interventions.

CONCLUSION

Avoiding weight gain over time is challenging whether following a diagnosis of breast cancer or in the general population. Interventions during chemotherapy may prove especially difficult due to fatigue and other chemotherapy-associated symptoms, but may be necessary to avoid weight gain and improve QoL in both the short- and long-term and enhance prognosis. The two years after treatment may contain "teachable moments" when breast cancer survivors are open to lifestyle counseling. In their 2012 overview, Caan *et al.*^[39] conclude "prevention of weight gain appears to be an evidence based public health goal for breast cancer survivors."

Evidence suggests that following modern day chemotherapy, the modest weight gain experienced by breast cancer survivors does not impact on survival. However, a minority of breast cancer patients gains substantial weight, and further studies on the prognostic effect of change in body composition and measures of metabolism, including insulin resistance will be important. As the numbers of breast cancer survivors continue to grow, promoting strategies to avoid weight gain are supported by the literature and make sense to optimize overall health.

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WJCO 5th Anniversary Special Issues (2): Breast cancer

Pathogenesis, prevention, diagnosis and treatment of breast cancer

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Core tip: This is a review of past and current literature/landmark trials in the etio-pathogenesis, diagnosis and management of breast cancer. We have attempted to cover this vast topic in review form and hope that it will serve as a reference for clinicians who treat patients with breast cancer.

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Abstract

Breast cancer is the most common cancer affecting women worldwide. Prediction models stratify a woman's risk for developing cancer and can guide screening recommendations based on the presence of known and quantifiable hormonal, environmental, personal, or genetic risk factors. Mammography remains the mainstay breast cancer screening and detection but magnetic resonance imaging and ultrasound have become useful diagnostic adjuncts in select patient populations. The management of breast cancer has seen much refinement with increased specialization and collaboration with multidisciplinary teams that include surgeons, oncologists, radiation oncologists, nurses, geneticist, reconstructive surgeons and patients. Evidence supports a less invasive surgical approach to the staging and management of the axilla in select patients. In the era of patient/tumor specific management, the advent of molecular and genomic profiling is a paradigm shift in the treatment of a biologically heterogeneous disease.

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INTRODUCTION

Breast cancer is the most common cancer and also the leading cause of cancer mortality in women worldwide. Approximately 1.38 million new breast cancer cases were diagnosed in 2008 with almost half of all breast cancer cases and nearly 60% of deaths occurring in lower income countries^[1]. There is a large variation in breast cancer survival rates around the world, with an estimated 5-year survival of 80% in high income countries to below 40% for low income countries^[2].

Low and middle income countries face resource and infrastructure constraints that challenge the goal of improving breast cancer outcomes by early detection, diagnosis and treatment^[3]. In high income countries like the United States, approximately 232340 women will be diagnosed and 39620 will die of breast cancer in 2013^[4]. For an American woman, the lifetime risk of developing breast cancer is 12.38% or 1 in 8^[4]. The significant decrease in breast cancer-related mortality in the United

States from 1975 to 2000 is attributed to continued improvement in both screening mammography and treatment^[5,6]. According to the World Health Organization, improving breast cancer outcome and survival by early detection remains the cornerstone of breast cancer control.

RISK FACTORS AND RISK PREDICTION

Age, reproductive factors, personal or family history of breast disease, genetic pre-disposition and environmental factors have been associated with an increased risk for the development of female breast cancer.

Age

The risk of developing breast cancer increases with age. By using the Surveillance, Epidemiology, and End Results (SEER) database, the probability of a woman in the United States developing breast cancer is a lifetime risk of 1 in 8; 1 in 202 from birth to age 39 years of age, 1 in 26 from 40-59 years, and 1 in 28 from 60-69 years^[4].

Personal history

A personal history of breast cancer is also a significant risk factor for the development of a second ipsilateral or contralateral breast cancer. In fact, the most common cancer amongst breast cancer survivors is a metachronous contralateral breast cancer^[7]. Factors associated with an increased risk of a second breast cancer include an initial diagnosis of DCIS, stage IIB, hormone receptor negative cancers, and young age^[8].

Breast pathology

Proliferative breast disease is associated with an increased risk of breast cancer. Proliferative breast lesions without atypia, including usual ductal hyperplasia, intraductal papillomas, sclerosing adenosis and fibroadenomas confer only a small increased risk of breast cancer development, approximately 1.5-2 times that of the general population^[9]. Atypical hyperplasia including both ductal and lobular, usually incidentally found on screening mammography, confers a substantial increased risk of breast cancer. Women with atypia have an approximately 4.3 times greater risk of developing cancer compared to the general population^[9,10].

Family history

A woman's risk of breast cancer is increased if she has a family history of the disease. In the Nurses' Health Study follow-up, women with a mother diagnosed before age 50 had an adjusted relative risk of 1.69 and women with a mother diagnosed at 50 or older had a relative risk of 1.37 compared to women without a family history of breast cancer. A history of a sister with breast cancer also demonstrated an increased relative risk of 1.66 if the diagnosis was made prior to age 50 and a relative risk of 1.52 if diagnosed after age 50 compared to patients without a family history^[11]. The highest risk is associated with in-

creasing number of first degree relatives diagnosed with breast cancer at a young age (under age 50). Compared with women who had no affected relative, women who had one, two or three or more affected first degree relatives had risk ratios of 1.80, 2.93 and 3.90, respectively^[12].

Genetic predisposition

Approximately 20%-25% of breast cancer patients have a positive family history but only 5%-10% of breast cancer cases demonstrate an autosomal dominant inheritance^[13,14]. Genetic predisposition alleles have been described in terms of clinical significance^[15]. High-risk predisposition alleles conferring a 40%-85% lifetime risk of developing breast cancer include BRCA1 and BRCA2 mutations, mutations in TP53 gene resulting in Li-Fraumeni syndrome, PTEN resulting in Cowden syndrome, STK11 causing Peutz-Jegher's syndrome, Neurofibromatosis (NF1) and (CDH-1) E-Cadherin^[16]. Half of the breast cancer predisposition syndromes are associated with mutations in BRCA1 and BRCA2. Women with BRCA1 or BRCA2 deleterious mutations have a significantly higher risk of developing breast cancer. Lifetime breast cancer risk ranges from 65% to 81% for BRCA1 mutation carriers and 45% to 85% for BRCA2 carriers^[17-19]. Moderate risk genes including homozygous ataxia-telangiectasia (ATM) mutations^[20], somatic mutations in tumor suppressor gene CHEK2, and BRCA1 and BRCA2 modifier genes BRIP1^[21] and PALB2^[22] confer a 20%-40% lifetime risk of breast cancer. Numerous low-risk common alleles have been identified largely through genome-wide association studies^[15] and the clinical application in the presence of these mutations is yet to be determined.

ENDOGENOUS HORMONE EXPOSURE AND REPRODUCTIVE FACTORS

The cycles of endogenous estrogen levels throughout a woman's lifetime have implications for the development of or the protection against breast cancer.

Early menarche

Early age at menarche is a risk factor among both pre- and postmenopausal women for developing breast cancer. Delay in menarche by two years is associated with corresponding risk reduction of 10%^[23]. Within the European Prospective Investigation into Cancer and Nutrition cohort, women who had early menarche (≤ 13 years) demonstrated a nearly twofold increase in risk of hormone receptor positive tumors^[24].

Parity and age at first full term pregnancy

Nulliparous women are at an increased risk for the development of breast cancer compared to parous women. Young age at first birth has an overall protective effect, whereas relatively advanced age at first birth confers a relative risk of breast cancer greater than that of a nulliparous woman. Compared to nulliparous women the

cumulative incidence of breast cancer in women experiencing their first birth at age 20, 25, and 35 years was 20% lower, 10% lower and 5% higher, respectively^[25].

Breast feeding

Evidence suggests that breast feeding has a protective effect against the development of breast cancer. Breast feeding may delay return of regular ovulatory cycles and decrease endogenous sex hormone levels. It has been estimated that there is a 4.3% reduction for every one-year of breast feeding^[26].

Testosterone

High endogenous sex hormone levels increase the risk of breast cancer in both premenopausal and postmenopausal women. High levels of circulating testosterone in postmenopausal women have been linked to increased risk of developing breast cancer [relative risk (RR), 2.86-3.28]^[27].

Age at menopause

Later onset of menopause has also been associated with increased breast cancer risk. Every year delay in the onset of menopause confers a 3% increase in risk and every five year delay in the onset of menopause confers a 17% increase in risk of breast cancer^[23,28].

EXOGENOUS HORMONE EXPOSURE

Evidence suggests a relationship between the use of hormone replacement therapy (HRT) and breast cancer risk. Breast cancers related to HRT use are usually hormone receptor positive. When compared with patients who do not use HRT, breast cancer risk is higher in HRT users^[29]. An international meta-analysis examining the risk of breast cancer with HRT found that in women who did not use HRT, RR increased by a factor of 1.028 for each year older at menopause, comparable to the relative risk of 1.023 per year in women who use HRT or for those who ceased to use HRT up to four years previously^[30].

In the Woman's Health Initiative randomized control trial, combined estrogen plus progestin in postmenopausal women with an intact uterus significantly increased the risk of breast cancer, delayed breast cancer detection and diagnosis, and significantly increased breast cancer mortality. The study was terminated early because of increased mortality in the combined estrogen plus progestin group. By contrast, the use of estrogen alone by postmenopausal women without a uterus did not interfere with breast cancer detection and statistically significantly decreased the risk of breast cancer^[31]. Data from the Nurses' Health Study, however, suggest that women who use unopposed postmenopausal estrogen increase their risk of breast cancer by 23% at age 70^[32].

Timing and duration of HRT seem to be important factors associated with breast cancer risk as well. Breast cancer risk from exogenous hormone exposure is inversely associated with time from menopause. Women

initiating hormone therapy closer to menopause have a higher breast cancer risk^[33]. Long term (> 5 years) combined HRT use has been associated with the highest risk whereas short-term use of combined estrogen-progestin therapy does not appear to confer a significantly increased risk (RR = 1.023 per year)^[30].

LIFESTYLE FACTORS

Modifiable risk factors including the excessive use of alcohol, obesity and physical inactivity account for 21% of all breast cancer deaths worldwide^[34].

Alcohol consumption

Alcohol consumption has been associated with increased breast cancer risk that is statistically significant at levels as low as 5.0 to 9.9 g per day, equivalent to 3 to 6 drinks per week (RR = 1.15; 95%CI: 1.06-1.24; 333 cases/100000 person-years). Binge drinking, but not frequency of drinking, was associated with breast cancer risk after controlling for cumulative alcohol intake. Alcohol intake both earlier and later in adult life was independently associated with risk^[35].

Physical activity

Consistent physical activity has been shown to reduce the risk of breast cancer in a dose dependent manner, with modest activity conferring a 2% decrease in risk and vigorous activity a 5% decrease in risk^[36].

Obesity

Obesity, specifically in postmenopausal women, has also been shown to increase a woman's risk of breast cancer. In the EPIC multicenter prospective cohort study, postmenopausal women who did not use HRT had elevated breast cancer risk with increasing weight, body mass index (BMI) and hip circumference^[29]. In this cohort, multivariate relative risk was 1.28 for overweight women (BMI 25.0-29.9) and obese women (BMI > 30.0) compared to women in the normal weight range. Lean women on HRT are incongruously at an increased risk of breast cancer (RR = 2.04) compared to their overweight (1.93) and obese (1.39) counterparts^[29].

Insulin resistance and hyperinsulinemia have been studied as a risk factor for the comorbidities associated with obesity including cardiovascular disease and diabetes. Insulin has anabolic effects on cellular metabolism and insulin receptor overexpression has been demonstrated in human cancer cells^[37]. Hyperinsulinemia has been shown to be an independent risk factor for breast cancer in non-diabetic postmenopausal women and may help to explain the relationship between obesity and breast cancer^[38].

Radiation

Radiation exposure from various sources including medical treatment and nuclear explosion increases the risk of breast cancer. Radiation to the chest wall for treatment of childhood cancer increases the risk of breast cancer

linearly with chest radiation dose^[39]. Survivors of childhood cancers who received therapeutic radiation are at a dose dependent risk for the development of breast cancer, and those treated for Hodgkin's disease are at highest risk ($RR = 7$)^[40]. Radiation effects on the development of female breast cancer have also been demonstrated in Japan post nuclear attack on Hiroshima and Nagasaki^[41] and positively correlate with age younger than 35 years at time of exposure. The incidence of breast cancer has also increased in areas of Belarus and Ukraine. A significant two fold increase was seen in the most contaminated areas around Chernobyl following the nuclear accident and manifest in women who were younger at the time of the exposure^[42].

PREDICTION MODELS

Prediction models are used to better stratify a person's risk for developing cancer based on the presence of known and quantifiable risk factors. There is great value in identifying high risk individuals to better tailor timing of screening modalities or prompt referral to a geneticist for counseling and testing. The concordance statistic or "c-statistic" quantifies the ability to distinguish patients who will develop cancer from those who will not. A c-statistic of 0.5 indicates that the prediction model is no better at discriminating patients who are at risk from those who are not than flipping a coin.

Gail model

The most well-known and widely used screening tool is the Breast Cancer Risk Assessment Tool (BCRAT) or the Gail model, developed by Dr Mitchell Gail^[43,44] at the National Cancer Institute (NCI). The initial model used age, age at menarche, age at first live birth, number of previous biopsies, and number of first degree relatives with breast cancer, modified to include history of atypical ductal hyperplasia, lobular carcinoma *in situ*, and predicts a woman's 5-year and lifetime risk of developing invasive breast cancer. It was developed with data from the Breast Cancer Detection Demonstration Project (BCDDP) and included white and black women over age 35 only. Screening tools rely on incidence of disease and the Gail model is updated as necessary and is easily accessed at www.cancer.gov/bcrisktool. The NCI's BCRAT is widely available to clinicians and is best used for women without a strong family history. The c-statistic for the Gail model has reported to be between 0.55-0.67^[45]. The Gail model may under-predict women with a strong familial predisposition.

Models that emphasize family history

A commonly used risk prediction model with an emphasis on family history, including maternal and paternal family history and age of onset is the Claus model, engineered by Dr. Elizabeth Claus. The model, which predicted an autosomal dominant gene that led to an increased risk for developing breast cancer, was published the same

year as the BRCA1 gene was cloned^[46]. This model used data from the Cancer and Steroid Hormone study to assess breast cancer risk in women with a family history of breast cancer^[47]. The c-statistic for the Claus model is approximately 0.56^[48].

Mendelian models outperform epidemiologic models, owing to the high penetrance of BRCA gene mutations. BRCAPRO is a computer model developed by the University of Texas Southwestern Medical Center and Duke University that incorporates six unique predictive models to assess a woman's risk of developing breast cancer or carrying a deleterious BRCA gene mutation^[49,50]. The c-statistic for BRCAPRO is 0.76-0.92^[51,52] but when compared to experienced risk counselors, sensitivity for identifying BRCA gene mutation carriers were similar^[53].

The Tyrer-Cuzick model incorporates personal, familial and genetic risk factors in a comprehensive way to compare a woman's personal risk of developing breast cancer in 10 years with that of the population^[54]. The model accounts for BRCA genes, low penetrance genes, family history and personal risk factors such as age, age at menarche, age at first birth, menopausal state, body mass index and use of hormonal therapy. This model is considered to be one of the most accurate models in predicting a woman's risk for cancer with a c-statistic of 0.762, but may overestimate risk in patients with atypia^[55,56].

Most risk factors for breast cancer are fairly weak, ubiquitous or not yet known, making the prediction models that examine epidemiological risk factors inherently difficult. Advances in genomic sequencing, biomarker identification and genetic testing may improve the accuracy of these quantitative risk prediction models in the future.

SCREENING

Breast self- and clinical breast examination

Utility of the breast self-examination (BSE) is controversial as the benefit in terms of decreased mortality has not been demonstrated^[57]. Most clinicians encourage women to perform monthly BSE to become familiar with their normal anatomy and empower them with regards to their own healthcare^[58]. The 2013 NCCN guidelines recommend annual clinical breast examination (CBE) for women of average risk > 40 years of age as well as BSE to develop and exhibit breast self-awareness^[59].

Mammography

One of the most important advances in the treatment of breast cancer is early detection of non-palpable masses. In the 1960's, the first randomized control trials comparing periodic mammography screening *vs* clinical examination demonstrated a decreased mortality by approximately one third in the experimental group. However there is still controversy regarding mortality from breast cancer in the subset of women aged 40-49 years^[60-62]. Contemporary randomized control trials have demonstrated the benefits from screening mammography in women aged 40 to 70

years^[63-65]. A 2013 Cochrane Review suggests that mortality is an outcome biased toward screening, routine mammography leads to undue stress and uncertainty in the face of false-positive results with increase in total numbers of lumpectomies and mastectomies but no decrease in mortality^[66]. Controversy surrounding mammography is related to the inherent lead time and length time biases in screening for disease. Lead time bias is an overestimation of survival among screen detected cases compared to clinically detected cases when true survival time actually remains unchanged. Length bias is an overestimation of survival time among screening-detected cases, which is caused by those slowly progressing cases that may never be clinically relevant. The 2013 NCCN guidelines recommends annual screening mammography in women ≥ 40 years of average risk and annual mammography at age 25 or individualized based on onset of cancer in proband in patients who are high risk by prediction models, known history or genetic predisposition syndrome as well as the counseling and education of risks and benefits related to participating in cancer screening^[59].

Magnetic resonance imaging

Mammography remains the gold standard for breast imaging but magnetic resonance imaging (MRI) has become an important modality in the detection, assessment, staging, and management of breast cancer in selected patients. Screening MRI is more sensitive but less specific for the detection of cancer in high risk women. The sensitivity of MRI is 0.77-0.79 compared to mammographic sensitivity of 0.33-0.39. Specificity of MRI is 0.86-0.89 compared to mammographic specificity of 0.95^[67,68]. In a systematic review, MRI and mammography demonstrated a combined sensitivity and specificity of 0.94 and 0.77, respectively^[67]. The 2013 NCCN guidelines recommend patients who have increased ($> 20\%$) lifetime risk of developing breast cancer undergo annual mammography and MRI starting at age 25 or an age tailored to the risk of the patient on an individual basis. MRI is valuable in the screening of select high risk patients, patients in whom breast augmentation prevents effective screening mammography, or in patients with equivocal findings on other imaging modalities.

Ultrasound

There are several studies supporting the use of adjunctive screening ultrasound in high risk patients with dense breast tissue, which imparts a substantial but accepted number of false positives^[69]. No randomized controlled trials have been conducted to evaluate the impact of screening ultrasonography on breast cancer mortality rates. Whole breast ultrasound may allow the clinician to screen for breast cancers not detected by traditional mammography, especially in dense breasts where mammographic sensitivity is lower^[70]. Single center studies have shown that the incremental detection of breast cancer by ultrasound following screening mammogram offers only marginal added benefit in women of average

risk^[71].

DIAGNOSIS

History and physical examination

The clinical history is directed at assessing cancer risk and establishing the presence or absence of symptoms indicative of breast disease. It should include age at menarche, menopausal status, previous pregnancies and use of oral contraceptives or post-menopausal hormone replacements. A personal history of breast cancer and age at diagnosis, as well as a history of other cancers treated with radiation. In addition, a family history of breast cancer and/or ovarian cancer in a first-degree relative should be established. Any significant prior breast history should be elucidated including previous breast biopsies. After the estimated risk for breast cancer has been determined (see above), the patient should be assessed for specific symptoms like breast pain, nipple discharge, malaise, bony pain and weight loss.

Physical examination should include a careful visual inspection with the patient sitting upright. Nipple changes, asymmetry and obvious masses should be noted. The skin must be inspected for changes such as; dimpling, erythema, peau d'orange (associated with local advanced or inflammatory breast cancer). After careful inspection and with the patient in the sitting position the cervical, supraclavicular and axillary lymph node basins are palpated for adenopathy. When palpable the size, number and mobility should be ascertained. Palpation of the breast parenchyma itself is performed with the patient supine and the ipsilateral arm placed over the head. The subareolar (central quadrant) and each quadrant of both breasts is palpated systematically. Masses are noted with respect to their size, shape, location, consistency and mobility.

DIAGNOSTIC IMAGING

The initial choice of imaging should be individualized to each patient based on the age and characteristics of the lesions. Diagnostic imaging and image-guided needle biopsies play a central role in the diagnosis, treatment planning, and staging of patients with breast cancer.

Mammography

Mammography remains the mainstay in breast cancer detection^[72]. Diagnostic mammograms are performed in women who have a palpable mass or other symptom of breast disease, a history of breast cancer within the preceding 5 years, or have been recalled for additional imaging from an abnormal screening mammogram. Diagnostic mammograms include special views such as focal compression of one area of the breast tissue or magnification images. The breast imaging reporting and database system (BI-RADS) is the standardized method for reporting of mammographic findings^[73]. Carcinomas present as masses, asymmetries, and calcifications (Table 1). By definition, a mass is a space-occupying lesion seen in

Table 1 Breast imaging reporting and database system^[73]

Category	Assessment	Follow up
0	Need additional imaging evaluation	Additional imaging needed before a category can be assigned
1	Negative	Continue annual screening mammograms (women older than 40 yr)
2	Benign	Continue annual screening mammograms (women older than 40 yr)
3	Probably benign	Initial short term follow-up (usually six month) mammogram (< 2% chance of malignancy)
4	Suspicious abnormality	Biopsy should be considered (2%-95% chance of malignancy)
5	Highly suggestive of malignancy	Requires biopsy (> 95% chance of malignancy)
6	Known cancer	Biopsy-proven malignancy

two different planes. This is distinguished from a density, which is seen only in a single plane. The shape of masses is described as round, oval, lobular, or irregular, while the margins are identified as circumscribed (with well-defined margins), indistinct, and spiculated (with densities radiating from the margins). Calcifications associated with benign disease are generally larger than those seen with malignancy and typically are coarse (round, lucent centered, or “layering” on medial lateral or lateral medial images). Clustered amorphous, indistinct, pleomorphic (or heterogeneous), or fine, linear, or branching calcifications are more typical of carcinomas.

MRI

Breast MRI has become an integral part of breast cancer diagnosis and management in selected patients. Current indications for breast MRI include evaluation of patients in whom mammographic evaluation is limited by augmentation (including silicone and saline implants and silicone injections), determining the extent of disease at the time of initial diagnosis of breast cancer (including identification of invasion of the pectoralis major, serratus anterior, and intercostal muscles), evaluation of inconclusive findings on clinical examination, mammography, and/or ultrasonography, screening of the contralateral breast in selected patients with newly diagnosed breast carcinoma, and asymptomatic screening of patients at very high risk of breast carcinoma (in conjunction with routine mammography). Other uses of breast MRI include evaluation of response to neoadjuvant chemotherapy with imaging before, during, and/or after treatment, and identification of residual disease in patients with positive margins after lumpectomy.

Ultrasound

The current indications for breast ultrasonography include palpable findings (including as the initial imaging test of palpable findings in patients who are younger than 30 years, pregnant, or lactating), abnormalities or suspected abnormalities on mammography or MRI, problems with breast implants, suspected underlying mass in the setting of microcalcifications or architectural distortion on mammography, supplemental screening in women at high risk for breast cancer who are not candidates for or do not have easy access to MRI, and suspected axillary lymphadenopathy. Real-time imaging is also possible with ultrasonography, making it ideal for interventional pro-

cedures. Breast ultrasound imaging should be performed with a high-resolution real-time linear array transducer with a center frequency of at least 10 MHz, using the highest frequency with which adequate penetration of the tissue is feasible.

PROGNOSTIC INDICATORS

Estrogen receptor and progesterone receptor status

Estrogen receptor (ER) and progesterone receptor (PR) represent weak prognostic factors for patients with breast cancer, but these receptors are the strongest predictive factors for response to endocrine therapy. ER and PR assays should be performed on all invasive breast cancers^[74]. Both ER and PR are assessed by immunohistochemistry (IHC) on paraffin sections. IHC allows assessment of the expression specifically in either invasive or *in situ* cancer. Positive interpretation requires at least 1% of tumor cells showing positive nuclear staining of any intensity. Receptor negative is reported if less than 1% of tumor cells show staining of any intensity^[75]. The cutoff to define positivity is 1% because patients with even 1% ER/PR-positive tumors may benefit from hormonal therapy. About 70% of all breast cancers are ER-positive and 60% to 65% of all breast cancers are PR-positive. For the patients with a “weak positive” result an Allred score helps differentiate positive from negative receptor status. The Allred score categorizes the percentage of cells (scored from 0 to 5) with the intensity (scored from 0 to 3) and adds these two scores to give a numerical score from 0 to 8^[76]. A score of 0-2 was regarded as negative and 3-8 as positive.

HER2 protein expression and gene amplification

HER-2/neu is a proto-oncogene that encodes for a transmembrane tyrosine kinase growth receptor, and it is involved in several regulatory pathways in breast, involving proliferation, survival, cell motility, and invasion. HER2 is usually assessed by IHC. Fluorescence *in situ* hybridization (FISH) assay of HER2 expression is usually performed when the evaluation by IHC is equivocal. HER2 is a prognostic factor for outcome in both node-negative and node-positive patients and is a predictive factor for response to certain therapies that target the HER-2/neu receptor such as trastuzumab (Herceptin), a monoclonal antibody targeted to the HER2 protein, and other newer anti-HER2 agents.

Overexpression/amplification is reported in 10% to 34% of invasive breast cancers. Gene over expression and amplification and surface membrane protein expression are concordant in more than 90% of cases^[77,78].

Commercially available gene assays

OncotypeDX (Genomic Health, Inc, Redwood City, California) is a reverse transcription polymerase chain reaction-based assay that can be performed on paraffin sections. It is based on analysis of the expression of 21 genes and provides a “recurrence score” that correlates with outcome. Although it was initially used to assess prognosis in ER-positive, node-negative patients^[79], data have indicated that it is an equally valuable prognostic indicator in ER-positive, node-positive patients.

Another molecular profiling product is the Amsterdam 70-gene profile, Mammaprint (Agendia, Amsterdam, Netherlands), in which a microarray analysis of gene expression is used on breast cancer tissue. It is used to determine the prognosis of patients with breast cancer and can be used for all tumors, including node-positive, *HER-2 neu*-positive, and ER/PR-negative disease^[80].

MANAGEMENT

After a breast cancer has been diagnosed, the patient is clinically staged using the American Joint Commission on Cancer (AJCC) guidelines (Tables 2 and 3).

Several landmark trials with decades of follow-up form the foundation of contemporary breast surgery. The National Surgical Adjuvant Breast and Bowel Project (NSABP) B-04 trial compared radical mastectomy (RM) to total mastectomy (TM) with or without radiation therapy in a prospective randomized fashion. In the TM arm, axillary dissection was performed only if lymph nodes were positive. The investigators reported no difference in either group with regard to disease-free survival, relapse-free survival, distant-disease-free survival, or overall survival, confirming no advantage to RM. The NSABP B-06 trial prospectively randomized women with tumors less than 4 cm to mastectomy, lumpectomy, or lumpectomy with radiation. All women had an ALND regardless of treatment assignment or nodal status; negative margins, defined as no tumor at ink, were required. The 20-year follow-up data were published in 2002; the investigators found no difference in disease-free, distant-disease-free, or overall survival between any of the treatment arms^[81,82]. The data did demonstrate, however, a significant reduction in local recurrence (LR) after lumpectomy with the addition of radiation therapy (39.2% *vs* 14.3%, $P < 0.001$). The National Institutes of Health (NIH) issued a Consensus Conference statement in 1990 recommending BCT as the preferred surgical treatment of women with early stage breast cancer^[83].

Contraindications to BCT exist and are classified as absolute or relative. Absolute contraindications include multicentric disease (tumors in more than one quadrant of the breast), diffuse malignant-appearing calcifications,

inflammatory breast cancer, prior radiation to the chest or breast or inability to receive radiation, persistent positive margins despite appropriate attempts for breast-conserving surgery, and the need for radiation during pregnancy. Skin dimpling, nipple and areolar retraction, and tumor location are not contraindications to BCT, yet these should be considered in the preoperative assessment, specifically with respect to the ability to achieve negative margins.

Achieving negative surgical margins is a hallmark of successful BCT because this is associated with a lower rate of LR. However, what constitutes a negative margin remains a matter of considerable debate. The NSABP has long defined a negative margin as “no tumor at ink” regardless of the proximity of the nearest tumor cell. Historically, other series have argued that margins of more than 1 mm, more than 2 mm, more than 5 mm, or even more than 10 mm provide better local control. A recent meta-analysis reviewed 21 studies and 14571 patients undergoing BCT^[84]. Data demonstrate a significant increase in LR for positive margins with an odds ratio (OR) of 2.42 ($P < 0.001$) compared with negative margins. Direct comparison between different margin widths found no statistically significant improvement in local control. Although a weak trend was identified suggesting declining LR with increasing margin distance, this trend disappeared after adjustments for radiation boost treatment and endocrine therapy.

Neo-adjuvant chemotherapy increases eligibility for breast-conserving surgery, especially in patients presenting with locally advanced breast cancer or in borderline cases whereby the tumor-to-breast size ratio will not allow for excision and acceptable cosmetic results. NSABP B-1840 established the efficacy of neo-adjuvant therapy randomizing women with early stage breast cancer to 4 cycles of neo-adjuvant or adjuvant doxorubicin plus cyclophosphamide. An updated analysis with more than 16 years of follow-up demonstrates no difference in overall survival, disease-free survival, or event-free survival between the two arms^[85]. Women receiving neo-adjuvant therapy had a higher rate of pathologic negative axillary lymph nodes at surgery and a higher rate of BCT.

Radiation therapy plays a crucial role in successful BCT and has long been recognized to reduce LR risk by approximately 50%. The 2005 Early Breast Cancer Trialists' Collaborative Group's (EBCTCG) overview analyses demonstrated the influence of local control on long-term survival^[86]. With regard to BCT, the EBCTCG collectively analyzed data from 10 trials of 7300 women and found the risk of LR at 5 years to be significantly reduced from 26% after lumpectomy alone to 7% after lumpectomy with radiation therapy, an absolute reduction of 19%. The EBCTCG recently updated this data in 2011, expanding their analysis to 17 randomized trials of 10801 women undergoing breast-conserving surgery with and without radiotherapy. This meta-analysis again confirmed that radiation therapy resulted in an overall absolute reduction in LR of 15.7% at 10 years compared with those

Table 2 American Joint Commission on Cancer guidelines—tumor node metastasis classification

Primary tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma <i>in situ</i>
Tis (DCIS)	Ductal carcinoma <i>in situ</i>
Tis (LCIS)	Lobular carcinoma <i>in situ</i>
Tis (Paget's)	Paget's disease of the nipple
T1	Tumor ≤ 20 mm in greatest dimension
T1mi	Tumor ≤ 1 mm in greatest dimension
T1a	Tumor > 1 mm but ≤ 5 mm in greatest dimension
T1b	Tumor > 5 mm but ≤ 10 mm in greatest dimension
T1c	Tumor > 10 mm but ≤ 20 mm in greatest dimension
T2	Tumor > 20 mm but ≤ 50 mm in greatest dimension
T3	Tumor > 50 mm in greatest dimension
T4	Tumor of any size with direct extension to the chest wall and/or to the skin (ulceration or skin nodules)
T4a	Extension to the chest wall, not including only pectoralis muscle adherence/invasion
T4b	Ulceration and/or ipsilateral satellite nodules and/or edema (including peau d'orange) of the skin, which do not meet the criteria for inflammatory carcinoma
T4c	Both T4a and T4b
T4d	Inflammatory carcinoma
Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed (for example, previously removed)
N0	No regional lymph node metastases
N1	Metastases to movable ipsilateral level I, II axillary lymph node(s)
N2	Metastases in ipsilateral level I, II axillary lymph nodes that are clinically fixed or matted; or in clinically detected ipsilateral internal mammary nodes in the absence of clinically evident axillary lymph node metastases
N2a	Metastases in ipsilateral level I, II axillary lymph nodes fixed to one another (matted) or to other structures
N2b	Metastases only in clinically detected ipsilateral internal mammary nodes and in the absence of clinically evident level I, II axillary lymph node metastases
N3	Metastases in ipsilateral/intraclavicular (level III axillary) lymph node(s) with or without level I, II axillary lymph node involvement; or in clinically detected ipsilateral internal mammary lymph node(s) with clinically evident level I, II axillary lymph node metastases; or metastases in ipsilateral supraclavicular lymph node(s) with or without axillary or internal mammary lymph node involvement
N3a	Metastases in ipsilateral/intraclavicular lymph node(s)
N3b	Metastases in ipsilateral internal mammary lymph node(s) and axillary lymph node(s)
N3c	Metastases in ipsilateral supraclavicular lymph node(s)
Distant metastases (M)	
M0	No clinical or radiographic evidence of distant metastases
cM0(i +)	No clinical or radiographic evidence of distant metastases, but deposits of molecularly or microscopically detected tumor cells in circulating blood, bone marrow, or other nonregional nodal tissue that are no larger than 0.2 mm in a patient without symptoms or signs of metastases
M1	Distant detectable metastases as determined by classic clinical and radiographic means and/or histologically proven larger than 0.2 mm

not receiving radiation (19.3% *vs* 35.0%, $P < 0.00001$, two-tailed); this translated into an absolute reduction in breast cancer death of 3.8% at 15 years^[87].

LR after BCT can be described as: (1) a true recurrence, one within the primary tumor bed; (2) a marginal miss, one within the same quadrant just outside of the tumor bed; and (3) an elsewhere recurrence, one in a separate quadrant of the breast. Generally, true recurrences and marginal misses account for 46% to 91% of all LRs and tend to occur earlier than elsewhere recurrences^[88]. The EBCTCG demonstrates that more than 75% of all recurrences occur within 5 years^[86]. Risk factors for LR include positive margins, young age, ER-negative receptor status, larger tumor size, positive nodes, and lymphovascular invasion^[89,90]. Systemic therapy, especially targeted therapy, reduces the risk of LR. For example, the adjuvant trastuzumab trials demonstrate that patients receiving trastuzumab had a 50% reduction in LR^[91].

Similarly, Mamounas and colleagues evaluated LR in estrogen receptor-positive patients enrolled in NSABP B-14 and NSABP B-20 according to the 21-gene recurrence score assay (Oncotype DX, Genomic Health, Redwood City, California, United States)^[92]. At 10 years, tamoxifen significantly reduced the risk of LR in the low-risk group from 10.8% to 4.3% ($P < 0.001$). The addition of chemotherapy further reduced LR to 1.6% in that group ($P = 5.028$).

LOCOREGIONAL TREATMENT OF CLINICAL STAGE I, II A, OR II B DISEASE OR T3, N1, M0

Lumpectomy with surgical axillary staging

Negative axillary nodes: Radiation therapy to whole breast with or without boost (by photons, brachytherapy,

Table 3 Clinical staging-American Joint Commission on Cancer guidelines

Stage 0	Tis	N0	M0
Stage I A	T1	N0	M0
Stage I B	T0	N1mi	M0
	T1	N1mi	M0
Stage II A	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
Stage II B	T2	N1	M0
	T3	N0	M0
	T4	N0	M0
Stage III A	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
Stage III B	T3	N2	M0
	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage III C	Any T	N3	M0
Stage IV	Any T	Any N	M1

or electron beam) to tumor bed or consideration of partial breast irradiation (PBI) in selected patients. Radiation therapy should follow chemotherapy when chemotherapy is indicated.

One-three positive axillary nodes: Radiation therapy to whole breast with or without boost (by photons, brachytherapy, or electron beam) to tumor bed following chemotherapy when chemotherapy is indicated. Strongly consider radiation therapy to infraclavicular region and supraclavicular area. Strongly consider radiation therapy to internal mammary nodes. Radiation therapy should follow chemotherapy when chemotherapy is indicated.

> Four positive axillary nodes: Radiation therapy to whole breast with or without boost (by photons, brachytherapy, or electron beam) to tumor bed, infraclavicular region and supraclavicular area. Strongly consider radiation therapy to internal mammary nodes. Radiation therapy should follow chemotherapy when chemotherapy is indicated.

Total mastectomy with surgical axillary staging ± reconstruction

No radiation therapy: Negative axillary nodes and tumor ≤ 5 cm and margins ≥ 1 mm.

Consider postchemotherapy radiation therapy to chest wall: Negative axillary nodes and tumor ≤ 5 cm and close margins (< 1 mm).

Strongly consider radiation therapy to internal mammary nodes: Negative axillary nodes and tumor > 5 cm or margins positive: Consider radiation therapy to chest wall \pm infraclavicular.

One-three positive axillary nodes: Strongly consider

postchemotherapy radiation therapy to chest wall + infraclavicular and supraclavicular areas; if radiation therapy is given, strongly consider internal mammary node radiation therapy.

\geq Four positive axillary nodes: Postchemotherapy radiation therapy to chest wall + infraclavicular and supraclavicular areas. Strongly consider radiation therapy to internal mammary nodes.

MASTECTOMY

Approximately 30% to 40% of breast cancer patients in the United States are candidates for mastectomy, either because they are not eligible for BCT or the patients choose mastectomy.

The types of mastectomy available are: TM or simple mastectomy: removal of the breast, overlying skin, and the nipple and areolar complex; SSM or skin-sparing: same as TM or simple mastectomy but sparing as much skin as possible and the infra-mammary fold for immediate or delayed reconstruction; MRM or modified radical mastectomy: same as TM but within continuity axillary lymph node dissection.

NSM: SSM technique also saving the areola and/or nipple; RM or radical mastectomy which includes removal of the pectoralis muscles and level III axillary nodes. Mastectomy is usually done in conjunction with sentinel node biopsy. Prophylactic mastectomy (PM) is a term that applies to mastectomy when there is no cancer in the breast.

MANAGEMENT OF THE AXILLA

The status of the axillary lymph nodes is one of the most important factors impacting overall prognosis and treatment decision-making for breast cancer. Complete axillary lymph node dissection (ALND), or removal of level I and II axillary nodes was the standard surgical approach to invasive breast cancer until recently. Now this operation is reserved for patients with clinically positive lymph nodes confirmed on needle biopsy at initial evaluation, or when a clinically negative axilla is evaluated by ultrasound, found to have a suspicious node and this is confirmed by needle biopsy. In patients with a clinically and radiologically negative axilla, a sentinel lymph node (SLN) biopsy can be performed safely at the time of mastectomy or lumpectomy, sparing patients the morbidity associated with ALND.

The sentinel node is based on the concept that breast cancers drain to a single node or nodes, the sentinel nodes, before draining to more distal nodes. One of the earliest randomized trials examining the use of SLN biopsy in breast cancer was reported by Veronesi *et al.*^[93] in 2003. They randomized 516 patients with breast cancer with tumors less than 2 cm in diameter to receive a SLN biopsy followed by routine ALND or SLN biopsy followed by an ALND only if the SLN contained metas-

tases. After 10 years of follow-up, no differences were observed between the groups for local axillary recurrence (0% in the SLN biopsy group *vs* 2% in the ALND group) or disease-free survival (89.9% *vs* 88.8%)^[94].

Recent research has questioned whether all patients with a positive SLN require a completion ALND. In patients with clinically node-negative disease, the SLN is the only involved node in 60% to 70% of patients, which raises the question as to whether ALND offers additional therapeutic benefit for all patients^[95].

This question was addressed prospectively in the ASOCOG Z0011 trial^[96]. Patients with T1 and T2 tumors undergoing lumpectomy who were found to have metastatic disease in the SLNs were randomized to undergo either ALND or no further treatment of the axilla. All patients were required to have negative margins in the breast excision, and went on to have whole-breast irradiation. Adjuvant treatment was per the primary team, with 96% receiving chemotherapy and 47% endocrine therapy. The trial closed early because of low accrual and events rates, reaching only 47% of its accrual goals (891 patients enrolled). Median follow-up for the evaluable patients was 6.3 years. At 5 years, the local recurrence rate was 1.6% in the SLN biopsy group compared with 3.1% in the ALND arm. There was also no difference in 5-year disease-free survival (89.9% *vs* 88.8%). The authors concluded that for select patients with node-positive breast cancer and low-volume axillary disease, a SLN biopsy alone does not result in inferior survival or inadequate local control.

ADJUVANT THERAPY

A multidisciplinary approach to the treatment of breast cancer has been fundamental for the recent advances in the management of this disease. A meta-analysis conducted by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG), which included randomized clinical trials conducted since adjuvant therapies became widely used in the 1990s, reported a decrease in annual relative risk of relapse and mortality of 23% and 17% respectively^[97]. The purpose of adjuvant systemic therapy is to improve the disease-free survival (DFS) and overall survival (OS) rates associated with treatment of BC by local therapies (surgery and/or radiation) alone. The high rates of recurrence are probably related to the presence of micrometastatic disease in 10%-30% of LN-negative and in 35%-90% of LN-positive patients at the time of diagnosis^[98,99]. Adjuvant chemotherapy helps eradicate residual local or distant residual microscopic metastatic disease. The addition of taxanes (paclitaxel and docetaxel) to the standard anthracycline based chemotherapy has been studied extensively and has shown a significant reduction of 17% in the risk of recurrence^[100,101]. A meta-analysis demonstrated that taxane-based regimens provide both DFS and OS benefit with an absolute 5-year risk reduction of 5% for DFS and 3% for OS when compared with standard anthracycline regimens irrespective of ER

status, LN status, and age. Additionally, the improvements in DFS and OS were similar for both paclitaxel and docetaxel^[102].

Current guidelines for adjuvant hormonal and chemotherapy after surgical treatment for invasive breast cancer vary depending on hormone receptor positivity or negativity and expression of HER-2/neu. Applicable practice guidelines are reproduced from the NCCN Breast Cancer Practice Guidelines below^[103].

Hormone receptor-positive

Her2-positive disease: pT1, pT2, or pT3; and pN0 or pN1mi (≤ 2 mm axillary node metastasis): (1) Tumor ≤ 0.5 cm or microinvasive (pN0, consider adjuvant endocrine therapy; pN1mi, adjuvant endocrine therapy or adjuvant chemotherapy with trastuzumab followed by endocrine therapy); (2) Tumor 0.6-1.0 cm, adjuvant endocrine therapy \pm adjuvant chemotherapy with trastuzumab; (3) Tumor > 1 cm, adjuvant endocrine therapy + adjuvant chemotherapy with trastuzumab; and (4) Node positive (one or more metastases > 2 mm to one or more ipsilateral axillary lymph nodes), adjuvant endocrine therapy + adjuvant chemotherapy with trastuzumab.

Her2-negative disease: pT1, pT2, or pT3; and pN0 or pN1mi (≤ 2 mm axillary node metastasis): (1) Tumor ≤ 0.5 cm or microinvasive (pN0, consider adjuvant endocrine therapy; pN1mi, adjuvant endocrine therapy or adjuvant chemotherapy with trastuzumab followed by endocrine therapy); (2) Tumor > 0.5 cm, consider 21-gene RT-PCR assay (not done, adjuvant endocrine therapy \pm adjuvant chemotherapy; low recurrence score (< 18); adjuvant endocrine therapy; intermediate recurrence score (18-30), adjuvant endocrine therapy \pm adjuvant chemotherapy; high recurrence score (≥ 31), adjuvant endocrine therapy + adjuvant chemotherapy; node positive (one or more metastases > 2 mm to one or more ipsilateral axillary lymph nodes), adjuvant endocrine therapy + adjuvant chemotherapy).

Hormone receptor-negative

Her2-positive disease: pT1, pT2, or pT3; and pN0 or pN1mi (≤ 2 mm axillary node metastasis): (1) Tumor ≤ 0.5 cm or microinvasive (pN0, no adjuvant therapy; pN1mi, consider adjuvant therapy with trastuzumab); (2) Tumor 0.6-1.0 cm, consider adjuvant chemotherapy with trastuzumab; and (3) Tumor > 1 cm, adjuvant chemotherapy with trastuzumab.

Her2-negative disease: pT1, pT2, or pT3; and pN0 or pN1mi (≤ 2 mm axillary node metastasis): (1) Tumor ≤ 0.5 cm or microinvasive (pN0, no adjuvant therapy; pN1mi, consider adjuvant chemotherapy); (2) Tumor 0.6-1.0 cm-Consider adjuvant chemotherapy; (3) Tumor > 1 cm-Adjuvant chemotherapy; and (4) Node positive (one or more metastases > 2 mm to one or more ipsilateral axillary lymph nodes), adjuvant chemotherapy.

More recently, the development of genomic profil-

ing techniques has identified gene expression patterns in breast tumors with distinct molecular profiles, pathologic features, and clinical outcomes^[104,105]. Expression patterns have defined 4 different subtypes: luminal A and B (estrogen-sensitive BC), HER2-enriched, and basal-like tumors (negative ER/PR and negative HER2). Luminal A tumors are classified by positive ER/PR, negative HER2, and low Ki-67, whereas luminal B tumors characteristically have positive ER/PR, negative HER2, and high Ki-67^[106,107]. The additive prognostic value of Ki-67, a cell proliferation marker, to steroid and HER2 receptors is accepted, as many significant genes in gene expression profiles are proliferation related. Ki-67 marks the difference between luminal A and B tumors; however, Ki-67 is not yet routinely available and standard cutoffs are not well defined.

Adjuvant hormone therapy is considered standard in all patients with endocrine-sensitive tumors defined by the expression of ER and PR by IHC. Approximately 70% of BCs have positive expression of the ER and are considered hormone sensitive. Treatment with tamoxifen for 5 years reduces the risk of recurrence by 41% and BC mortality by 34%^[97].

In postmenopausal women, an Aromatase Inhibitor may be substituted because of the proven efficacy and the low risk of for development of endometrial cancer with this drug. The arimidex, tamoxifen, alone or in combination (ATAC) trial, is a pivotal trial in adjuvant hormone therapy^[108]. The ATAC trial compared the adjuvant use of anastrozole plus tamoxifen either alone in postmenopausal women with early-stage BC. At 10 years, anastrozole as initial therapy showed increased DFS (HR 0.86, $P = 5.003$), time to local and distant recurrence (HR 0.79, $P = 5.0002$; HR 0.85, $P = 5.02$, respectively), and reduced indices of contralateral BC (HR 0.62, $P = 5.003$) compared with tamoxifen. However, there was no significant difference in overall mortality between the 2 groups^[109].

Approximately 15% to 20% of all BCs present with amplification of the HER2 gene^[110]. HER2 over-expression is reported to be an independent predictor of poor prognosis. This can be addressed by the incorporation of anti-HER2 therapy with trastuzumab, a monoclonal antibody targeting the extracellular domain of the HER2 protein, which in the adjuvant setting has shown significant improvement in clinical outcomes from adjuvant chemotherapy plus trastuzumab compared with chemotherapy alone. Based on results from randomized clinical trials, a trastuzumab-containing regimen for up to 1 year is now considered standard for all patients with HER2-positive tumors larger than 1 cm^[111,112].

There are rapid advances being made with respect to systemic therapy targeting specific molecular targets like phosphatidylinositol 3-kinase vascular endothelial growth factor receptor, epidermal growth factor receptor and poly polymerase.

performed by de Bock *et al.*^[113] identified that 40% of recurrent cancers are diagnosed in asymptomatic patients during routine visits. This data stresses the importance of follow up and surveillance. Clinical evaluation including history and physical exam is recommended every four to six months for five years, then every twelve months with annual mammography. Women on tamoxifen should undergo annual gynecologic assessment if the uterus is present. Women on an aromatase inhibitor or who experience ovarian failure secondary to treatment should have monitoring of bone health with a bone mineral density determination at baseline and periodically thereafter. Patients should also be instructed to augment modifiable risk factors such as decreasing alcohol consumption, increasing physical activity and decreasing BMI.

CONCLUSION

There is a greater refinement in breast cancer care with increased specialization and collaboration amongst surgeons, oncologists, radiation oncologists, nurses, geneticist, reconstructive surgeons and patients. The effectiveness and benefits of a multidisciplinary approach to the treatment of breast cancer has been empirically demonstrated^[114,115]. In the future, there will be great value in genomic sequencing and proto-identification of women at risk for developing breast cancer.

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SURVEILLANCE AND FOLLOW-UP

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WJCO 5th Anniversary Special Issues (2): Breast cancer

Evolution of breast cancer therapeutics: Breast tumour kinase's role in breast cancer and hope for breast tumour kinase targeted therapy

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Abstract

There have been significant improvements in the detection and treatment of breast cancer in recent decades. However, there is still a need to develop more effective therapeutic techniques that are patient specific with reduced toxicity leading to further increases in patients' overall survival; the ongoing progress in understanding recurrence, resistant and spread also needs to be maintained. Better understanding of breast cancer pathology, molecular biology and progression as well as identification of some of the underlying factors involved in breast cancer tumourigenesis and metastasis has led to the identification of novel therapeutic targets. Over a number of years interest has risen in breast tumour kinase (Brk) also known as protein tyrosine kinase 6; the research field has grown and Brk has been described as a desirable therapeutic target in relation to tyrosine kinase inhibition as well as disruption of its kinase independent activity. This review will outline the current "state of play" with respect to targeted therapy for breast cancer, as well as discussing Brk's role in the processes underlying tumour development and metas-

tasis and its potential as a therapeutic target in breast cancer.

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Key words: Breast tumour kinase; Protein tyrosine kinase 6; Breast neoplasms; Targeted molecular therapy; Intracellular signaling peptides and proteins; Protein kinase inhibitors

Core tip: Breast tumour kinase/protein tyrosine kinase 6 is overexpressed in up to 86% of invasive breast cancers. It plays a key role in regulating a number of cell processes that are involved in the metastatic process. As a kinase involved in both epidermal growth factor and insulin like growth factors signaling, inhibiting its activity could prove to be an effective way to enhance the effects of current targeted treatments. In addition, disrupting the protein-protein interactions central for the kinase-independent aspects of its function may generate an alternative mechanism for selective targeting of breast cancer cells.

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INTRODUCTION

Exactly how breast cancer therapeutics has changed over the years is an intriguing aspect of the progress and evolution that has been made in research for cancer cures and treatments. Looking at the incidence and

mortality rates over the years in the United Kingdom and worldwide indicates the significant impact that continued improvements in detection and treatment of breast cancer have made. Statistically breast cancer incidence rates have been increasing since the 1970s^[1]. Approximately 75000 cases of breast cancer were diagnosed in 1975 compared to approximately 126000 in 2010^[1]. The increased frequency may be due to improved diagnostic techniques that contribute towards the rise in incidence; thus detecting cancers that may have remained unnoticed until much later. Breast cancer is the most common cancer in the United Kingdom with more than 48000 people being diagnosed each year^[1] and is the most recognized cause for the mortality rates of women aged between 33 and 55. This indicates room for improvement in relation to enhanced and effective therapeutic techniques that are patient specific, relatively non-toxic and contribute towards patients overall survival. Not only that, but there is ongoing progress needed towards understanding recurrence, resistant and spread^[2].

A closer look at breast cancer pathology, molecular biology and progression may lead to a better understanding of the underlying factors involved in breast cancer tumourigenesis and metastasis. A particular interest has risen in breast tumour kinase (Brk) also known as protein tyrosine kinase 6 (PTK6). Over a number of years the Brk research field has grown and Brk has been hinted at, as being a desirable therapeutic target in relation to tyrosine kinase inhibition as well as disruption of its kinase independent activity. Here, we outline Brk's role in the pathways essential for breast tumour development and discuss its potential as a therapeutic target in breast cancer.

BRK

Brk, also known as PTK6, was originally identified in a study involving human melanocytes and subsequently isolated from breast cancer cell lines in a study identifying novel kinases with therapeutic potential^[3]. The *ptk6* gene encodes the non-receptor tyrosine kinase, which consists of SH2, SH3 a linker region and catalytic domains. Total activity of Brk is significantly higher in malignancy than in normal mammary tissue, and over-expression of the protein has been noted in more than 80% of invasive ductal breast tumors^[4]. Brk expression so far has been detected in the majority of breast cancer cell lines with differing intensities^[5]. Gene sequencing indicated similarities with the SRC-family of protein tyrosine kinases, however there are distinct differences such as the lack of N-terminal extension and consensus sequences for fatty acylation and membrane association^[6]. Furthermore, its genomic structure is quite distinct from the SRC-family PTKs, which demonstrates an evolutionary divergence^[7]. Brk has also been shown to have a significant degree of similarity with the *Drosophila* src related gene known as Dsrc41, with six out of seven of Brk's exon boundaries

conserved with the Dsrc41 gene which has 9 exons. This could indicate Brk is likely to share a common ancestor with Dsrc41. The *ptk6* gene comprises 8 exons and encodes a 451 amino acid protein and FISH studies indicated localisation to chromosome 20q13.3^[6]. The protein product has a predicted molecular weight of 50 kDa, which generally resolves to around 48 kDa on an SDS-PAGE gel.

SH3 domains are small protein nodules made up of β -sheets. SH3 domains allow the assembly of specific protein complexes *via* proline-rich peptide binding, reviewed in^[8]. Brk's SH2 and SH3 domains are used for substrate recognition^[9,10]. The Brk SH3 domain has been known to undergo conformational changes due to pH fluctuations indicating that its structure could determine substrate and protein interaction, thus influencing its varied role in diverse cellular environments. The SH3 domain may have a role in enzyme regulation^[11]. The SH2 domain contains α/β folds and a phosphotyrosine binding surface with two α -helices opposite a central $\beta\alpha$ -sheet made up of four anti-parallel strands^[12]. This domain plays a role in protein-protein interactions and is important for regulation of catalytic activity^[10]. Due to the lack of myristoylation and a nuclear localization sequence, Brk's regulation is difficult to determine which allows for more flexibility with its subcellular localization, reviewed in^[13].

REASONS FOR NEW CANCER THERAPIES

Chemotherapy and radiotherapy have been recognised to target normal rapidly dividing cells such as bone marrow, gastrointestinal tract or hair follicles; the range and intensity of adverse effects reduces the specificity and increases toxicity of these therapies. Both these types of treatments therefore have a limited therapeutic index and can often be palliative in use as reviewed in^[14]. Hormonal therapies have also been in use; for example Tamoxifen, which has been used for early stage and metastatic breast cancer since its licence in 1972^[15]. Although proven to be effective against breast cancer, especially those that are oestrogen receptor positive, there are still many issues that need to be overcome for maximum effect of the drug to be achieved, whether by itself or as combination therapy. These include the diverse adverse toxicities such as thrombosis, strokes and development of secondary cancers. Other issues include resistance against Tamoxifen and subsequent recurrence in some patients. Furthermore, this drug is only effective against oestrogen or progesterone receptor positive breast cancers thus making it unsuitable for other types of breast cancer such as HER2 positive/ER/PR negative and triple negative breast cancers (reviewed in^[16]). However there are treatments available for HER2 positive cancers such as Herceptin, a monoclonal antibody that binds to HER2 thus negatively effecting receptor function, as well as a number of kinase inhibitors. Unfortunately the more advanced stages of

breast cancer do not always respond to Herceptin therapy and those that do, often progress in 12 mo from the start of the treatment^[17]. In addition resistance may occur due to the involvement of a number of signaling pathway molecules such as activation of the PI3K/AKT pathway, loss of PTEN and activation of PIK3CA, reviewed in^[18]. Brk is expressed in a wide range of cancer types and its expression appears to be independent of ER/PR/HER2 positivity, thus making it an ideal candidate for therapeutic intervention^[5,19].

TYROSINE KINASE INHIBITION

Targeted therapy is largely directed specifically towards tumour cells thus reducing many side effects and providing a wider therapeutic window. They can also be used in combination with traditional chemotherapy or radiotherapy to enhance anticancer effects. Further patient benefit includes convenience with oral consumption rather than intravenous administration as is the case for many chemotherapy drugs.

Tyrosine kinases have been implicated in a range of cancers; they are involved in cellular signalling and play an important role in growth factor signalling. In their active forms tyrosine kinases can promote tumour cell proliferation, growth and induce anti-apoptotic effects, as well as promote angiogenesis and tumour cell metastasis. Since most of these cellular events contribute to tumour progression and decreased patient prognosis, tyrosine kinases are considered ideal candidates for targeted therapy.

There are two main types of kinases; these can be categorized as receptor protein kinases that are generally membrane spanning, or non-receptor protein kinases that relay intracellular signals from the receptors and, of which, Brk is one. Briefly, when ligands bind to their cognate receptors, they stimulate receptor dimerization followed by autophosphorylation and activation of tyrosine kinase activity. As a result, multiple signalling pathways are activated and intracellular mediators in these pathways transduce signals from membrane receptors through the cytosol and into the nucleus which ultimately alters DNA synthesis and cell division as well as a wide range of biological processes as reviewed in^[14]. Protein tyrosine kinase activity within breast tumour tissues has been reported to be significantly higher in comparison with benign or normal breast tissues^[20]. For example, the tyrosine kinase activity of the product of c-src proto-oncogene has been found to be elevated in human breast tumors^[21], and several tyrosine kinase receptors have also been implicated in breast cancer development and progression.

These include members of the erbB Type 1 transmembrane receptor family such as epidermal growth factor receptor (EGFR) and HER2/neu transmembrane tyrosine kinase receptor, as well as receptors for insulin like growth factors (IGF) such as IGF-1R^[22,23]. Others include fibroblast growth factor receptor^[24] and met receptor tyrosine kinase^[25]. Of the substrates and proteins interacting with Brk^[13], many are linked to signalling from

these receptors, thus indicating a role for Brk in these signalling pathways.

EXAMPLES OF TYROSINE KINASE INHIBITORS IN BREAST CANCER- LAPATINIB, ERLOTINIB AND GETFINTIB

Gefitinib is an EGFR inhibitor, the first of its kind, that is also known to have an effect in HER2-overexpressing cell lines probably due to the reduction in phosphorylation of HER2/EGFR heterodimer^[26]. Gefitinib is especially effective in ER-positive and Tamoxifen resistant tumours indicating a target group of patients for this type of treatment^[27]. Erlotinib works in much the same way as Gefitinib, as a reversible EGFR inhibitor. It is clinically effective in locally advanced and metastatic non-small cell lung cancer^[28] but its benefit in breast is only recently becoming clearer, and there is some indication for Erlotinib in triple negative breast cancers^[29]. Erlotinib inhibits triple negative breast cancer as shown by Ueno and Zhang^[30] when they generated a SUM149 xenograft model by implanting luciferase expressing SUM149 cells into mammary pads of athymic nude mice. The results indicated significant inhibition of tumour growth at doses of 50 and 100 mg/kg.

There is a strong correlation between HER2 and EGFR in terms of dimerization, expression as well as activity^[31] since HER2 is EGFR's most common heterodimerization partner and HER2 potentiates EGFR signalling by enhancing EGF binding affinity^[32]. A dual inhibitor for HER2 and EGFR may therefore be of greater clinical benefit than individual therapies. When using an inhibitor with a single mode of action, therapeutic resistance can be increased whereas dual inhibition may reduce the chance of this happening^[32]. Other benefits of dual inhibition may include targeting a wider range of cancers since single inhibitors are, in some cases, specific for one particular type of cancer as well as a more effective inhibition in cancer cell growth overall^[33].

One such inhibitor already in use is Lapatinib, which reversibly inhibits the tyrosine kinase activity of both HER2 and EGFR, reviewed in^[32]. It is an orally available inhibitor that binds to EGFR in its inactive form in comparison to other EGFR inhibitors such as Erlotinib and Gefitinib, which bind EGFR in its active form. This allows for a greater duration of effect at the target site^[34]. So far Lapatinib has proven to be well tolerated with rare occurrences of high-grade toxicities. In relation to Brk, Lapatinib has been recognised to have reduced efficacy with increased levels of Brk in HER2-transfected mammary epithelial cells^[35]. This may suggest a role for Brk in acquired resistance against HER2 targeted therapy when both proteins are over-expressed. Targeting Brk alongside HER2 inhibition could overcome resistance as well as increase efficacy of HER2-targeted treatment.

Both Gefitinib and Erlotinib initially indicated no real

clinical benefit for metastatic breast cancer patients and had little efficacy in phase II studies^[27]. This indicates lack of correlation between EGFR expression level with response to treatment even though many breast cancer cells express EGFR^[36]. Previously there was strong evidence for combination therapy with tyrosine kinase inhibitors and hormone therapy^[32], thus allowing for a greater clinical benefit. However more recently, combination therapy using Gefitinib and anti-hormonal therapy (fulvestrant and anastrozole) demonstrated only a modest increase in clinical benefit compared to Gefitinib or hormonal therapy alone^[37].

Erlotinib resistance is linked to CDK2 activity; cell proliferation is induced in Erlotinib treated cells when CDK2 is expressed and, conversely, there is increased sensitivity to Erlotinib when CDK2 activity is suppressed. Erlotinib has also shown to upregulate p27 and nuclear translocation in association with cell growth inhibition in non-small cell lung cancer^[38]. Cyclin dependent kinases regulate cell cycle progression from quiescence to mitosis by activating the transcription factor E2F leading to activation of genes needed for progression from G₁ to S phase^[36]. Furthermore, in relation to breast cancer it has been shown that inhibition of the erbB2 pathway by EGFR inhibitors in erbB2-overexpressing breast cancer cell lines caused G₁ phase arrest with accumulation of p27 and reduced Cyclin D1^[39]. This is further verified by Nahta *et al.*^[40] who showed that downregulation of p27 in breast cancer cells was accompanied by an increased S-phase fraction and increased resistance to Herceptin therapy. The induction of p27 is therefore necessary in Erlotinib inhibition of cell growth.

Since cell cycle deregulation is implicated in development of neoplasia and may contribute towards development of breast cancer, as well as response to therapy the discovery that Brk is involved in cell cycle regulation was of importance^[41]. Brk deregulates cell cycle progression; an inverse correlation was shown between Brk expression levels and p27 expression levels. With increasing Brk expression there was reduced p27, and in cells lacking p27 and Fox03a, Brk induced a decreased level of cell proliferation indicating it may need the p27/Fox03a pathway to promote cell growth. This indicates the importance of Brk's role in cell cycle progression and suggests a role for Brk in mediating cell responses to EGFR/HER2 inhibitors through regulation of p27.

BRK AND HER2 INTERACTIONS

In cancer cells, alterations in HER receptors or in their downstream signalling components are known to occur; HER2 is a negative prognostic factor, the presence of which indicates aggressive phenotype and reduced overall survival rate as reviewed in^[42], although it should be noted that survival rates for HER2 positive cancers are improving due to the introduction of targeted therapies^[43].

HER2 is overexpressed in approximately 20%-30% of breast cancers^[44] and it has been suggested that Brk may be involved in regulation of signal transduction from HER tyrosine kinases. Several studies have shown a link between HER2/neu expression and Brk/PTK6^[35,45].

This is of interest because, as discussed above, HER2 targeted therapy although clinically proven to be effective does pose some restrictions such as lack of effect in some HER2/neu positive cancers as well as resistance. Therefore, Brk targeted therapy maybe of clinical benefit especially when used in combination with existing HER2/neu targeted therapy. Due to the strong correlation between PTK6 and HER2, there is evidence suggesting that it may also be linked with prognosis thus indicating a role for Brk as a prognostic factor in breast cancer. PTK6 expression studied in 426 breast cancer cases further supports its potential as a independent prognostic factor regardless of morphological and molecular markers such as lymph node involvement, tumour size and HER2 status^[46]. Co-expression of Brk and HER2 has been linked to co-amplification of both the *ErbB2* and *ptk6* genes^[35], although this is not a consistent finding in other patient studies. It is worth noting that although HER2 is over expressed in 20%-30% of breast cancers^[44], Brk is over expressed in upto 86% of breast cancers^[47], which indicates that in the majority of breast cancers Brk is over expressed independently of HER2 status.

At a protein level, Aubele and colleagues showed that PTK6 forms protein complexes with HER2 in paraffin tissues from invasive breast carcinomas^[48]. Recently, the effect of simultaneous knockdown of PTK6 and HER2 has been analysed in the herceptin-resistant cell line JIMT-1^[49]. The results indicated significant reduction in phosphorylation of important signalling intermediates, namely MAPK, ERK and p38MAPK and PTEN, which are involved in tumourgenesis. Furthermore there was reduced migration and invasion of JIMT-1 cells when expression of both proteins was suppressed. HER2 may also be involved in elevating Brk levels *via* upregulating calpastatin and inhibiting calpain-1 activity in breast cancer cells^[50].

These combined data further support the study of dual inhibition of Brk and HER2, firstly for a greater clinical effect and secondly to overcome anti-HER2 therapeutic resistance.

BRK AND EGFR INTERACTIONS

ErbB signalling in breast tumour progression has been extensively documented. EGFR tyrosine kinases have been involved in regulation of normal and abnormal cellular proliferation and survival (reviewed in^[51]). Both EGFR and HER2 are intrinsically linked; HER2 potentiates EGFR signalling by enhancing binding ability of EGF and reducing its degradation and studies have indicated that HER2 signalling is reduced *via* EGFR-specific inhibitors^[52].

The EGF receptor family are linked to the tumour-genic transformation of breast epithelial cells. When Brk Transfected mammary epithelial cell lines, MCF10A and Hb4a, were treated with human growth factors (EGF), mitogenic activity of Brk was observed. This reveals Brk's role in sensitizing human mammary epithelial cells to growth factors such as EGF^[53]. Furthermore, Brk association with the EGF receptor has also been detected, even in the absence of EGF, leading to proliferation of epithelial cells. EGFR expression also plays a role in keratinocyte differentiation^[54], a process in which Brk is involved^[55]. Inhibition of EGFR signalling during differentiation induces growth arrest, however Brk's promotion of EGFR signalling suggests that one of its roles may involve promoting cell survival during early differentiation.

EGF stimulation results in rapid phosphorylation of Brk indicating the involvement of Brk in the EGF signalling complex^[56]. EGF binding to its receptor, EGFR, not only induces its phosphorylation but also the phosphorylation of other EGFR family members (HER2, 3 and 4). The expression of Brk therefore not only increases phosphorylation of EGFR and HER2 but also of HER3 (erbB3) in breast cell lines^[56]. It may do this *via* direct interaction with an erbB3-containing complex in response to EGF stimulation. Brk's ability to enhance EGFR signalling could be mediated by inhibition of EGFR down-regulation^[57], which is achieved by phosphorylation of either ARAP-1^[58] or c-Cbl^[59], thereby prolonging EGFR signalling.

Expression of Brk enhances EGF-induced ErbB3 phosphorylation and recruitment of P13K to ErbB3 thus inducing P13K activity^[56]. Since the P13K pathway is implicated in breast cancer and is linked with resistance to HER2 targeted therapies, its interaction with Brk may give a wider overview of the mechanisms involved in developing resistance. P13K has been a desirable therapeutic target of its own and current therapies, as reviewed in^[60], focus on anti-mTOR agents, tyrosine kinase inhibitors and P13K inhibitors. However to create a greater clinical effect, combinations of these treatments including a potential Brk tyrosine kinase inhibitor maybe more useful and reduce the activation of compensatory feedback loops which could decrease efficacy of single agents. Since Akt is a known substrate of Brk, and Brk negatively regulates its phosphorylation in epithelial cells, the potential consequences of this therapy may need to be fully assessed^[61]. Nonetheless, due to most of the normal cells that express Brk being outside the proliferative areas of the tissues Brk targeted therapy may still be highly specific^[62].

Overall Brk has shown to prolong EGFR signalling, sensitise tumour cells to EGF stimulation, inhibit degradation of EGFR and reduce sensitivity of EGFR inhibitors in Brk overexpressing cells. This suggests Brk inhibition could reduce tumour proliferation and growth *via* reduced EGFR signalling and increase EGFR inhibitor sensitivity.

BRK AND IGF INTERACTIONS

Insulin receptor substrate 4 (IRS-4) is part of the insulin receptor substrate family, which also contains IRS-1, IRS-2 and IRS-3. Their function includes a variety of biological effects such as cell proliferation, growth, survival and differentiation downstream of insulin and insulin-like growth factor 1 (IGF-1) receptors^[63]. IRS-4, upon IGF-1 stimulation, is phosphorylated which leads it to binding to P13K activating the MAPK pathway. The link between insulin-like growth factor (IGF) and breast cancer has been well documented; the IGF-1 receptor is significantly overexpressed in tumour cell lines compared to normal breast cancer epithelial tissue and benign tumours^[64] and there is also a clear correlation between poor breast cancer prognosis and overexpression of IGF-1R^[65]. In the MCF-7 breast carcinoma cell line, IGF stimulation results in Akt activation leading to cell proliferation through phosphorylation of Raf kinase. Along with this, IGF stimulation leads to resistance to anoikis^[66], a process whereby epithelial cells undergo apoptosis due to loss of interaction with neighbouring cells and the basement membrane^[67]. Overexpression of IGF-1R is also implicated in resistance against breast cancer therapy, especially against Herceptin^[17].

Immunoprecipitation and mass spectrometry have shown IRS-4 substrate interaction with Brk in co-transfected HEK 293 cells^[68]. In addition to this interaction with IRS-4, Brk also co-precipitates with IRS-1 and IGF-IR^[66]. The interaction of Brk with IGF-IR as well as EGFR and HER2 gives an indication of the range of Brk activity in regulating signalling. EGFR, HER2 and IGF-1R are overexpressed in different subsets of breast cancers whereas Brk is overexpressed in majority of breast cancers and has a role in each of these signalling pathways, thus making it an attractive therapeutic target for disrupting signalling cross-talk. Since Brk interacts with IGF-1R, disrupting this association may also reduce the chance of developing resistance against current breast cancer therapies such as Herceptin, through HER2-IGF-R1 heterodimer formation resulting in phosphorylation and activation of HER2 as reviewed in^[69].

BRK AND ITS INTERACTION

ALTERNATIVELY SPLICED ISOFORM, ALT-PTK6

During the characterisation and chromosome mapping of Brk, an alternatively spliced short isoform was identified in T47D cells, which was originally called $\lambda m5$ and then later renamed to ALT-PTK6^[6,70]. ALT-PTK6 is 134 amino acids in size (15 kDa) and is expressed from an alternatively spliced transcript that has a 122 base pair deletion at the 3' end of the SH3 coding region; thus it lacks the SH2 and kinase domains and has an alternative C-terminal proline rich sequence (Figure 1). The protein

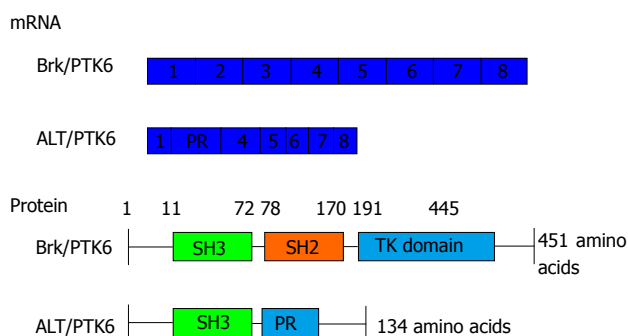


Figure 1 Breast tumour kinase domain structure. Full length Brk/PTK6 and short isoform ALT-PTK6 structure indicating the SH3 domain (green), SH2 domain (orange) and a tyrosine kinase domain or proline rich sequence (blue). Underneath is indicated the cDNA structure of both forms, with each box indicating each exon (total of 8 exons). ALT-PTK6 has deletion at exon 2 and lacks SH2 domain due to shift in open reading frame, which gives a proline rich sequence at exon 3 instead. PTK6: Protein tyrosine kinase 6; Brk: Breast tumour kinase.

level of ALT-PTK6 was lower than full length Brk in T47D breast cancer cells^[6]. There is still speculation of the role of ALT-PTK6 and the extent of its effect in the overall process of cancer pathology, however, there has been indication that the short isoform may act as a competitive inhibitor for the SH3 binding partners^[70] since both contain the SH3 domain^[6].

The Tyner laboratory were the first to describe a function of ALT-PTK6, albeit in prostate cancer cell lines^[70]. β -catenin has been identified as a direct substrate of Brk^[71] and ALT-PTK6 may influence Brk's ability to regulate β -catenin/TCF transcription^[70]. The Wnt/ β -catenin pathway promotes cancer cell growth and its deregulation is involved in pathogenesis of various cancers including breast cancer, reviewed by^[72]. ALT-PTK6 has also been shown to inhibit the phosphorylation of Brk to some extent, since the presence of ALT-PTK6 reduced PTK6 interaction with tyrosine-phosphorylated proteins in what appears to be a dose-dependent manner^[70].

Further to this, in co-transfection experiments, increased ALT-PTK6 expression resulted in an increase of constitutively active form of Brk in the nucleus with a decreased proportion at the membrane^[70]. There was also reduced proliferation in ALT-PTK6 expressing prostate tumour cells compared to those without this short isoform. These data therefore suggest that ALT-PTK6 may contribute towards limiting Brk localisation to the nucleus and acting as a competitive inhibitor thus reducing the ability of Brk to interact with its substrates that promote tumour cell growth.

The localisation of Brk therefore may play an important role in development of cancer since Brk's arrangement in the cellular environment may affect its role due to the variety of substrates available in nucleus and cytoplasm^[13]. Also Brk's role in various tissue types has shown to be dramatically different, for example, in normal tissues it is involved in regulation of differentiation process whereas in tumour cells it promotes cell proliferation and survival. Therefore, Brk's function may depend on the

tissue it is expressed in, its intracellular location and the substrate it interacts with^[70].

BRK'S ROLE IN CELL MIGRATION, TUMOUR FORMATION AND METASTASIS

To a large extent Brk is involved in breast cancer cell proliferation^[73], however it is also involved in cell migration. A reduced ability for T47D and JIMT-1 breast cancer cell migration was observed in response to Brk suppression^[49]. Paxillin is a multidomain protein that is recruited to edges of the cell once migration is initiated, as reviewed in^[74], and Brk has been recognised as a novel paxillin tyrosine kinase that binds to, as well as phosphorylates, paxillin^[75]. Brk also acts a mediator of EGF-induced paxillin phosphorylation, thus promoting activation of Rac1 and stimulating cell migration and invasion. KAP3A is a subunit of the kinesin-2 heterotrimeric complex and binds microtubule-based subunits KIF3A/3B to various cargo proteins, which enable membrane morphogenesis^[76]. KAP3A has also been identified as a substrate of Brk and is phosphorylated at its C-terminus, a process required for Brk-induced cell migration^[77]. Along with this, p190, another substrate of Brk, once phosphorylated is associated with p120 leading to Rho inactivation and Ras activation^[78]. Migratory effects of Brk are greatly impaired in cells lacking p190. These interactions indicate an important function of Brk in regulating tumour cell migration *via* various systems, thus a Brk-targeted therapy could potentially cause disruption in these processes.

STAT3 and STAT5 are also recognised as substrates of Brk and are involved in cellular processes leading to cell proliferation, migration and survival^[79-81]. STAT3 is regarded as an oncogene, with tyrosine phosphorylation of STAT3 connected to breast cancer development as discussed in^[79]. Activation of STAT3 by Brk may contribute towards cell transformation and uncontrolled growth in early stages of breast cancer. Brk also mediates STAT3 regulation in established tumours^[80], and constitutive activation of Brk accelerated cell migration and tumour growth *in vivo*^[82].

Angiogenesis has been recognised as an essential process in survival of cancerous cells *in vivo*; it is involved in tumour growth, progression and metastasis^[83]. One of the main pro-angiogenic factors involved in promoting tumour angiogenesis is vascular endothelial factor (VEGF)^[84]. Osteopontin is a secreted non-collagenous chemokine-like protein that regulates VEGF expression *via* a Brk/nuclear factor-kappaB (NF- κ B)/ATF-4 signalling cascade^[85]. Higher levels of expression of Brk, NF- κ B and ATF-4 correlated with higher tumour grades. As osteopontin is secreted from the bone and expressed in brain^[86,87], which are frequent sites for breast cancer metastasis^[88], this study provides a mechanism whereby Brk could be involved in the formation of metastases.

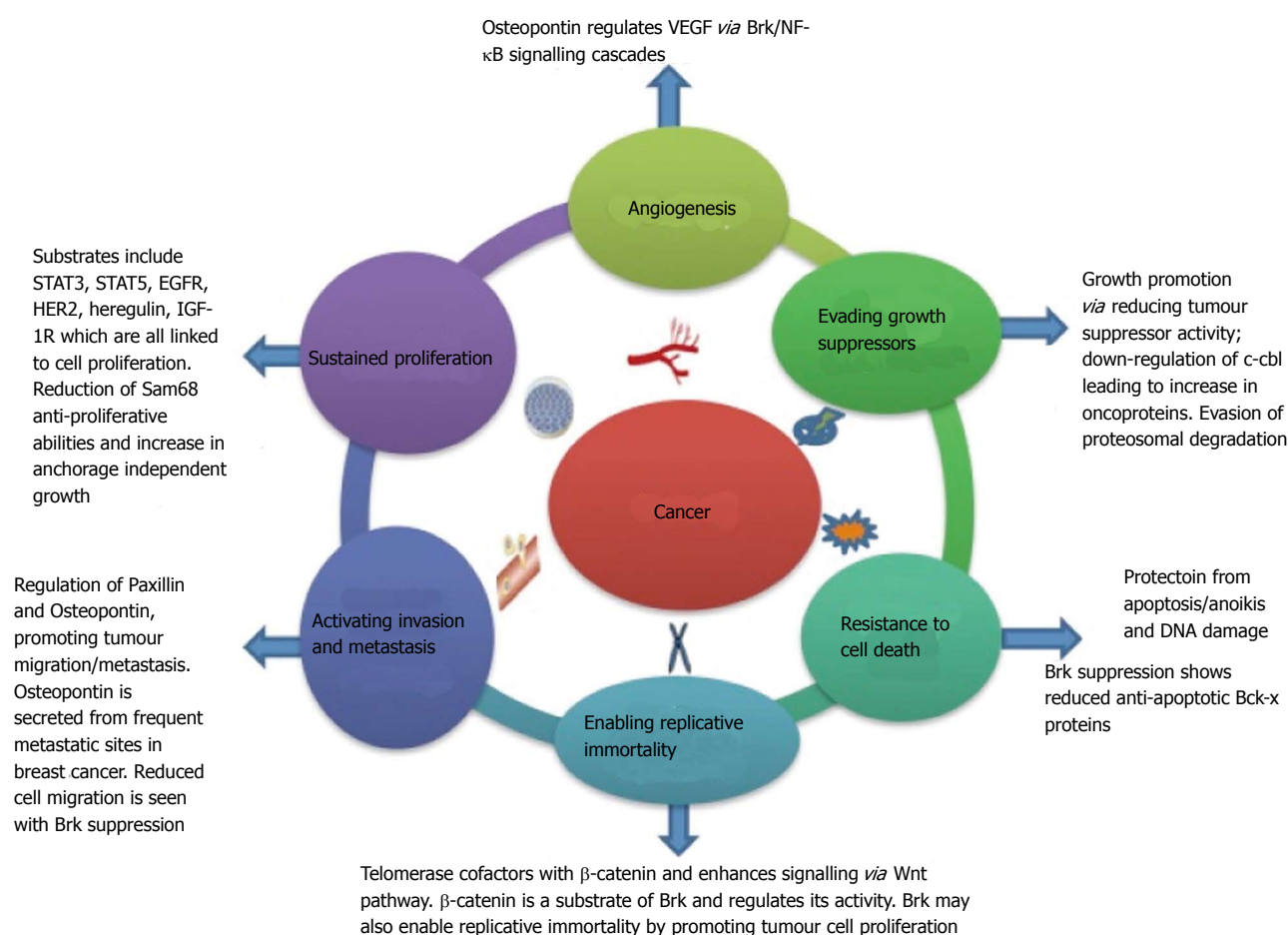


Figure 2 Breast tumour kinase and cancer hallmarks. Brk and cancer hallmarks. The original six cancer hallmarks described by Hannahan and Weinberg (2001)^[86] are illustrated along with a summary of Brk's role in their regulation. Brk: Breast tumour kinase.

BRK AND CELL DEATH

Brk's proliferative ability when coupled with its ability to protect cancerous cells from cell death promotes tumour cell survival. Brk has shown association with the P13-K/Akt pathway which is not only involved in cell proliferation but in apoptosis; thus activation of this pathway may reduce the ability of cells to undergo apoptosis^[56]. Furthermore, Brk can protect breast cancer cells from autophagy; reduced expression of Brk coupled with suspension of culture increased the number of dead cells compared to controls^[89]. Brk also increases phosphorylation of p38 MAPK which is associated with pro-survival cell phenotypes in breast cancer^[90]. Brk's ability to protect cancer cells from cell death is further enhanced due to its ability to protect cells from DNA damage-induced apoptosis in colon cancer cells. Knockdown of Brk in HCT116 cells led to increased apoptosis following γ -irradiation^[91]. In addition p53 was recognised as a possible positive regulator of Brk/PTK6 activity in response to DNA damage. Reduced expression of p21 and STAT3 were also noticed in Brk-suppressed cells, leading to increased apoptosis and decreased survival^[91]. This gives indication for PTK6 inhibitors reducing tumour cell growth and survival.

As discussed above, Brk also protected cells from anoikis *via* IGF-I signalling^[66]. Further to this, recently PTK6 was described to protect cells from anoikis *via* direct phosphorylation of focal adhesion kinase (FAK) and activating Akt^[92]. Knockdown of PTK6 in PC3 prostate cancer cell line disrupted FAK and Akt activation which in consequence promoted anoikis thus indicating important promotional role of Brk in anchorage independent survival.

Within a different cellular context such as non transformed rat fibroblasts, Brk has shown a role contradictory to that seen in breast cancer since it sensitises cells to apoptosis^[93]. Furthermore, it also promotes apoptosis in crypt epithelial cells in response to DNA damage^[94]. This may show a role for Brk as a damage sensor that promotes apoptosis in response to cellular stress. These differences in Brk's role may again depend on its cellular localisation, substrates and protein interaction as well as the tissue in which it is expressed^[74].

FUTURE PERSPECTIVES

Given that Brk's roles extend to regulation of several cancer hallmarks (Figure 2), Brk has promise as an ideal therapeutic target in breast cancer. Brk's therapeutic po-

tential could also extend to non-small cell lung cancer, prostate and colon cancer therapy^[70,91,95].

It was recognised as one of the tyrosine kinases expressed in breast cancer due to the immense interest in tyrosine kinase inhibitors^[23]. So far tyrosine kinase inhibitors have proven to be well tolerated with manageable side effects, selective and relatively effective in a range of cancers^[97]. PTK6-null mice have shown to grow relatively healthily into adulthood indicating potential tolerability to Brk inhibitors in breast cancer patients^[61]. These properties still make tyrosine kinase inhibition an attractive prospect despite Brk's kinase independent functions. Nonetheless, many of Brk's activities involve its kinase catalytic domain including EGFR signalling, cell migration and cell death^[13], thus targeting Brk's kinase activity may still prove to be effective. Furthermore, in comparison to HER2, EGFR, IGFR and p53 its expression is much higher in many types of breast cancer indicating targeting Brk may benefit a wider range of patients^[62].

Further study into the regulation of Brk may provide greater understanding in its therapeutic value. So far studies focusing on discovery of selective Brk inhibitors have shown effective inhibition with imidazo[1,2-a]pyrazin-8-amines^[98]. Recently, Brk's importance in anti-cancer therapy was uncovered in a study involving heat shock protein 90 (Hsp90) which has shown to regulate Brk since it is involved in folding and stability of many proteins^[99]. A potent Hsp90 inhibitor, geldanamycin decreased PTK6 expression in T47D and BT474 cell lines indicating the requirement of Brk's interaction with Hsp90 for stability. Further investigations in a clinical setting regarding these therapies may show their efficacy in treating subtypes of breast cancers including triple negative basal like breast cancer.

Brk has many substrates and protein interactions; some have been reported to require Brk's kinase domain whereas others may require protein-protein interactions and some through indirect association *via* a third party^[13]. Therefore, Brk kinase-independent activity does need consideration when looking at tyrosine kinase inhibitors. Interfering with Brk's protein-protein interactions by disrupting its conformational activation may also be therapeutically effective, including disruptions in the SH2 and SH3 domains^[62].

Additionally, ALT-PTK6 the short isoform of Brk may contribute towards its cellular localisation as increased ALT-PTK6 expression resulted in decreased Brk at the membrane and an increase in nuclear Brk^[70]. Normally Brk is active in differentiating, non-dividing epithelial cells in the small intestine where it is a negative regulator of growth, however, due to an external stimuli such as DNA damage by irradiation, it contributes towards apoptosis^[94]. This shows Brk's differing role within the same tissue but in different conditions. Thus Brk's functions may also depend on external stimuli and the short isoform ALT-PTK6. This needs further investigation and clarification within breast cancers.

Brk's role as a potential oncogene in relation to breast

cancer is further supported by a recent study, which indicated Brk catalytically phosphorylated c-Cbl, a E3 ubiquitin ligase that downregulates oncoproteins, resulting in c-Cbl degradation *via* auto-ubiquitination^[59]. Of the multiple signalling pathways that Brk is involved in, this is yet another pathway in which Brk plays a key role thereby enhancing tumour survival. Therefore Brk's involvement is extensive which suggests targeting Brk may allow targeting of a wider range of pathways that lead to tumour growth, survival and progression.

In the immediate future, most progress could probably be made in combination treatments. Brk's role in mediating cell responses to current EGFR/HER2 therapies is becoming known^[35,49]. Further work in this area could pave the way for improvement to these therapies. Investigations into Brk's role in tumour progression should allow for pre-clinical studies of breast cancer metastasis and determine whether any therapeutic intervention has potential clinical benefit.

CONCLUSION

Brk has been implicated in tumorigenesis, survival and progression as well as diagnosis and prognosis of breast cancer despite the fact there is no indication of expression of Brk in normal mammary tissue. Thus making it a viable target of breast cancer therapy and an interesting protein to investigate. Brk expression changes have not been associated with solely with an increase in *ptk6* gene copy number, which allows for study of alternative mechanisms by which Brk expression and breast cancer develops. Ongoing research may still reveal more associations of Brk with, as yet, uncharacterised substrates. A wider view of Brk's interacting proteins and substrates therefore will prove to be useful in developing anti-Brk therapies for breast cancers.

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WJCO 5th Anniversary Special Issues (2): Breast cancer

Epithelial-mesenchymal transition transcription factors and miRNAs: "Plastic surgeons" of breast cancer

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Abstract

Growing evidence suggests that breast cancer cell plasticity arises due to a partial reactivation of epithelial-mesenchymal transition (EMT) programs in order to give cells pluripotency, leading to a stemness-like phenotype. A complete EMT would be a dead end program that would render cells unable to fully metastasize to distant organs. Evoking the EMT-mesenchymal-to-epithelial transition (MET) cascade promotes successful colonization of distal target tissues. It is unlikely that direct reprogramming or trans-differentiation without passing through a pluripotent stage would be the

preferred mechanism during tumor progression. This review focuses on key EMT transcriptional regulators, EMT-transcription factors involved in EMT (TFs) and the miRNA pathway, which are deregulated in breast cancer, and discusses their implications in cancer cell plasticity. Cross-regulation between EMT-TFs and miRNAs, where miRNAs act as co-repressors or co-activators, appears to be a pivotal mechanism for breast cancer cells to acquire a stem cell-like state, which is implicated both in breast metastases and tumor recurrence. As a master regulator of miRNA biogenesis, the ribonuclease type III endonuclease Dicer plays a central role in EMT-TFs/miRNAs regulating networks. All these EMT-MET key regulators represent valuable new prognostic and predictive markers for breast cancer as well as promising new targets for drug-resistant breast cancers.

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Key words: Embryonic transcription factors; Epithelial to mesenchymal transition; Breast cancer; MicroRNAs; Dicer; Feedback loop

Core tip: Epithelial-mesenchymal transition (EMT) and the reverse mesenchymal-epithelial transition (MET) are both involved in breast cancer plasticity. Embryonic transcription factors and miRNAs are key players regulating the balance between these two processes allowing cells that underwent EMT to transiently re-acquire epithelial phenotype. Here we highlighted the complex transcription factors/miRNAs regulation networks involved in EMT-MET during breast cancer progression and the central role played by Dicer, the key enzyme of miRNAs processing, in EMT process. These key regulators of EMT-MET may represent predictive markers and potential therapeutic targets for breast cancers.

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EMT AND MET REPROGRAMMING DURING BREAST CANCER PROGRESSION

During embryonic development, a complex organism is formed from a single starting cell. Growth and differentiation are driven by large transcriptional changes, directed by the expression and activity of transcription factors (TFs). Cancer is often suggested to imperfectly resemble the development process by re-expressing certain embryonic TFs. Links between normal embryonic development and cancer biology have been postulated, but no defined genetic/epigenetic basis has been established. During normal development, cells divide, align themselves, and specialize to form discrete tissues and organs. For the body to develop properly, cells must coordinate their migratory patterns and the process by which they differentiate or evolve from less-specialized cells into more-specialized cell types. A lack of such coordination leads to disordered development and, in some cases, cancer.

The mammary gland is an organ that undergoes distinct and complex developmental stages after birth. Post-natally, mammary ducts elongate into the mammary fat pad. Terminal end buds, the highly proliferative structures found at the tips of the invading ducts, expand and increase greatly after birth. By puberty, the mammary ducts have invaded to the end of the mammary fat pad. At this point, the terminal end buds become less proliferative and decrease in size. Side branches form from the primary ducts and begin to fill the mammary fat pad. Ductal development decreases with the arrival of sexual maturity and undergoes estrous cycles. As a result of estrous cycling, the mammary gland undergoes dynamic changes where cells proliferate and then regress. During each estrus cycle, the density of ductal branches and alveolar buds increases. During pregnancy, the alveolar buds formed on the ductal tree give rise to large, lobulo-alveolar differentiated structures capable of milk production. Understanding how the mammary tissue develops and functions is of great importance in determining how its control mechanisms break down in breast cancer. The leucine-rich repeat containing G protein-coupled receptor 4 (*Lgr4*) has been implicated in mammary development and stem cell activity, with *Lgr4*^{-/-} mice showing delayed ductal development, fewer terminal end buds, and decreased side-branching mediated by the Wnt/ β -catenin/Lef1 pathway and Sox2^[1]. An article from the Breakthrough Breast Cancer Research Centre has recently compared an embryonic mammary epithelial signature with *Brca1*-deficient mouse mammary tumors and human breast cancer signatures. Specific subsets of embryonic mammary genes were found over-expressed both in mouse *Brca1*^{-/-} tumors and in human basal-like

cancers^[2]. Reactivation of a small network of embryonic mammary programs within differentiated tumor cells may elicit cell behavior associated with a stem-like, highly plastic state. The EMT-mesenchymal-to-epithelial transition (MET) cascade, although an intrinsic part of normal developmental processes during organogenesis, is also recognized as a critical event for metastasis of carcinomas^[3,4]. EMT allows tumor cells to de-differentiate and to acquire motility and invasive properties in order to spread into distant organs, and the MET program then reboots an epithelial program to establish new tumors at the sites of dissemination^[5]. It is not entirely known how and when EMT and MET programs, and the genes associated with these processes, are coordinated. The hallmark of EMT is the loss of adherent junctions through loss of E-cadherin (*CDH1*) expression. E-cadherin repressors fall into two groups, direct or indirect regulators, depending on whether or not they bind the *CDH1* promoter.

EMT INDUCERS

The powerful direct repressors of *CDH1*, playing a major role in EMT, originate from three distinct families: The Snail family comprises three members, Snai1, 2 and 3 (also termed Snail, Slug and Smuc), the Zeb family (*Zeb1/2*), which are zinc-finger transcription factors that recognize a consensus E-box type element, and the b-HLH family (*TWIST1/2*) which also bind to a consensus E-Box sequence, as homo- or heterodimers. These factors also repress the transcription of several other junctional proteins, including claudins and desmosomes. The other group of *CDH1* repressors (indirect regulators) comprises FoxC2, Goosecoid, TCF4, paired mesoderm homeobox protein 1 (*PRRX1*), and some Sox family members. FoxC2 is a winged helix/Forkhead domain transcription factor, which lies downstream of Twist, Snail and Goosecoid, and affects E-cadherin expression by promoting its cytoplasmic localization. They increase the invasiveness of epithelial cells (Table 1). The third member of the Snail family, (Smuc) does not play a major role during EMT, while Zeb1 is a strong motility driver. The Snail, Zeb and TWIST families operate within a complex regulatory network where they activate or repress each other. EMT inducers, such as EMT-TFs (*TWIST1*, Snail, Slug, and Zeb1), can also confer 'stemness' as demonstrated in several studies, where the induction of EMT enhances self-renewal and the acquisition of CSC (Cancer Stem Cell) characteristics^[6-8] (Table 1). In contrast, several studies show that tumor cells with an epithelial phenotype survive in the circulation and form distant metastases^[9-12]. It has been demonstrated that the mesenchymal phenotype does not facilitate metastatic progression; rather, most cancers invade and travel through lymphatic and blood vessels *via* cohesive epithelial migration, and do not undergo EMT-MET^[13]. Interestingly, Zvelebil *et al*^[2] showed that enrichment for the mammary mesenchymal gene signature (*TGF β 1*, *TWIST2*, *ZEB2*) was correlated with large tumor size, but no significant association with

Table 1 Involvement of epithelial-mesenchymal transition-transcription factors in breast carcinogenesis

EMT-TF	Transcription factor type	Deregulated in breast cancer	Association with biological and clinico-pathological features in breast cancer
SNAI1 (Snail)	Zinc finger	High levels ^[75]	Lymph node metastasis, effusion, distant metastasis, recurrence
SNAI2 (Slug)	Zinc finger	High levels ^[76]	Effusion, distant metastasis, recurrence, stemness capacities
TWIST1	Basic Helix-loop-Helix	Up-regulated ^[77]	Primary transformation, escape from failsafe programs, invasion, bone metastasis, angiogenesis, poor prognosis
ZEB1	Zinc finger E-box-binding homeobox 1	High levels ^[76,77]	Invasion, distant metastasis
ZEB2	Zinc finger E-box-binding homeobox 2	High levels ^[76,77]	Invasion, distant metastasis, stemness capacities
FoxC2	Forkhead-related protein FKHL14, FKHL-14, mesenchyme fork head protein 1	High levels ^[78]	Stemness capacities, distant metastasis
Oct3/4	Octamer-binding transcription factor 4, POU domain, class 5, transcription factor 1S homeodomain transcription factor of the POU family	Up-regulated ^[79]	Stemness capacities, invasion, migration
Sox2	Sex determining region Y-box 2 highly conserved DNA binding domains High-mobility group box domains	Up-regulated ^[80]	Tamoxifen-resistance, lymph node metastasis, stemness capacities
Prrx1	Paired related homeobox 1	High levels ^[15]	Metastasis, poor prognosis
TCF4	Basic Helix-loop-Helix immunoglobulin transcription factor 2	Up-regulated ^[81,82]	Metastasis, poorer prognosis in patients with high levels of osteopontin and better prognosis with low levels of osteopontin

EMT-TF: Epithelial-mesenchymal transition-transcription factors.

overall survival was observed in patients whose breast cancers showed activation of the embryonic mesenchymal signature. Four transcription factors (Bcl11a, Grhl3, Prox1, Sox11) activated in *Bra1*^{-/-} mouse tumors and basal-like human breast cancers were confirmed to be embryonic-enriched and highly expressed by some tumors. Increasingly, evidence points towards the transient involvement of an activated EMT program in the invasive front of tumors rather than in dissemination of cancer cells. The mesenchymal transcriptomic program is found associated with metaplastic breast carcinoma (MBC), a rare tumor with a carcinosarcoma-like aspect, with a larger tumor size, accounting for < 1% of all breast cancers. Histologic subtypes identified were chondroid (24%), spindle (20%), sarcomatoid (16%), squamous (11%) and mixed (29%), with the origin of the "mixed" subtype hypothesized to be from the "differentiation" of immature breast glandular epithelial cells into non-glandular mesenchymal tissue. In prostate cancer cell lines, subpopulations with a strong epithelial gene program were enriched in highly metastatic tumor-initiating cells (TICs), whereas mesenchymal subpopulations showed reduced TIC^[12]. Are epithelial cancer cells expressing EMT-TFs without experiencing a full EMT program the most prominent to metastasize? The answer is no for some factors, as it was recently shown that TWIST1 down-regulation and a subsequent re-differentiation (MET) at the distant site is necessary to allow colonization and macrometastasis^[14]. Intriguingly, the EMT-inducer Prrx1 suppresses stemness traits^[15] and a knockdown of Prrx1 and TWIST1 increased lung metastasis after tail vein injection.

The question of whether the MET is stable in the metastases or if these cells show ongoing phenotypic plasticity leading to a second EMT is also an open question. Collectively, these results illustrate the plasticity governing self-renewal and mesenchymal gene interactions

(Figure 1).

INVOLVEMENT OF DICER IN EMT

Genes central to gene regulatory networks (GRNs) may have a huge impact on cell plasticity. The ribonuclease type III endonuclease Dicer, involved in the RNA interference process, belongs to this gene category. RNA interference (RNAi) and microRNA (miRNA) pathways are conserved, post-transcriptional gene silencing mechanisms in which single-stranded guide RNAs bind to cognate mRNAs and direct their endonucleolytic cleavage or translational repression by RNA-induced silencing complexes (RISCs). An important function of Dicer is to process miRNA precursors into approximately 22-nucleotide non-coding small RNAs. As a master regulator of miRNA biogenesis, Dicer is involved in EMT, cancer cell plasticity and tumor progression. We have found that Dicer mRNA expression was variable in breast carcinoma samples and that lower levels were more frequent in patients with metastatic relapse, indicating that Dicer mRNA levels are clinically relevant as reported by Grelier *et al*^[16]. In accordance with other studies, we have found a global decrease of miRNA expression in correlation with the decrease of Dicer expression^[17]. Levels of Dicer are tightly controlled to maintain the homeostasis of miRNA production, largely at the post-transcriptional level. Dicer is a highly conserved protein that is found in almost all eukaryotic organisms. Some organisms contain multiple *Dicer* homologues, whereby different Dicer isoforms have distinct roles, for instance *D. melanogaster* Dicer-1 is required for miRNA biogenesis, whereas Dicer-2 functions in siRNA production. Contrary to other organisms, mammals have a single *Dicer* gene, but its expression is a highly regulated process with spliced Dicer mRNAs putatively encoding both spliced and full-length pro-

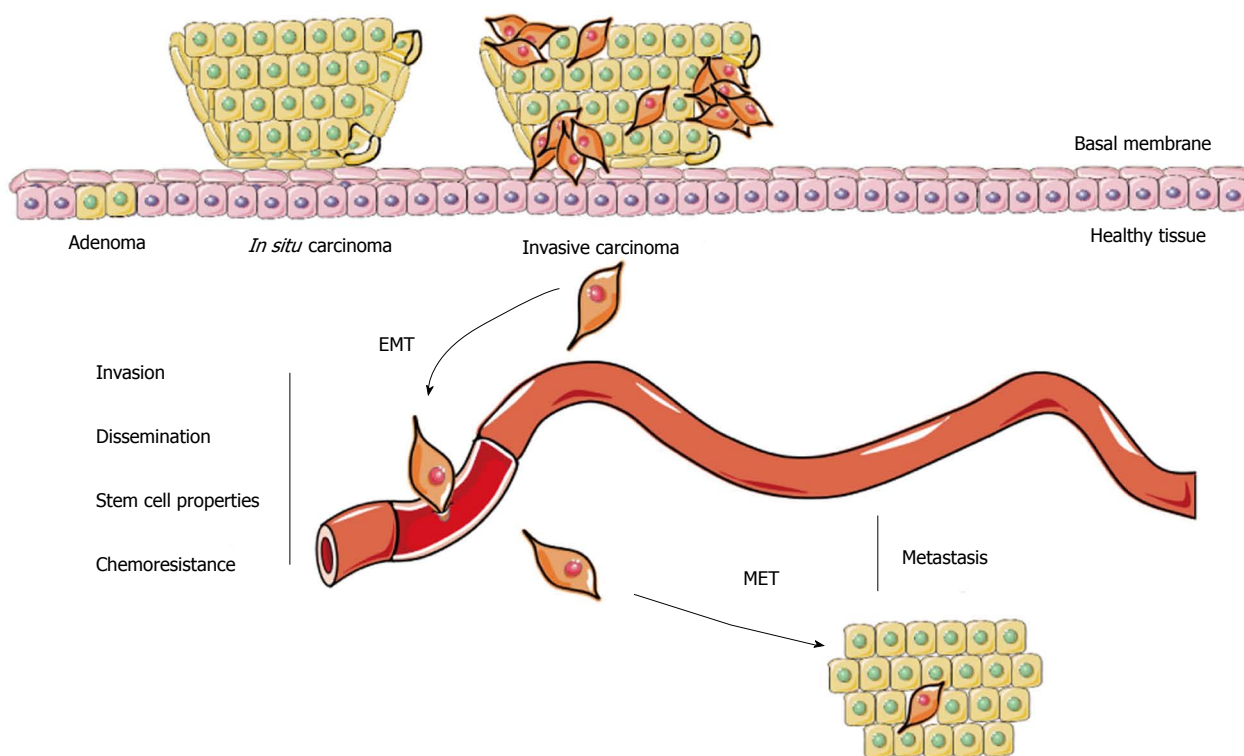


Figure 1 Epithelial to mesenchymal transition and mesenchymal to epithelial transition during breast tumor progression. During tumor progression, cancer cells undergo apithelial to mesenchymal transition (EMT) to acquire invasive, dissemination, chemoresistance and stem cell properties. Thus, an *in situ* carcinoma progresses to an invasive carcinoma and cells disseminate throughout the entire body via the blood and the lymphatic vessels. After dissemination, cells must undergo an mesenchymal to epithelial transition (MET) to colonize distant organs.

teins. In humans, there are 3 full-length isoforms showing considerable differences in their 3'UTR sequence. Only two variants exhibit a long 3'UTR sequence, while the third variant exhibits a very short 3'UTR lacking all predicted miRNA target sites^[18,19]. Moreover, we identified two splice variants which were highly expressed in some breast cancer cell lines, yet totally absent in others. Theoretically, these isoforms may be functional as they both contain the ribonuclease III domain and the dsRNA binding domain, while one isoform contains only a PAZ domain^[18]. We have shown that the full-length Dicer protein decreased during the EMT process^[16]. The presence of spliced forms was correlated with epithelial/mesenchymal phenotype. Indeed, in almost all cell lines that exhibit a complete or partial mesenchymal phenotype, these truncated isoforms were not detectable by western blot as shown by Hinkal *et al.*^[18]. Conversely, epithelial cells expressed easily-detectable levels of the two variants. Furthermore, we have found decreased expression of these variants during EMT using immortalized human epithelial mammary cells transfected by RAS. These data imply an integral role for internal site miRNA regulation of Dicer isoforms, but the physiological relevance of these data remains to be clarified. Thomas Duchaine's group has shown the presence of a truncated form of Dicer in *C. elegans*, corresponding to a C-terminal fragment (sDCR-1). They demonstrated that sDCR-1 operates independently of full-length Dicer in two distinct RNAi pathways; it enhances exogenous RNAi (exoR-

NAi) and concurrently acts as a negative regulator of microRNA (miRNA) biogenesis^[20]. Interestingly, one of the spliced form variants we identified in epithelial breast cancer cell lines encodes a protein sharing the same domains as sDCR-1. By ectopically expressing this isoform in HEK293T cells, they have found that, similar to the function of sDCR-1, there was a decrease in accumulation of mature-to-precursor forms for some miRNA but not all, showing that this function is miRNA-specific^[20]. Deciphering the role of the highly conserved Dicer variants is of great importance since Dicer acts as a tumor suppressor in specific cancers^[16,21,22]. As a miRNA target, full-length Dicer was also shown to be directly repressed by miR-103/107 and this repression enhanced breast cancer metastasis^[23], whereas transcriptional induction of Dicer by Tap63 suppressed metastasis^[24].

The nearly global decrease in miRNAs observed across a range of human tumors suggests that Dicer loss could be necessary for tumor progression. To better understand how cancer cells respond to loss of miRNA expression, Philip Sharp and collaborators^[25] have characterized the effects of homozygous deletions of *Dicer1*-conditional alleles on the tumorigenicity of murine sarcoma cells and on the cellular phenotype of immortalized murine mesenchymal stem cells (MSCs). *Dicer1*^{-/-} cells survived and proliferated without recovery of miRNA processing. Interestingly, their two models are mesenchymal, corroborating our results that show a repression of Dicer during the EMT process. Inactivation of p53,

a common feature in both the sarcoma and MSC models, may facilitate, or be indispensable for, viability in the absence of Dicer. p53 loss was shown to allow primary MEFs to bypass an immediate senescence phenotype induced by *Dicer1* loss^[26].

miRNAS AND BREAST CANCER PLASTICITY

As a consequence of Dicer loss, tumors of epithelial origin should express more miRNAs than mesenchymal tumors. If we compare development and cancer progression, a parallel can be drawn between miRNA biogenesis during embryogenesis with repression of miRNAs synthesis in stem cells, and global miRNA loss during acquisition of cancer cell stemness. In embryonic zebrafish development, most miRNAs were expressed in a highly tissue-specific manner during segmentation and later stages, but not early in development. This suggests that they do not play a role in tissue fate establishment, but rather in differentiation or maintenance of tissue identity^[27]. The link between deregulated miRNA expression and cancer has been well established, with miRNA profiling studies revealing distinct expression profiles in various cancers that could help in the diagnosis of these malignancies^[28-30]. Only a small subset of specific oncogenic miRNAs has been found to be upregulated in cancer.

Different miRNA signatures have been identified in the different breast cancer subtypes. Do these signatures reflect cell lineage of origin? miRNAs have been implicated in the development of murine mammary gland, which showed seven distinct temporal clusters during mammaryogenesis^[10,31]. Among them, miRNAs clusters over-expressed during puberty and gestation in normal tissue of mammary gland, for example the miR-17-92 cluster, have been shown to play a crucial role in breast cell proliferation and have also been found in aggressive cancers, such as the basal-like subtype^[32]. As it has been found for other types of cancer, miR-21 appeared to predominantly act as an oncogene and its expression is inversely correlated with the tumor suppressor PTEN (Phosphatase and TENSin homolog) expression^[33]. Another potent miRNA oncogene, miR-191 was positively regulated by estrogen and was shown to promote proliferation and invasion^[34]. miR-155 was also categorized as an oncomiR, as it was implicated in TGF- β -induced EMT, cell migration and invasion. Let-7 was the diametric opposite of miR-21, acting as a general tumor suppressor, and was found down-regulated in breast cancers. Let-7 has been described as a regulator of self-renewal and a pro-differentiation miRNA of breast cancer cells repressed by the Wnt- β -catenin pathway^[35], targeting oncogenes including RAS, HMGA2 and MYC. miR-21, miR-155 and let-7 are involved early in tumorigenesis and were found deregulated in benign breast tumors^[36]. In contrast to miR-191, miR-206 was negatively regulated by estrogens and decreased miR-206 levels are associated with breast cancer of advanced clinical stage and shorter overall survival. Different studies

have profiled the expression of miRNAs as a function of intrinsic breast cancer subtype. A clear miRNAs signature was identified in luminal breast cancer, with over-expression of miR-191 and miR-26 and down-regulation of miR-206. Interestingly, based on miRNAs signatures, the tumors can be easily classified as luminal A, luminal B, normal-like, HER²⁺ and basal-like^[32].

EMT-TFs signatures were more often found in Triple Negative Breast cancer (TNBC), a very aggressive cancer subtype representing, however, a very heterogeneous group of breast cancers with the only common phenotype being ER-, PR- and HER2-negative. Further transcriptomic studies allowed the sub-classification of TNBC by identifying additional entities such as the Claudin-low subtype, characterized by low expression of claudin proteins, proliferation genes and luminal markers, and high expression of EMT markers and CSC-like features^[37]. Interestingly, as previously mentioned for epithelial-mesenchymal dichotomy of miRNA expression, the highly undifferentiated nature of TNBC is correlated with a global down-regulation of microRNAs^[38]. However some miRNA are readily expressed in stem cells, such as the miR-302 cluster, "stemness miRNA cluster," in ES cells which decreases upon cell differentiation, and is undetectable in somatic cells. An Oct4/Sox2-miR-302-cyclin D1 regulatory network governing ES cell pluripotency and self-renewal properties has been proposed^[39]. miR-302 over-expression converts cancer cells into ES-like pluripotent stem cells associated with high expression of Oct3/4, SSEA-3, SSEA-4, Sox2, and Nanog^[40]. The group of Carlos Caldas also identified miR-301a as a hub of pluripotency in breast cancers, and demonstrated that mRNA relationships altered in miR-301a high/low tumors showed a link between immune and EMT pathways, illustrated by the immunoglobulin superfamily member ALCAM, the EMT-TF ZEB2 and Claudin-3. miR-301a directly targeted and suppressed the tumor suppressor PTEN, one negative regulator of the Wnt/ β -catenin signaling cascade, which promotes breast cancer invasion and metastasis. In the Claudin-low subtype, while the global decreased miRNAs expression can be assigned to a repression of Dicer expression, the down-regulation of miRNAs targeting transcription factors implicated in EMT and cancer stem cells may result from a transcriptional repression of their promoters. The miRNAs targets can directly drive this repression (Figure 2).

FEEDBACK LOOPS INVOLVING miRNAS DURING EMT-MET

EMT is driven both by transcriptional and post-transcriptional changes. Because of the reversible nature of EMT, miRNAs functioning as co-repressors or co-activators are key players in this plasticity, specifically involved in regulation networks with EMT-TFs. miRNAs are categorized as either EMT-inducers or EMT-repressors, inversely involved in MET.

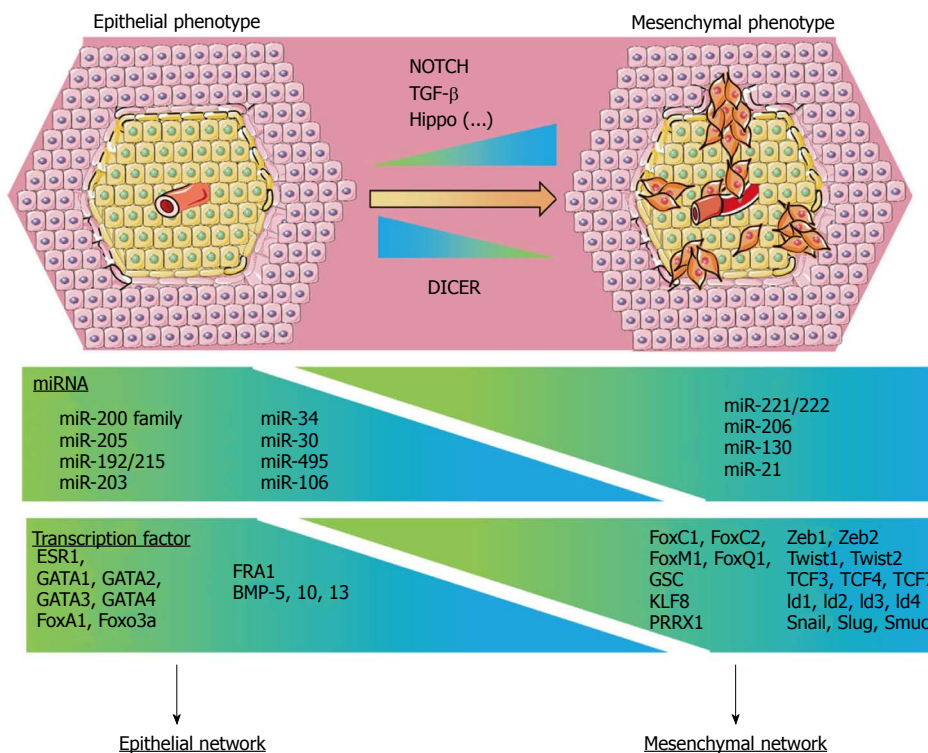


Figure 2 Transcription factors and miRNA epithelial and mesenchymal expression networks in breast cancer. Through activation of different signaling pathways such as TGF- β , Notch or Hippo pathways and the down-regulation of Dicer, epithelial cells undergo the epithelial to mesenchymal transition. Transcription factors and miRNAs act together to be the "plastic surgeons" of the epithelial or mesenchymal phenotype. Regulation networks between these two main actors drive cells to plasticity.

miRNAs with EMT inducer activities

The well-known oncomiR miR-21 was identified as an EMT-inducer, similarly to miR-103/107 which represses Dicer and PTEN expression during breast tumor initiation. PTEN is a major miR-21 target that negatively regulates EMT and CSC phenotypes. miR-10b was also identified as a positive regulator of EMT as it was demonstrated to be a positive effector of TWIST. It was shown to induce migration and invasion capacities in breast cancer cells *via* the direct targeting of the HOXD10 transcript. HOXD10 is a known repressor of genes involved in cell migration and extracellular matrix remodeling, including RHOC, α 3 integrin, matrix metalloproteinase-14 and urokinase-type plasminogen activator receptor^[41].

The oncomiR miR-206 expressed in aggressive breast tumors is involved in a double-negative feedback loop with ER α and participates in EGFR-mediated abrogation of estrogenic responses in MCF-7 cells, thus contributing to a Luminal-A- to Basal-like phenotypic switch^[42]. ER α is also involved in a simple negative feedback with the miR-18a (17-92 cluster), where ER α induced the expression of the miR-17-92 which in turn targets ER α with miR-18a. miR-17-92, an miRNA polycistron also known as oncomir-1, is among the most potent oncogenic miRNAs. Genomic amplification and elevated expression of miR-17-92 was found in several types of tumor, including mammary. miR-17-92 carries out pleiotropic functions during both normal development and malig-

nant transformation, as it acts to promote proliferation, inhibit differentiation, increase angiogenesis, and sustain cell survival^[43]. ER α functions in a forward positive feedback loop with miR-375. Inhibiting miR-375 in ER α -positive MCF-7 cells resulted in reduced ER α activation and cell proliferation. Researchers have identified RASD1 (Dexamethasone-induced Ras-related protein 1), a small G protein of the Ras family, as a potential miR-375 target. Mechanistic investigations revealed that miR-375 regulates RASD1 by targeting the RASD1 3'UTR and RASD1 negatively regulates ER α expression^[44]. miR-206, which contributes to a Luminal-A- to Basal-like switch, targets KLF4 (Kruppel-like factor 4) a pivotal transcription factor that is associated with both tumor suppression and oncogenesis. In untransformed cells, KLF4 likely acts as a potent inhibitor of proliferation. Conversely, in transformed cells, KLF4 suppresses the expression of p53 by directly acting on its promoter; consistently, KLF4 depletion from breast cancer cells restores p53 levels and causes p53-dependent apoptosis^[45]. To further complicate the function of KLF4, it was shown that a co-operative binding of KLF4 and p53 to the DNA binding sites of some p53 targets, contributes to p53 target selectivity^[46]. miR-206 levels were KLF4-dependent in breast cancer cells, and a KLF4-miR-206 feedback pathway was identified that negatively regulates protein translation in normal cells and cancer cells^[47]. Very recently, KLF4 was evoked in a feedback loop involving p21. The tumor suppressor p21 has been shown to regulate gene expression by func-

tioning as a transcription co-repressor. Li and collaborators^[48], have identified p21-regulated miRNAs, among them, the miR-200 family and the miR-183-96-182 cluster, that were down-regulated in p21-deficient cells.

miRNAs with EMT repressor activities

miR-200 family members were identified as the guardians of the epithelial phenotype in many types of cancers, including breast cancers^[49-51]. The miR-200 family activates the Sec23a-mediated tumor cell secretome which leads to secretion of metastasis-suppressive proteins. Predictably, loss of miRNA-200a is frequently observed in breast cancers, especially tumors with high-grade histology, but this loss does not predict tumor recurrence or patient survival^[52]. miR-200 family members (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) encoded from two clusters, directly target the mRNAs of the E-cadherin transcriptional repressors ZEB1 and ZEB2. Interestingly, Thomas Brabletz' group and others^[50,53] have shown that both promoter regions are repressed in mesenchymal cells by ZEB1 and ZEB2 through their binding to a conserved pair of ZEB-type E-box elements, located proximal to the transcription start site. These findings establish a double-negative feedback loop controlling ZEB1-ZEB2 and miR-200 family expression. During EMT induced by TGF- β , miR200s are inhibited mainly by ZEB1^[50]. The induction and maintenance of a stable mesenchymal phenotype requires the establishment of autocrine transforming growth factor- β (TGF- β) signaling to drive sustained ZEB expression. Prolonged autocrine TGF- β signaling induced reversible DNA methylation of the miR-200 loci, demonstrating the existence of an autocrine TGF- β /ZEB/miR-200 signaling network that regulates cancer cell plasticity^[54]. But the activity of this miRNA family is a doubled edged sword during cancer progression, as it has been shown to promote MET through E-cadherin up-regulation, allowing migrating cancer cells to colonize distant tissues. Intriguing, the role of the miR-200 family during metastatic colonization can be partly elucidated by the oncogene c-Myb, which was shown to activate the expression of all five members of the miR-200 family. The transcriptional activation of miR-200 by c-Myb occurs through binding to myb binding sites located in the promoter regions of miR-200 genes on human chromosomes 1 and 12. Furthermore, when c-Myb and the transcriptional repressor ZEB1 are co-expressed, such as at the onset of EMT, the repression by ZEB1 prevails over the activation by c-Myb, and miR-200s are repressed. Researchers have also shown a positive correlation between the expression of c-Myb and miR-200 members in a dataset of breast cancer patients^[55].

Interestingly, another EMT-TF, Slug (SNAI2, Snail2) is transcriptionally regulated by c-Myb and induces vimentin, fibronectin, and N-cadherin expression and membrane ruffling *via* actin polymerization, consistent with the acquisition of partial but not complete mesenchymal-like phenotype^[56]. Both expression of c-myb

and miR-200 members lead to simultaneous expression of vimentin, N and E-cadherin. These data support the concept that, during distinct phases of tumor progression, the role of the genes involved in the EMT process may change in relation to the expression of other regulators and to epigenetic changes. Are both mesenchymal and epithelial traits required for metastatic progression at distant sites? The complex relationship between miR-200 and ZEB during tumor progression was also investigated in a xenograft orthotopic model of breast cancer metastasis, where ectopic expression of members of the miR-200b/200c/429, but not the miR-141/200a, limits tumor cell invasion and metastasis. Despite modulation of the ZEB1-E-cadherin axis, restoration of ZEB1 in miR-200b-expressing cells was not sufficient to alter metastatic potential, suggesting that other targets contribute to this process^[48].

Other feedback loops between EMT-TFs and miRNAs were identified during breast tumor progression. miR-183 and miR-96 repressed common targets, including Slug, ZEB1, and KLF4. Re-introduction of miR-200, miR-183 or miR-96 into p21-/- cells inhibited EMT, cell migration and invasion. p21 forms a complex with ZEB1 at the miR-183-96-182 cluster promoter to inhibit transcriptional repression of this cluster by ZEB1, suggesting a reciprocal feedback loop. ZEB1 and ZEB2 are also involved in a negative feedback loop with miR-205 through the E-box motifs present in the miR-205 promoter sequences^[57]. During Snail-induced EMT in MCF7 breast cancer cells, miR-203 and miR-200 family members were repressed in a correlated manner. Importantly, miR-203 repressed endogenous Snail, forming a double negative miR-203/Snail feedback loop^[58]. miR-203 is also able to target Slug (SNAI2). In parallel with the TGF- β /ZEB/miR-200 negative loop, TGF- β induced Slug to promote EMT by repressing the miR-203 promoter to inhibit its transcription. SNAI2 and miR-203 thus form a double negative feedback loop. It was found that miR-203 was significantly down-regulated in highly metastatic breast cancer cells, and the restoration of miR-203 in these cells inhibited tumor cell invasion *in vitro* and lung metastatic colonization *in vivo* by repressing Slug^[59].

The miR-34 family is one of the most studied tumor suppressor miRNAs and comprises miR-34a, miR-34b and miR-34c. miR-34 is implicated in the inhibition of EMT mediated by p53. It was reported that activation of p53 down-regulates the EMT-inducing transcription factor Snail *via* induction of the miR-34a/b/c genes. Suppression of miR-34a/b/c caused up-regulation of Snail and EMT markers, and enhanced migration and invasion. Ectopic miR-34a induced MET and down-regulation of Snail. miR-34a also down-regulated Slug and ZEB1, as well as the stemness factors BMI1, CD44, CD133, OLFM4 and c-MYC. Conversely, the transcription factors Snail and ZEB1 bind to E-boxes in the miR-34a/b/c promoters, thereby repressing miR-34a/b/c expression. miR-34a prevents TGF- β -induced EMT, and the repression of miR-34 genes by Snail and related factors is part

of the EMT program^[60]. miR-34 and SNAIL represent a double-negative feedback loop controlling cellular plasticity, governed by p53.

Transcription factors/miRNAs regulating networks

What are the targets of miRNAs during EMT/MET and what is the mode of TF-miRNA co-operation during pluripotency reprogramming? Sass *et al.*^[61] demonstrated that miRNAs which target the same protein complexes are frequently co-expressed. They experimentally verified that the miR141-200c cluster simultaneously targets several protein components of the CtBP (C-Term binding proteins)/ZEB complex (CtBP are conserved transcriptional co-repressors), implying an efficient regulation of a protein complex by a cluster of miRNAs. There is also evidence of functional redundancy among miRNAs resulting, in part, from miRNAs existing in large families sharing common seed sequences that can be co-expressed in the same cell. Redundancy also occurs at the level of co-targeting, where multiple distinct miRNAs with different sequences commonly target a single transcript through non-overlapping sites^[62]. The miRNA-regulated protein complexes are mainly involved in regulation of transcription and chromatin modification. Conversely, house-keeping functions, such as translational elongation, are under-represented, meaning that miRNAs are "regulators of regulators"^[61].

An important goal is to elucidate how complex TFs/miRNAs networks evolve in cancer. TFs and miRNAs are the two largest families of trans-acting, gene regulatory molecules in multicellular organisms, and they share a common regulatory logic. TFs generally do not work in isolation, but instead, together with co-regulators, they form large networks of co-operating and interacting transcription factors. The term "motif" was used to describe a small group that illustrates the regulation patterns of an miRNA, a TF, and their target genes. Common motifs, such as feedforward loops (FFLs) and feedback loops (FBLs) have been found to play crucial roles in cancer, such as the miR-17 cluster, E2F1, and c-Myc that modulate cellular proliferation^[63]. However, the miRNA-TF synergistic effect may not be limited only to the FFLs or FBLs. Non-loop forms, such as the cascaded form, which have helped in understanding the regulatory mechanism, are also candidates^[48]. miRNAs can also antagonize the function of other miRNAs, for example, miR-22 can suppress the expression of miR-200 *via* direct targeting of chromatin remodeling enzymes such as TET family members, which leads to the hypermethylation of the miR-200 promoter^[64].

How can we decipher miRNA-regulating networks composed of proteins with opposite functions? For example, let-7 acts as a protective miRNA that inhibits RAS and transcriptional factors thus leading to cell commitment during development, but paradoxically Dicer, the master regulator of miRNA maturation is a hub for let-7 targeting. A recent finding may help us to understand this paradox; ZEB2 transcript was shown to function as

a competitive endogenous RNA (ceRNA) for PTEN miRNAs. ZEB2 loss during MET can lead to repression of PTEN^[65] and this regulation, that may appear counter-intuitive at first glance, may explain how MET could be intricately linked to stemness acquisition (Figure 3).

EMT, miRNAS AND CHEMORESISTANCE

Chemotherapeutics and radiotherapy effectively reduce tumor bulk but have little effect on cancer stem cells (CSC) that stimulate tumor recurrence, emphasizing the importance of identifying CSC-specific pathways that may be exploited to selectively target these resistant cells. Induction of EMT can activate some CSC state-specific signaling transcriptomic networks and the therapeutic resistance associated with CSCs. It was shown that EMT could be induced by chemotherapeutic agents and patients receiving neo-adjuvant therapy were more likely to express EMT-TFs in their circulating tumor cells (CTCs)^[66]. Adriamycin treatment has been seen to induce EMT in a Twist-dependent manner in breast cancer cells. Additionally, irradiation, a common treatment modality in breast cancer, can increase EMT and CSC characteristics^[67]. Tam and colleagues^[68] found that EMT stimulated a switch between two main kinase pathways, through the protein kinase C α (PKC α). PKC α was activated following EMT by a shift from EGF receptor (EGFR) signaling, which predominated in non-CSCs, to autocrine platelet-derived growth factor receptor (PDGFR) signaling in mesenchymal stem-like cells and basal breast cancer cell lines. Up-regulation of PKC α resulted in induction of the transcription factor FRA1 (FOS-like antigen 1), which was required for CSC viability and FRA1 expression was directly induced by the EMT transcription factors TWIST and Snail in triple-negative breast cancer (TNBC).

The mechanism of action of miRNAs in drug-induced EMT remains mainly unknown. miR-21 up-regulation has been associated with taxol resistance in breast cancer cells^[69] and suppression of the oncogenic miR-21 sensitizes cancer cells to chemotherapy. Results by Li and collaborators^[70] reported that miR-448 is the most strongly down-regulated miRNA following chemotherapy. Suppression of miR-448 correlated with EMT induction in breast cancer *in vitro* and *in vivo*. miR-448 suppression induces increasing epidermal growth factor receptor (EGFR)-mediated TWIST1 expression, as well as nuclear factor κ B (NF- κ B) activation. The authors have also demonstrated that the adriamycin-activated NF- κ B directly binds the miR-448 promoter, suppressing its expression, suggesting a positive feedback loop between NF- κ B and miR-448. It was shown that the loss of miRNA-200c correlated with the acquired resistance of breast cancer cells to adriamycin^[71]. In breast CSC, the Wnt- β -catenin pathway suppresses mature let-7 miRNAs by up-regulating Lin28, a negative let-7 biogenesis regulator. Loss of function of Lin28 impairs Wnt- β -catenin-pathway-mediated let-7 inhibition and breast cancer stem

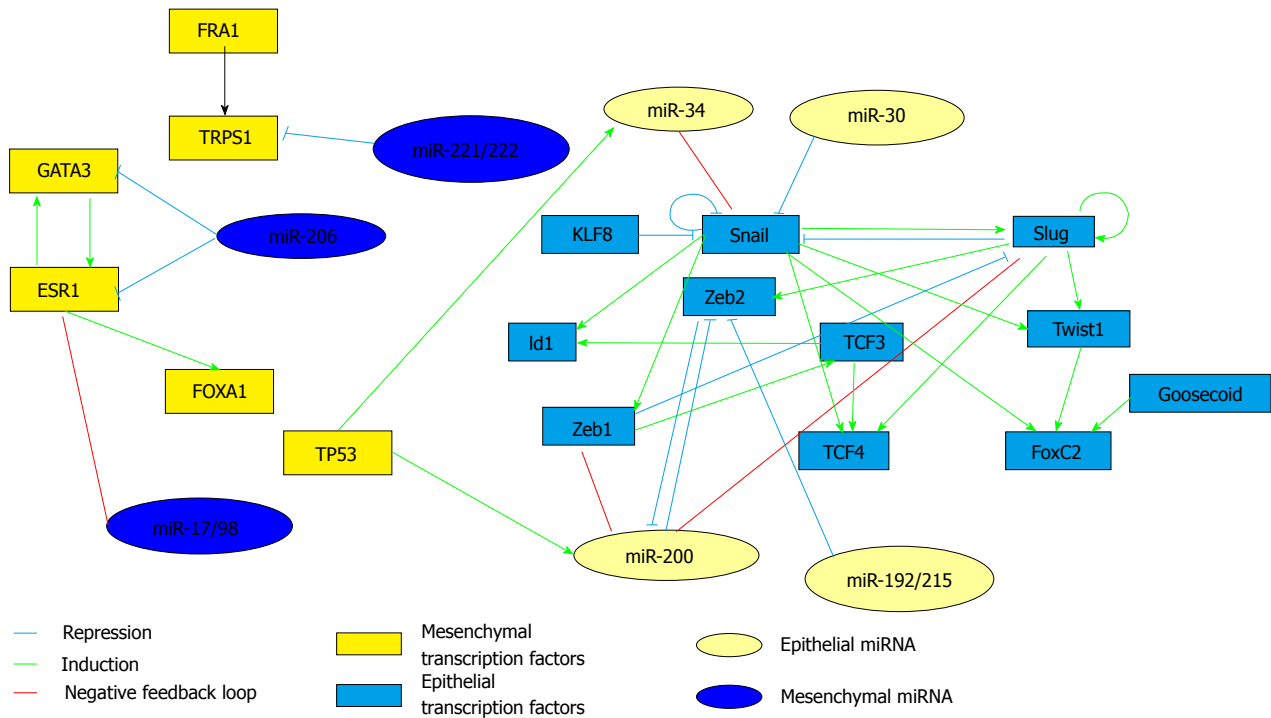


Figure 3 Feedback and feedforward loops existing between transcription factors and miRNA during breast cancer progression. Epithelial and mesenchymal regulators modulate their own expression creating regulatory feedback and feedforward loops.

cell expansion; enforced expression of let-7 blocks the Wnt- β -catenin pathway-stimulated breast CSC phenotype. Another study has shown that Lin28 expression was dramatically increased in tumor tissues after neoadjuvant chemotherapy, in local relapse and in metastatic breast cancer tissues^[72].

CONCLUSION

Due to their implication in tumor development and metastasis, miRNAs represent potential therapeutics tools. Several studies either inhibiting or re-introducing miRNAs involved in EMT and CSCs regulation, are currently ongoing. Cai *et al.*^[35] have delivered a let-7a agomir into the pre-malignant mammary tissues of MMTV-wnt-1 mice and shown that it resulted in a complete rescue of the stem cell phenotype driven by the Wnt- β -catenin pathway. An interesting approach to neutralize miRNAs is to saturate them with target mRNAs. These artificial targets are called "miRNA sponges", expressing an mRNA containing multiple tandem binding sites for an endogenous miRNA and thus prevent the association of the miRNA with its endogenous targets^[73]. We can hypothesize that introducing a ZEB2 miRNA sponge transcript in metastatic breast cancer cells may lead to a de-repression of PTEN transcripts.

In order to better evaluate the TFs--miRNAs regulatory relationships during mammary cancer progression, the next step is to identify specific combinations of epithelial and mesenchymal TFs--miRNAs networks co-existing in metastatic and stem-like cells in breast cancers. It is crucial to discriminate between the aberrant

dynamics of epithelial-mesenchymal transitions during tumorigenesis and normal programs of embryonic development and wound healing. Interestingly, a synthetic analysis has shown that a core regulatory unit composed of two highly interconnected modules, the miR-34/SNAIL and the miR-200/ZEB double negative feedback loops, participate in the regulation of stemness, genome stability, cell-cell communication, and cellular motility. The authors have shown that the miR-200/ZEB loop exhibits tristability (the existence of three distinct stable states: epithelial, hybrid and mesenchymal) and that the miR-34/Snail circuit exhibits monostability (existence of a single stable state)^[74]. Regarding breast tumorigenesis, the miR-200/ZEB circuit is likely involved in cell plasticity and the miR-34/Snail in the stabilization of metastatic phenotype.

Manipulation of the EMT-TF-miRNAs feedforward and/or feedback loops may provide new therapeutic targets for breast cancers.

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WJCO 5th Anniversary Special Issues (2): Breast Cancer

Regulation of the mRNA half-life in breast cancer

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Abstract

The control of the half-life of mRNA plays a central role in normal development and in disease progression. Several pathological conditions, such as breast cancer, correlate with deregulation of the half-life of mRNA encoding growth factors, oncogenes, cell cycle regulators and inflammatory cytokines that participate in cancer. Substantial stability means that a mRNA will be available for translation for a longer time, resulting in high levels of protein gene products, which may lead to prolonged responses that subsequently result in over-production of cellular mediators that participate in cancer. The stability of these mRNA is regulated at the 3'UTR level by different mechanisms involving mRNA binding proteins, micro-RNA, long non-coding RNA and alternative polyadenylation. All these events are tightly interconnected to each other and lead to steady state levels of target mRNAs. Compelling evidence also suggests that both mRNA binding proteins and regulatory RNAs

which participate to mRNA half-life regulation may be useful prognostic markers in breast cancers, pointing to a potential therapeutic approach to treatment of patients with these tumors. In this review, we summarize the main mechanisms involved in the regulation of mRNA decay and discuss the possibility of its implication in breast cancer aggressiveness and the efficacy of targeted therapy.

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Key words: mRNA stability; Breast cancer; RNA binding proteins; MicroRNA; Alternative polyadenylation

Core tip: This review article is dedicated to the understanding of the mechanisms involved in the regulation of mRNA half-life. mRNA relative stability is an important way to rapidly increase or decrease the level of a given gene. This process is a much more rapid mechanism compare to transcriptional regulation. Since many genes implicated in cancerous processes are regulated at the level of their half-life, the proteins and/or small non coding RNA implicated in this regulation may serve as relevant prognosis markers or predictive markers of the efficacy of chemotherapeutic agents.

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GENERAL INTRODUCTION TO mRNA STABILITY

In the last few decades, our knowledge of the complexity of the regulation of gene expression in eukaryotes has expanded considerably. Control of regulation is exerted through several mechanisms that take place in

the nucleus or in the cytoplasm. In the past, the majority of studies focused on transcription, but modulation of post-transcriptional events has gaining a lot of interest and represents a rapid and plastic way of regulating gene expression. In particular, regulation of mRNA stability determines the spatial and temporal expression of many genes and plays a fundamental role in determining the outcome of gene expression^[1,2].

Most mRNA regulatory elements involved in this process are situated within the 5' and 3'untranslated regions (UTRs), where they act as platforms for the assembly of protein complexes and other regulatory factors. Whereas the 5'UTR is primarily involved in controlling mRNA translation^[3], the 3'UTR regulates multiple aspects of mRNA metabolism, including nuclear export, cytoplasmic localization, translational efficiency and mRNA stability^[4].

Tight regulation of mRNA stability is essential for cells to perform their normal functions. Substantial stability means that a mRNA will be available for translation for a longer time, resulting in high levels of protein gene products. The lengths and structures of the 3'UTR vary substantially and more than half of the mammalian genes produced by alternative splicing and alternative polyadenylation (APA) multiple the number of mRNA isoforms that differ in their 3' UTRs and therefore in regulatory sequences within their 3' UTRs^[5-7].

Modulation of the mRNA half-life plays a central role in inflammatory diseases and various cancers^[8-17]. Aberrant stabilization of mRNAs may lead to prolonged responses that subsequently result in undesirable states, including over-production of growth factors, oncogenes and other mediators that participate in cancer. The steady state levels of mRNA-binding proteins and regulatory RNAs are often associated with invasion and aggressiveness^[18,19].

We are only beginning to define the mechanisms coordinating the mRNA half-life and to understand its involvement in tumorigenesis. The fate of a transcript is determined by the complex interplay of *cis*-acting sequences within the 3'UTR of the mRNA and *trans*-acting factors, such as RNA binding proteins (RBPs) and regulatory RNAs (microRNA (miRNA) and long non coding RNA (lncRNA)) that bind directly or indirectly to the *cis*-acting elements and promote the deadenylation and degradation of the mRNA.

In this review we will discuss the role played by all these factors on mRNA stability, focusing on breast cancer and the recent advances made in evaluating cancer aggressiveness and the efficacy of targeted therapy. We will focus on the effect of these key factors on only the mRNA half-life and not on the efficiency of protein translation (for more comprehensive studies see^[20-22]). We will also briefly discuss new topics such as shortening of 3' UTR by alternative polyadenylation.

A BRIEF OVERVIEW OF THE MECHANISMS INVOLVED IN THE REGULATION OF THE mRNA HALF-LIFE

The stability of common RNAs is regulated by a variety of signals acting on specific sequences within the RNA, which are recognized by *trans*-acting factors such as mRNA binding proteins, miRNA and lncRNA. All these factors are post-transcriptional gene regulators that bind mRNA and can regulate both mRNA stability and translation.

AU-rich sequences and mRNA binding proteins

The most conspicuous among the different *cis*-acting destabilizing elements identified so far are the AU-rich elements (AREs), located in the 3'UTR of a variety of short-lived mRNAs such as those for cytokines and proto-oncogenes^[23,24]. Estimated to represent approximately 7% of all transcripts, ARE-mRNAs (see public database at: <http://rna.tbi.univie.ac.at/AREsite>) encode a functionally diverse group of proteins involved in the inflammatory and immune response, transcription, cell proliferation, RNA metabolism, development, and signaling^[25,26]. This functional enrichment of ARE-genes correlates with the observed rapid patterns of mRNA decay, particularly of those involved in transcription and signaling^[27]. These elements are recognized by *trans*-acting factors, such as mRNA-binding proteins that bind directly or indirectly to the *cis*-acting elements and promote the deadenylation and degradation of the mRNA. These proteins may have opposite effects on mRNA stability. Some of them, such as AU-rich element RNA-binding protein 1 (AUF1), tristetraprolin (TTP) and KH-type splicing regulatory protein (KSRP), promote mRNA degradation, while others such as embryonic lethal abnormal vision (ELAV)-like protein 1 (HuR) and polyadenylate-binding protein-interacting protein 2 (PAIP2) act as mRNA stabilizing proteins^[10,28,29].

Briefly, AUF1 binds with high affinity to RNA that contain ARE sequences such as *MYC*, *FOS* and *GM-CSF* mRNAs. In contrast, it does not bind with high affinity to RNA sequences that lack a ARE^[30]. Over-expression of AUF1 correlates with rapid degradation of ARE-containing mRNAs^[31-33].

TTP (also named *ZFP36*) is characterized by two tandem repeat zinc finger motifs through which it binds to AREs and mediates mRNA decay^[34,35]. Some of the well-established targets of TTP include TNF alpha mRNA^[36], granulocyte/macrophage colony-stimulating factor (GM-CSF)^[37], cyclooxygenase 2^[38], vascular endothelial growth factor (VEGF)^[39], interleukin-1^[40], interleukin-8^[41,42] and the hypoxia-inducible factor-1 (HIF-1)^[43]. HuR is a member of the ELAV family of proteins found in mammalian cells. HuR selectively binds and stabilizes ARE-containing mRNAs of proto-oncogenes, cell cycle regulators, cytokines and other early-response genes^[44,45]. For a more

comprehensive review and detailed description of RNA binding proteins see^[10,28,30,46,47].

Regulatory non-coding RNA

RNAs have long been considered as an intermediate between DNA sequences and proteins that execute cellular functions. However, recent genome-wide analyses suggest that protein-coding genes represent only 2% of the human genome while there are at least thousands of non-coding RNAs (ncRNAs) transcribed from mammalian genomes. For many of them, a clear role in regulation of gene expression has been demonstrated^[48,49].

There are two major classes of ncRNAs; the small ncRNA, such as miRNAs and lncRNAs. miRNA act by pairing to the mRNAs of protein-coding genes to direct their repression, while lncRNAs show different mechanism of action, varying from chromatin remodeling, transcriptional responses and RNA processing^[50,51].

miRNAs, a small class of ncRNAs approximately 18-25 nucleotides in length, are able to regulate gene expression at the post-transcriptional level, by binding to partially homologous sequences to the 3'UTR of target mRNAs, and thereby causing a block in translation and/or mRNA degradation^[52]. Although miRNAs were first identified in the early 1990s, it is only during the past decade that their potential has been more widely explored. Several studies have demonstrated that miRNAs are highly specific for developmental stages and that they play important roles in essential processes, such as differentiation, cell proliferation, stress response and cell death^[53,54]. Gene regulation by miRNAs is important for the onset and progression of several human cancers^[55-57].

Recent profiling studies have identified miRNAs that are aberrantly expressed in human cancers and miRNAs are now widely believed to play an essential role in many malignancies, acting as either tumor suppressors or oncogenes. This classification is based on repression of their target genes, which means that certain miRNA will be tumor suppressive if its target gene is an oncogene or a tumor suppressor^[58,59]. miRNA usually act by pairing to complementary sequences in their 3'UTR and promoting mRNA deadenylation or a translational block^[20,60].

lncRNA are a new class of ncRNA, with a length ranging from 200 bp to 100 kbp, which have recently caught a lot of attention. The latest GENCODE project has annotated 14880 lncRNAs from 9277 loci^[61], but only a few of them have been characterized. Studies demonstrated that lncRNAs play major biological roles in embryonic stem cell biology and cellular development and show developmental and tissue specific expression patterns^[62-65]. lncRNA are involved in numerous biological roles such as imprinting^[66,67], epigenetic regulation^[68], apoptosis and cell cycle control^[69], transcription^[49] and post-transcriptional regulation, splicing^[70] and aging^[71].

Therefore, aberrant lncRNA expression can cause various human diseases including cancer^[69,72]. Currently, dozens of lncRNAs have been identified to play critical roles in the development and progression of cancer, act-

ing as potential onco- or tumor-suppressor RNAs^[73,74]. The mechanisms of action of lncRNAs are varied and include the creation of secondary RNA structures, binding to DNA and RNA binding proteins, or hybridization with complementary sequences of RNAs^[75,76].

Accumulating evidence of deregulated lncRNA expression in numerous cancer types suggests that this type of regulation may open new avenues to identification of therapeutic targets for cancer^[73,77]. For more a detail discussion on short and long RNA see also^[59,78-80].

Alternative polyadenylation

Alternative polyadenylation (APA) is emerging as a widespread mechanism used to control gene expression but the mechanisms/steps governing both global and gene-specific APA are only starting to be deciphered.

APA consist of two steps, cleavage and polyadenylation of RNAs, which are maturation events that cut and add an oligo(dA) tail to the 3' end of the nascent transcript. This processing protects mRNAs from degradation and increases their stability. The specific cleavage position and the efficiency of the process depend on the interaction between *trans*-acting polyadenylation factors and *cis*-elements present in the pre-mRNAs, such as the central sequence motif AAUAAA, identified in the mid 1970s and subsequently shown to require flanking, auxiliary elements for both 3'cleavage and polyadenylation pre-mRNA^[2,81-83]. Previous studies have indicated that more than half of the human genes possess multiple polyadenylation sites^[84], called APA, which may produce mRNA isoforms with different protein-coding regions or 3'UTRs of variable length (when APA occurs in the last exon). The differential recognition of polyadenylation signals leads to long or short 3'UTR of the transcripts. Usage of alternative poly(A) sites influences the fate of mRNAs in several ways, for example, by altering the availability of RNA binding protein sites and miRNA binding sites. Usage of APA and alterations in polyadenylation are beginning to be discovered and studied in human diseases^[85,86] and it is now clear that APA has several functional consequences in cancerogenesis.

INTERACTIONS BETWEEN THESE MECHANISMS

Our knowledge of the complexity of post-transcriptional regulation has expanded continuously and it is now clear that there is a strict connection between all the mechanisms involved in determining mRNA half-life. Evidence collected so far show a growing number of connections between miRNAs and RNA-binding proteins, underlying a new level of complexity of regulation of gene expression^[87,88]. As described above, miRNAs and RBPs are post-transcriptional gene regulators that bind mRNA and can regulate both mRNA stability and translation.

Bioinformatic analyses showed that ARE motifs, normally recognized by RBPs, are over-represented in

miRNA target sites of transcripts and might antagonize or cooperate with miRNA-dependent gene regulation^[89]. This *in silico* analysis provides support for other studies in which close interactions between ARE-BPs and miRNAs were found^[88,90]. In fact, recent studies demonstrated co-operative interactions between miRNA and ARE-BPs in the modulation of gene expression. TTP and miR16, a miRNA containing a UAAAUAUU sequence that is complementary to the ARE sequence, were shown to depend on each other to efficiently suppress the mRNA of TNF alpha^[88]. In other cases, ARE-BPs and miRNA compete for a binding site on the mRNA, thereby counteracting the functions of each other. The binding of HuR to the 3'UTR of the cationic amino acid transporter 1 (CAT1) mRNA prevents miR122-mediated repression of CAT-1 expression, thereby resulting in enhanced expression of the CAT-1 gene^[91]. Other studies revealed that HuR sites are enriched near predicted miRNA sites in mRNAs and frequently overlap with them^[92,93].

There are also other examples, miRNAs can regulate the expression of RBPs, or the converse, where an RNA-binding protein specifically regulates the expression of a specific miRNA, and some of them have been described in detail in a recent review^[94].

Recently an interaction between the two major classes of regulatory RNAs has been demonstrated: miRNA and lncRNA. It has long been assumed that miRNAs can only target protein-coding mRNAs in the cytoplasm. However, recent studies have revealed that miRNAs are also transported from the cytoplasm to the nucleus, where they function in a non-canonical manner to regulate lncRNAs^[95]. These results suggest that certain miRNAs might affect the expression level of many genes through modulating the biogenesis and function of lncRNAs^[96]. Recently, a dynamic interplay between alternative polyadenylation and miRNA regulation has also been reported. Since APA often results in mRNA isoforms with different 3'UTRs lengths, these isoforms of an mRNA are differentially regulated by miRNAs^[97].

The connection between all the mechanisms involved in determining mRNA half-life underlies the great potential of fine-tuning post-transcriptional control of gene expression. A careful revision of the literature has made us realize that alterations and dysfunction in all these mechanisms may be involved in breast cancer.

BREAST CANCER

Breast cancer is the leading cause of cancer-related deaths among women and its incidence is increasing worldwide. This neoplasia is a multi-factorial disease in which several factors contribute to initiation of the disease such as a genetic predisposition, chronic inflammation, exposure to toxic compounds, abundant stress factors, and others. The cumulative effects lead to a high incidence of breast cancer in populations worldwide^[98]. In the last few years post-transcriptional regulation has been demonstrated to play a major role in breast cancer. Below, we will provide

an overview and update of the dysfunction or alteration involved in the control of the mRNA half-life. Therefore, a better understanding of these mechanisms may help exploit the full potential of mRNA stability with respect to cancer diagnosis, treatment, and therapeutics.

RBPS IN BREAST CANCER

Modulation of the mRNA half-life plays a central role in breast cancer. Evidence collected so far has demonstrated the important role for RNA binding proteins in regulation of the mRNA half-life of several genes involved in cancer progression such as oncogenes, cytokines, and growth factors that are often involved in tumorigenesis^[10,99,100]. In particular, TTP and HuR have often been shown to be deregulated in breast cancer and can be proposed as prognostic markers.

Several studies performed on breast cancer cell lines showed that TTP is deficient in breast cancer cells when compared with normal cell types, suggesting the involvement of TTP as a tumor suppressor in breast cancer^[101,102]. Similar results were confirmed by analysis of samples from breast cancer patients.

In 2009, a gene array data set of 251 breast tumors, showed a negative correlation between TTP mRNA levels and tumor grade, with more advanced tumors typically showing the weakest TTP expression^[103]. Moreover, patients with intermediate or low tumor TTP mRNA levels were 2- to 3-fold more likely to die from recurrent breast cancer than patients whose tumors strongly expressed TTP, suggesting that suppressed TTP expression may represent a negative prognostic indicator in breast cancer. The same findings were confirmed by other studies, which also found that TTP expression is higher in normal breast tissue and benign lesions than in infiltrating carcinomas. Moreover a strong positive association of TTP expression and mammary differentiation was identified in normal and tumor cells, with mammary differentiation inducing expression of TTP^[104]. Loss of TTP also enhances infiltration of monocytes/macrophages into the tumors, which is typically associated with poor prognosis in breast cancer^[105]. Recently, TTP has been showed to be involved in mammary differentiation both in normal and tumor cells, suggesting that this protein might play specific and relevant roles in the normal physiology of the gland^[104]. Regarding HuR, a lot of evidence showed a direct role in breast carcinogenesis. HuR seems to enhance breast cancer cell growth and invasion^[106], even though the same group showed that breast cancer patients expressing high levels of HuR had a favorable prognosis^[107]. This finding contradicts two recent studies showing that cytoplasmic HuR expression is elevated in ductal *in situ* carcinomas, when associated with a high tumor grade^[108], and a negative prognostic indicator for survival in patients with breast cancer^[109]. Moreover, in breast cancer cell lines HuR specifically regulates the Forkhead box O (FoxO) transcription factor FOXO1, an important tumor suppressor involved in apoptosis,

the cell cycle, DNA damage repair and oxidative stress. Recently, it was demonstrated that cytoplasmic HuR is associated with reduced survival in invasive breast cancer and can be used as an independent prognostic marker in breast cancer patients undergoing chemotherapy^[110].

REGULATORY RNA IN BREAST CANCER

As described above, regulatory RNAs include miRNAs and lncRNA that play important gene-regulatory roles. While lncRNAs show different mechanisms of action, miRNA act by pairing to the mRNAs of protein-coding genes to direct their repression. In this way, they can decrease the translational efficiency and/or decreased mRNA levels of gene targets^[50]. Even though most of miRNA act by lowering mRNA levels^[20], after reviewing the vast amount of literature on miRNAs, we found that most of the published articles do not investigate the mechanisms by which the miRNA act. Most of them reported the binding of the miRNA to the 3'UTR of the gene or the effect obtained with a luciferase assay, without clarifying if the miRNA affect the mRNA stability or efficiency of translation of the target gene. Since the effect on the efficiency of translation goes beyond this review, we will focus here on miRNA that affect the mRNA half-life and not the global number of miRNA involved in breast cancer. This topic has been extensively covered by other reviews^[111-114]. We will briefly discuss some examples of miRNA demonstrated to directly affect mRNA stability. By acting on mRNA stability, miR125a and miR125b decrease the expression of HER2 and HER3, two genes crucial in breast carcinogenesis and c-RAF, another gene that plays a crucial role in cancer^[115-117]. Another important miRNA is miR206, which targets ER alpha and represses both ER alpha mRNA and protein expression^[118,119]. miR200 regulates epithelial-mesenchymal transition (EMT) targeting Zfhx1b mRNA probably by mRNA deadenylation and destabilization^[120] while miR506 is always involved in EMT by increasing the levels of E-cadherin (CDH1)^[121]. miR34 suppresses invasion and metastatic potential of breast cancer cells by directly targeting Fos related antigen 1 (Fra1) by reducing both the mRNA and protein level^[122]. miR31 decreases the mRNA levels of many genes involved in breast cancer metastasis^[123], while miR203 plays a crucial role in triple negative breast cancer by targeting baculoviral IAP repeat-containing protein 5 (BIRC5) and Lim and SH3 domain protein 1 (LASP1) at the RNA level^[124]. miR21 is another miRNA that affects mRNA stability and is involved in decreasing mRNA levels of Programmed cell death 4 (PDCD4)^[125], while miR26b decreases *Prostaglandin-endoperoxide synthase-2 (PTGS2)* levels in breast cancer^[126] and miR124 affects the cd151 mRNA level^[127].

It is interesting to note that some miRNA affect the mRNA half-life of target genes by perturbing the levels of RNA binding proteins. In 2009, miR29a was reported to suppress TTP in cancer cell lines^[128]. The same miRNA is abundant in invasive breast cancer cells, where it

increases stabilization and subsequent over-expression of HuR. In this way the TTP: HuR ratio was perturbed and associated with cancer invasion^[129]. Moreover, other miRNAs bind to AREs and, thus, interact with ARE-binding proteins (ARE-BPs) to regulate transcript levels. miR3134 mediates an up to 4-8-fold increase in the levels of SOX9, VEGFA, and EGFR, which contain ARE in their 3'UTR, and are also regulated by HuR. Both miR3134 and HuR act together in a general mechanism of regulation of gene expression, which enhances transcript stability^[130].

Another example of interactions is the one between miR125 and HuR. Guo *et al.*^[115] observed that the expression of miR125a inversely correlated with HuR in several different breast carcinoma cell lines. They demonstrated that HuR was translationally repressed by miR125a. This result suggested that miR125a may function as a tumor suppressor for breast cancer, with HuR as a direct and functional target.

To date, the emerging literature on lncRNA includes only one example of regulatory RNA that effect mRNA stability in breast cancer. Two studies reported the effect of a lncRNA on the half-life of the HIF-1 mRNA. HIF-1 is a heterodimeric transcription factor that regulates the expression of genes associated with adaptation to reduced oxygen pressure. HIF-1 is considered to be a reliable prognostic and diagnostic marker for an increasing number of cancers from various origins, including breast cancer^[131]. A natural antisense of HIF-1 transcript (aHIF) that is complementary to the 3'untranslated region of the HIF-1 mRNA has been described in breast and renal carcinoma. The mechanism of action is not clear but it seems that aHIF could expose AU rich elements present in the 3' untranslated region of the HIF-1 mRNA and thus increase the rate of degradation of the HIF-1 mRNA^[132]. aHIF has been proposed as a marker of poor prognosis^[133], but further studies are clearly necessary.

ALTERNATIVE POLYADENYLATION IN BREAST CANCER

APA gives rise to mRNA isoforms with 3'UTR of variable length. There are few examples of the usage of alternative poly(A) in breast cancer in control of gene expression. One of the best examples is the shortening of the 3'UTR, which has been reported by Lembo *et al.*^[134] to correlate with poor prognosis. These authors showed that shorter 3'UTR can contain less regulatory elements that are normally involved in mRNA decay, such as miRNA binding sites or ARE sequences. In this way, miRNA and RBPs may increase the degradation rate of long isoforms of the gene, altering the ratio between the long and short isoforms and causing a shift from the normal to cancerous state^[134,135]. Recently, it has been reported that estrogens, which play a major role in breast cancer initiation and progression, can induce APA

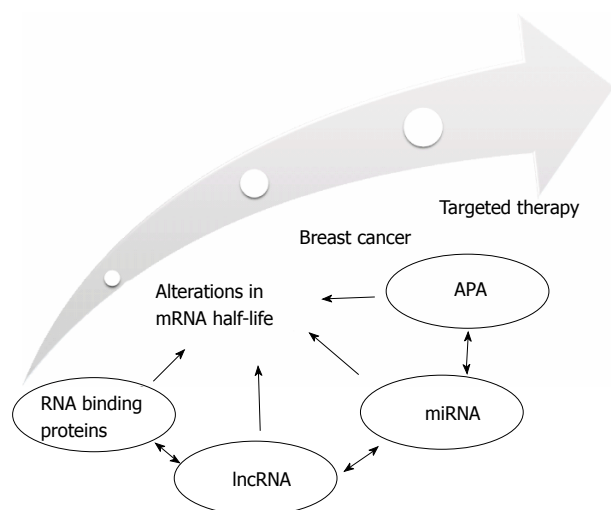


Figure 1 The regulation of the mRNA half-life and targeted therapy in breast cancer. Modulation of mRNA half-life depends on the interaction and balance between RNA binding proteins, regulatory RNAs (miRNA and lncRNA) and alternative polyadenylation in the 3'UTR of genes.

in breast cancer cells. In particular, estradiol up-regulates the 3'UTR shortening of CDC6, an essential regulator of DNA replication^[136]. Further investigations are necessary to establish if the expression ratio of alternative 3'UTR can be a predictor of survival in breast cancer patients.

PROGNOSTIC FACTORS AND TARGETED THERAPEUTICS

In the last few years several studies have proposed evaluating post-transcriptional regulation as a prognostic factor for breast cancer aggressiveness and for development of targeted therapy.

Regarding miRNA, Iorio and coauthors identified several miRNA associated with breast cancer, which points to their role in the development of this neoplasia and to the impact on putative innovative therapeutic approaches^[113]. Among the miRNA that have a direct impact on mRNA half-life of genes involved in breast cancer, miR206 and miR125a and 125b are surely of great interest. These miRNA are involved in specific networks, such as in the HER family-driven or ER-mediated signaling, and could likely influence the response to chemotherapy or to targeted therapies, such as trastuzumab, the monoclonal antibody directed against HER2, or anti-estrogens, such as tamoxifen. Recently Hong L and coauthors investigated the role of miR210 in predicting survival. A total of 511 cases of breast cancer were examined in a global meta-analysis that showed that high expression levels of miR210 predicted poor survival in patients with breast cancer^[137], confirming previous results obtained with miR210^[138].

Several miRNA involved in the regulation of the mRNA half-life in breast cancer, such as miR200, miR26b, miR21, miR34a are also modulated by estradiol^[139], which plays a major role in a hormone-dependent cancers such

as breast cancer.

Regarding RBPs, it is now generally assumed that loss of TTP and gain of HuR expression represent useful negative prognostic indicators in breast cancer. Monitoring mRNA or protein levels of these RBPs is being discussed, but it seems that at least for TTP, monitoring protein levels would provide a better negative correlation with breast cancer invasiveness than quantifying transcript levels^[101,104]. Recently, it has been suggested that the ratio between TTP and HuR be evaluated, since it is perturbed in invasive breast cancer patients, and to correlate it with cancer invasion^[108,109,129,140,141]. Due to the pivotal role played by TTP and HuR in stabilizing mRNA of key factors and cytokines involved in carcinogenesis and subsequent cancer progression, their clinical implication and therapeutic potential in cancer have been thoroughly investigated and recently reviewed by Ross *et al.*^[46], Eberhardt *et al.*^[142], Srikantan *et al.*^[47] and Abdelmohsen *et al.*^[143].

Recently, Wang *et al.*^[110] investigated the predictive and prognostic value of HuR expression in women with breast cancer who underwent neo-adjuvant chemotherapy followed by surgical resection. They evaluated the relationship between the HuR expression level and pathologic complete response (pCR), and found that cytoplasmic expression of HuR was an independent prognostic marker in breast cancer patients undergoing chemotherapy^[110].

Promising data have been obtained from the study of regulatory lncRNA or alternative polyadenylation, but need further investigation and large-scale studies on cohorts of breast cancer patients.

It is of interest to point out the genetic polymorphisms in TTP and HuR, which may represent prognostic factors for breast cancer. Since the two RBPs play a central role in post-transcriptional control of genes involved in breast cancer, some studies analyzed the frequency of germline polymorphisms in breast cancer patients compared to healthy controls. A synonymous polymorphism in the TTP gene showed a statistically significant association with a lack of response to Herceptin/trastuzumab in HER2-positive breast cancer patients^[101]. This polymorphism was associated with a decrease in translational efficiency. Moreover, another genetic variation in the promoter of the gene drastically reduced the amount of TTP mRNA and was significantly associated with poor prognosis. No association between polymorphisms of the HuR gene and breast cancer have been found^[144].

Several polymorphisms in miRNA have been described but meta-analysis has shown that they can be used for prediction of breast cancer risk in healthy population but not as prognostic markers in breast cancer patients^[145-148].

FINAL REMARKS

In recent years the importance of post-transcriptional control in breast cancer has become recognized. This process involves several steps including mRNA degrada-

Table 1 mRNA half-life modulators in breast cancer

mRNA half-life modulators	Main target	Prognostic factor	Ref.
RBP			
TTP	GM-CSF, cox2, VEGF, IL8, HIF1a, TNF- α	Yes	Griseri and Pagès
HuR	GM-CSF, cox2, VEGF, IL8, TGFB, TNF- α	Yes	Griseri and Pagès
microRNA			
miR125a, miR125b	HER2, HER3, cRAF, HuR	Possible	[115-117]
miR206	ER	Unknown	[118,119]
miR200	Zfhx1b	Yes	[120,137,138]
miR506	cdh1	Unknown	[121]
miR34	Fra1	Unknown	[122]
miR31	Fzd3, ITGA5, MMP16, RDX, RhoA	Unknown	[123]
miR203	BIRC5, LASP1	Unknown	[124]
miR21	PDC4	Unknown	[125]
miR26b	PTGS2	Unknown	[126]
miR124	cd151	Unknown	[127]
miR29a	TTP	Unknown	[128]
miR3134	SOX9, VEGFA, EGFR	Unknown	[130]
miR125	HuR	Unknown	[115]
lncRNA			
aHIF		Possible	[132,133]

tion and translation. Here we have focused on the regulation of the RNA half-life in breast cancer, focusing on several mechanisms that control this process, all involving the 3'UTR of genes: mRNA binding proteins, regulatory RNA such as short and long RNAs, alternative polyadenylation. All these mechanisms lead to a broad range of rates in gene decay. A diagram summarizing the involvement of different proteins in regulating the balance between stabilization and degradation of mRNA is given in Figure 1. In this review, we have discussed how alteration of mRNA stability mediated by all these mechanisms can contribute to the development of breast cancer. The goal is to elucidate the molecular mechanisms involved in breast cancer and to identify molecules that are useful as bio-markers of diagnosis or prognosis.

Full knowledge of this process may help to develop potential diagnostic and therapeutic strategies against tumor progression. The available data strongly suggest the use of RBPs or miRNAs as markers of diagnosis and prognosis, and eventually as new targets or tools in specific therapy. Table 1 shows the key players involved in control of mRNA half-life in breast cancer as discussed in this review, documenting the functional importance in defining the aggressiveness of breast cancer cells. A better understanding of the pathways they modulate and how this dictates pathological processes will surely help to obtain a better understanding of the pathogenesis of breast cancer and will in the future open doors for better classification, prognosis and direct treatment.

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WJCO 5th Anniversary Special Issues (2): Breast cancer**Molecular pathogenesis of bone metastases in breast cancer: Proven and emerging therapeutic targets**

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Core tip: Breast cancer cells preferentially colonize bone sites during their metastatic diffusion. Bone microenvironment and tumor cells operate a reciprocal selective pressure that results in the formation of osteolytic lesion and tumor progression. Targeting the molecular factors involved in this interaction has been demonstrated to be an effective strategy in preventing the clinical morbidity associated with bone metastases. In this review, we summarize physiopathologic aspects of bone metastases from breast cancer and discuss the most promising therapeutic targets for their future clinical management.

Abstract

Metastatic occurrence is the principal cause of death in breast cancer patients. The high osteotropism makes breast cancer the most common primary tumor type associated with metastatic bone disease. The peculiar clinical aspects associated with metastases limited to the skeletal system suggest considering these cases as a distinctive subset of metastatic patients with a better prognosis. Because bone is frequently the first metastatic site in disease relapse, it is feasible that the next improvement in therapeutic options for bone metastatic disease could be associated with an improvement of survival expectation and quality of life in breast cancer patients. Study of the molecular basis of bone remodeling and breast cancer osteotropism has allowed identification of several therapeutic candidates involved in formation and progression of bone metastases. These targets are frequently the determinants of positive feedback between the tumor and bone cells whose clinical outcome is osteolytic lesions. In this review, we discuss the physiopathologic features underlying targeted therapeutic strategies aimed at interfering with the aberrant bone remodeling associated with breast cancer metastases.

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CLINICAL ASPECTS

Progress in the diagnosis and treatment of primary breast carcinoma (BrCa) has not led to an overall improvement in survival. The main reason is that residual disease always generates distant metastases, eventually leading to a substantial reduction in life expectancy. As revealed by autopsy studies, undiagnosed metastases in BrCa patients are more frequent than expected, and without an effective strategy aimed at reducing the spread of cancer cells, clinically relevant metastases are estimated to remain the main cause of death associated with BrCa. Although

the presence of metastasis at diagnosis is uncommon in BrCa, the risk for this complication increases considerably within a few years after first treatment. Metastases involving multiple organs, mainly the lung, liver, and bone, are the most frequent situation, while the incidence of isolated metastasis is thought to be lower than 5%^[1].

Large autopsy series have demonstrated that bone metastases are present in 54%-73% of BrCa patients who died of the cancer^[2]. In addition, the high incidence of latent bone metastases at autopsy and the frequent detection of disseminated tumor cells in the bone marrow by sensitive methods suggest that bone colonization by BrCa cells is commonly associated with an indolent disease course and clinical dormancy^[3]. For this reason, study of the molecular determinants associated with predominance of dormancy over aggressive metastatic growth will have an important impact on clinical management of BrCa patients.

Although BrCa is the most common primary tumor type associated with metastatic bone disease, bone-only metastasis is infrequent and about 2% of these patients are diagnosed at the time of initial treatment^[4,5]. Also, according to autopsy records the chance of having bone-restricted metastasis is very low. Patients with disease confined to bone are more likely to be older at diagnosis and to have well differentiated tumors. Bone metastases are frequently associated with lung and/or liver metastases, and the cases with bone-only metastases are about 5%^[1]. In a distinctive way, patients with bone metastasis are more likely to have metastases in the central nervous system (CNS, about 27%) with respect to patients without bone metastasis (about 10%)^[6], and 18% of patients with CNS disease have bone as the initial site^[7]. In addition, lung and liver metastases are frequently found in more widespread disease with respect to bone metastasis^[6].

The predilection of BrCa cells for bone is confirmed by the fact that in more than 50% of BrCa patients, bone is the first site of distant relapse^[5,8]. The median period from BrCa diagnosis to the development of first metastatic bone lesion is about 3 years. However, 49% of these relapsing patients tend to develop extraosseous metastases, and 56% of patients with solitary bone metastasis tend to develop multiple metastatic bone lesions.

Also, for BrCa patients with bone-only disease the presence of multiple bone metastases at first diagnosis is common (59%), and the more frequent (> 50%) anatomical sites are thoracic and lumbar spine, the sternum, and pelvis. The sternum was described as the site of solitary metastasis in a significant percentage of BrCa patients at the time of diagnosis and this could be explained by anatomical proximity with the primary tumor^[5,9].

The median survival after the first recurrence of BrCa in bone is markedly higher with respect to the survival of those with first recurrence of cancer in extraosseous sites (20 mo *vs* 3 mo)^[8]. Although bone-only metastasis demonstrates a relative good prognosis, skeletal involvement is frequently associated with considerable morbidity. This

includes hypercalcemia, fractures, impaired mobility, and spinal cord compression and pain, which require higher and higher doses of analgesics.

According to the histological and clinical features, bone metastases can be classified as osteolytic, osteosclerotic, or mixed, the latter when both features coexist in the same metastatic district^[10]. BrCa bone metastases are quite exclusively osteolytic, characterized by bone destruction due to an exacerbated activity of osteoclasts, the bone cells physiologically devoted to resorb bone matrix. Indeed, the first theories speculated that the osteolytic lesion was the result of physical pressure of the tumor on the bone or bone resorption activity of the tumor cells themselves. It has also been highlighted that cancer cells induce lymphocytes to produce factors such as prostaglandins, which in turn could stimulate destruction of the bone^[11]. To date, more and more evidence has conclusively shown that cancer cells are not able to directly destroy the bone, but they release factors that directly or indirectly activate the formation and activity of osteoclasts^[10].

Due to the prevalent osteolytic nature of BrCa bone metastasis, skeletal progression of the disease can be monitored by the measurement of specific biochemical markers derived from the breakdown of type I collagen^[12]. These peptides include crosslinked C-terminal telopeptide isomers (CTX) and crosslinked N-terminal telopeptide (NTX), which can be measured in serum and urine.

MOLECULAR BASES

The high osteotropism of BrCa cells has been widely demonstrated in preclinical studies. The predilection of tumor cells for bone tissue could not be explained simply by anatomical features, and the so-called "seed and soil" theory, postulated by Steven Paget more than 100 years ago, is still valid today^[13]. This theory emphasizes that the process of colonization requires an interaction between tumor cells, which represent the seed, and the bone microenvironment, a deposit of calcium and growth factors released in response to bone resorption that provides the fertile "soil" in which cancer cells can proliferate.

These considerations have supported an intense investigation of the molecular determinants of osteotropism. However, the frequent widespread presentation of metastases in BrCa patients indicates that the pure osteotropic signature is restricted to a limited number of bone-only cases. Today, histologic analysis of BrCa subtypes drives prognosis, and in many cases permit prediction of the metastatic propensity of the primary tumor^[14]. Although much effort has been made to find a link between molecular profiles and metastatic site, this has not been fully established. In addition, molecular subtyping of the primary tumor is often not repeated in the metastases and the assumption that the metastatic tumor has identical marker expression as the primary tumor is currently debated^[15]. Moreover, it has to be considered that the bone microenvironment, with its peculiar characteristics,

may exert strong selective pressure on tumor cells, thus influencing the resulting phenotype of clinically relevant metastases. For example, parathyroid hormone-related protein (PTHrP), which was previously considered an effective predictor for identifying patients who are at high risk of developing bone metastases and which is expressed in the large majority of BrCa bone metastases and in 60% of primary BrCa, is thought to be stimulated in BrCa cells in response to transforming growth factor- β (TGF- β) released in the bone^[16].

Estrogen receptor (ER) expression represents the best consolidated marker associated with the risk for bone metastasis^[17]. ER+ breast tumors relapse preferentially to the bones over a delayed period^[18]. However, because there is significant loss of ER expression at many metastatic sites including bone, the role of ER in driving pathogenesis of bone metastasis needs to be verified^[6].

THERAPEUTIC OPTIONS

Bone-only metastatic cases have attracted clinical attention due to their good response to treatment and their association with extended patient survival. This aspect suggests considering patients diagnosed with metastases limited to the skeletal system as a distinctive subset of metastatic BrCa patients. The median survival of patients with metastatic BrCa involving extraskelatal sites is significantly shorter with respect to that of patients with bone-only metastasis, independent of the number of sites of skeletal involvement^[19]. Over the past two decades, bisphosphonates have emerged as a safe and effective component of treatment of bone metastatic disease from different cancers. The use of bisphosphonates in managing bone metastases had a profound beneficial effect on the frequency and severity of skeletal morbidity, resulting in improvement of quality of life^[20]. Bisphosphonates are potent inhibitors of osteoclast function that are recommended for long-term treatment^[21]. These compounds bind to exposed bone mineral and then are internalized by bone-resorbing osteoclasts, inhibiting bone resorption with different modes of action. Nitrogen-containing bisphosphonates (*e.g.*, zoledronic acid and pamidronate) act by inhibiting farnesyl diphosphate synthase, while other bisphosphonates are involved in the formation of cytotoxic metabolites in osteoclasts^[22,23]. In addition, recent studies have suggested that bisphosphonates may directly affect tumor cell invasion and survival^[24] and inhibit tumor-induced angiogenesis^[25].

Beyond the use of bisphosphonates, considered the most effective treatment for cancer-induced skeletal complications, other therapy choices include orthopedic intervention, radiation therapy, and cyclooxygenase-2 inhibitors or nonsteroidal anti-inflammatory drugs for reducing bone pain^[26]. Novel agents in clinical development are bone-seeking radionuclides that have been demonstrated to be safe and potentially useful in combination therapy with bisphosphonates or radiosensitizing drugs^[27].

PHYSIOLOGY OF BONE REMODELING

Understanding the molecular mechanisms leading to cancer-induced bone metastases requires knowledge of the physiology of bone tissue which, in contrast to its “hard” feature, is extremely dynamic and undergoes continuous renewal throughout the life of each individual, through a process known as bone remodeling^[28]. In particular, it has been estimated that about 10% of the bone is renewed every year^[29]. This phenomenon guarantees the following crucial functions: (1) Regulation of calcium homeostasis; (2) Renewal of old bone; (3) Substitution of primary infantile bone with mechanically competent bone; and (4) Repair of ischemic and microfractured bone.

Bone remodeling is the result of a perfect balance between the function of bone resorption performed by osteoclasts and osteogenesis accomplished by osteoblasts. Both cell types are the principal cells of bone tissue. This balance is crucial for maintenance of a proper bone mass and the lack of synchrony between the two functions is the starting point for skeletal diseases. As described in Figure 1, bone remodeling takes place according to the following phases:

Activation phase

This phase is so called because the lining cells, which are quiescent osteoblasts, respond to different stimuli (*i.e.*, growth factors, alteration of mechanical loading, and micro fractures) by increasing the expression of factors that stimulate osteoclast differentiation.

Resorption phase

Mature osteoclasts polarize on the bone surface, adhere to it and, by a process of acidification and subsequent release of proteolytic enzymes, such as the cathepsin K and the metalloproteinase (MMP)-9, degrade the bone matrix^[30]. Once they have accomplished their function, osteoclasts undergo apoptosis, a physiologic consequence necessary to avoid exacerbated bone resorption.

Reverse phase

This phase is so called because of the presence of reverse cells, macrophage-like cells that are likely responsible for removing the debris produced during bone matrix degradation.

Formation phase

The players of this phase are osteoblasts, recruited by the growth factors that are usually stored in the bone matrix but that are released after its degradation by osteoclasts. Once recruited, osteoblasts produce a new bone matrix, initially not calcified (osteoid), and then they provide mineralization, thus completing the bone remodeling process.

PLAYERS IN BONE REMODELING

The principal cells of the bone involved in bone remodeling

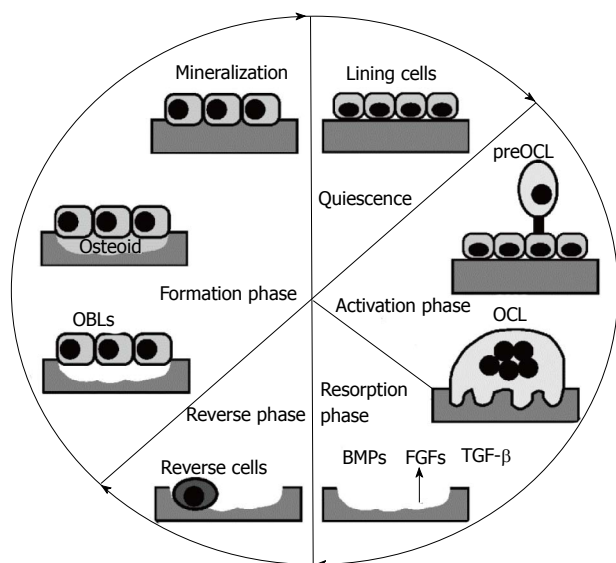


Figure 1 Phases of bone remodeling. Starting from the quiescence phase, bone remodeling is triggered by different stimuli that lead to activation of lining cells, which increase surface expression of receptor activator of nuclear factor kappa-B ligand (RANKL). This cytokine, by binding to its receptor RANK, promotes osteoclast differentiation (activation phase). Next, mature osteoclasts resorb bone (resorption phase), thus allowing the release of factors usually stored in the bone matrix (BMPs, TGF- β and FGFs) that recruit osteoblasts in the reabsorbed area. Once recruited, osteoblasts form the bone matrix and ensure its mineralization (formation phase), completing the bone remodeling process. BMPs: Bone morphogenetic proteins; TGF- β : Transforming growth factor- β ; FGFs: Fibroblast growth factor; OCL: osteoclast; OBL: osteoblast.

eling, osteoblasts and osteoclasts, also play a key role in the development of bone metastases.

Osteoclast formation and function

Osteoclasts arise from the monocyte/macrophage lineage^[31] and are multinucleated cells formed by the fusion of mononuclear precursors^[30]. Starting from a pluripotent hematopoietic stem cell, the transcription factor PU.1, along with macrophage colony stimulating factor (M-CSF), drives the commitment of a common progenitor for macrophages and osteoclasts. In particular, M-CSF stimulates proliferation of osteoclast precursors and upregulates receptor activator of nuclear factor kappa-B (RANK) expression, while PU.1 positively regulates the expression of c-Fms, the M-CSF receptor^[32]. With the appearance of c-Fms and RANK receptors, the precursors become fully committed to an osteoclast lineage. The RANK/receptor activator of nuclear factor kappa-B ligand (RANKL) pathway is mandatory for osteoclast differentiation and function, although it is not the only player in correct osteoclastogenesis.

Once differentiated, multinucleated osteoclasts need to adhere to the bone matrix and polarize in order to resorb bone. The first step of resorption requires the dissolution of the inorganic component of the matrix, which is hydroxyapatite. This can be achieved by the release of hydrochloric acid into the area to be resorbed, called resorption lacuna^[33], and also requires sealing of the underlined bone matrix, which is achieved through a cytoskeletal rearrangement and subsequent formation of an actin

ring. This is a circumferential structure formed by several dynamic and dot-like structures called podosomes, each of which consists of an actin core surrounded by the $\alpha_v\beta_3$ integrin and associated cytoskeletal proteins^[34].

Dissolution of mineral crystals allows digestion of the bone matrix organic component, which is performed by MMPs and lysosomal cathepsins. Among the latter, cathepsin K has a crucial role, as its deletion in mice leads to several skeletal diseases^[35]. Regarding the MMPs, osteoclasts mainly produce the MMP-9 isoform and, to a lesser extent, MMP-14^[36].

Osteoblasts

They arise from a mesenchymal stem cell (MSC) lineage consisting of pluripotent cells following a specific program of gene expression that may give rise to different tissue-specific cells including osteoblasts, chondrocytes, fibroblasts, myocytes, and adipocytes^[37]. The initial step of osteoblastogenesis is the commitment of MSCs towards an osteo/chondro-progenitor, which relies on the activation of two principal pathways: the Wntless-int (Wnt) pathway and the pathway triggered by bone morphogenetic proteins (BMPs). One of the earliest factors mandatory for osteoblast differentiation is Runt-related transcription factor 2 (RUNX2), along with osterix (OSX), which is downstream of RUNX2^[38]. Committed pre-osteoblasts are identifiable because they express alkaline phosphatase (ALP), one of the earliest markers of the osteoblast phenotype. As the pre-osteoblasts cease to proliferate, a key signaling event occurs for development of the large cuboidal differentiated osteoblasts. The active osteoblast is highly enriched in ALP and secretes bone matrix proteins such as collagen I and several non-collagenous proteins including osteocalcin, osteopontin, osteonectin, and bone sialoprotein II.

REGULATION OF BONE REMODELING

Several factors, systemic as well as local, regulate bone remodeling. Moreover, it is well known that the two principal players of bone remodeling talk to each other to reciprocally regulate their functions. In particular, osteoblasts produce RANKL mainly in the transmembrane form and, to a lesser extent, as soluble cytokine, which interacts with its receptor RANK, expressed by osteoclast precursors, eventually activating the intracellular pathway that stimulates osteoclast differentiation^[39]. Osteoblasts also produce osteoprotegerin (OPG), a soluble protein with the same extracellular structure as RANK but lacking the transmembrane domain. This allows it to act as a decoy receptor, since it binds RANKL, thus preventing its interaction with RANK and inhibiting osteoclastogenesis. Therefore, osteoclast differentiation relies on a proper RANKL/OPG ratio^[40].

BONE REMODELING PERTURBATION AND THE ONSET OF THE VICIOUS CYCLE

The physiology of bone remodeling is drastically dis-

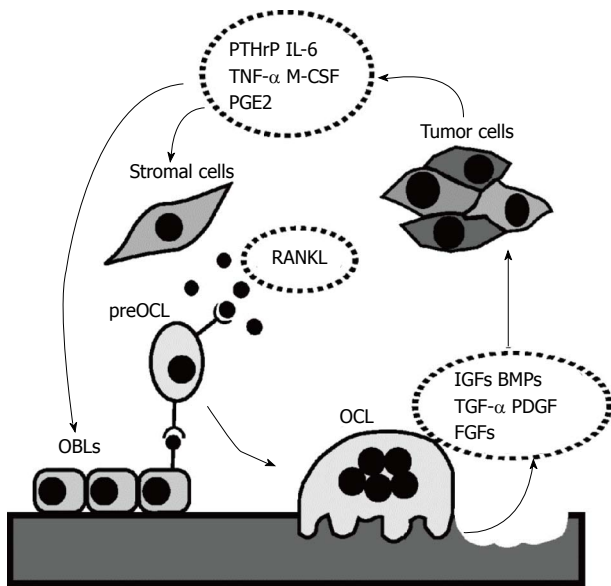


Figure 2 Schematic representation of the vicious cycle. Under physiologic conditions, osteoblasts produce factors that regulate osteoclastogenesis (RANKL/OPG). Mature osteoclasts erode the bone matrix, allowing the release of factors (IGF-1, BMPs, TGF- β , PDGF, and FGFs). Tumor cells perturb this homeostasis by producing factors (PTHrP, IL-6, TNF- α , M-CSF, and PGE2) that favor osteoclastogenesis, with subsequent bone resorption and release of the growth factors stored in the bone matrix, which in turn enhance tumor growth. BMPs: Bone morphogenetic proteins; TGF- β : Transforming growth factor- β ; FGFs: Fibroblast growth factor; IGFs: Insulin-like growth factors; PDGF: Platelet-derived growth factor; M-CSF: Macrophage colony stimulating factor; PGE2: Prostaglandin E2; OCL: osteoclast; OBL: osteoblast.

rupted by tumor cells once they colonize the bone milieu, eventually leading to the so-called vicious cycle (Figure 2). Indeed, tumor cells produce factors that, directly or indirectly through osteoblasts, induce exacerbated osteoclastogenesis, which in turn increases bone resorption and osteolysis. This means that tumor cells are not able to destroy bone *per se*, but they constrain resident cells to support their growth by creating new spaces inside the bone and allowing the release of several growth factors stored herein, such TGF- β , VEGF, insulin-like growth factors, platelet-derived growth factor, and BMPs. Thus, synergy between osteoclasts and tumor cells is created that fuels the vicious cycle, with an inexorable increase in both bone destruction and tumor growth^[10].

The ability of tumor cells to release in the bone microenvironment osteoclastogenic factors, usually produced by osteoblasts, further feeds the vicious cycle. Among the osteoclastogenic factors is PTHrP, produced by tumor cells under the stimulation of TGF- β , which in turn elicits RANKL expression and inhibits OPG production by bone marrow stromal cells and osteoblasts. Evidence for a role of PTHrP in this context arose some years ago from studies by Theresa Guise showing, in a mouse model of bone metastasis, that treatment with PTHrP-blocking antibody reduced BrCa cell-induced osteolysis as well as cancer growth. Moreover, tumor cells also produce M-CSF and prostaglandin E2, as well as several pro-inflammatory cytokines such as interleukin (IL)-1 β , IL-6, IL-11, and tumor necrosis factor α (TNF α),

which directly stimulate osteoclast formation and function^[41]. Finally, evidence from the vicious cycle tells us that, in order to fight it, we could act on two fronts: interfering with the release of specific factors by tumor cells or inhibiting aberrant osteoclastogenesis, and these two phenomena are strictly related.

THERAPEUTIC TARGETS

RANK/RANKL pathway

Given the crucial role of this pathway in osteoclast differentiation, it is not surprising that it has been considered one of the most promising therapeutic targets, leading to the development of denosumab, a human monoclonal antibody directed against membrane-bound and soluble RANKL. Denosumab prevents the binding of RANKL to its receptor, eventually leading to the inhibition of osteoclastogenesis. This drug is currently in clinical trials for the treatment of post-menopausal osteoporosis and bone metastases. In particular, denosumab administered subcutaneously every 4 wk at a dose of 120 mg showed a higher efficiency compared with zoledronic acid in delaying the onset of skeletal-related events in patients with BrCa and in reducing the levels of the bone resorption marker NTX^[42]. However, there was no difference between the two drugs in terms of the effect on patient survival. With regard to the potential side effects, we should not forget the role played by RANKL in the immunologic contest. A few cases of osteonecrosis of the jaw^[43] as well as frequent occurrence of hypocalcaemia^[44] have been reported.

Cathepsin K

Cathepsins are a family of cysteine proteases with 11 members in humans (cathepsin B, C, H, F, K, L, O, S, V, W, and X/Z) with different functions, such as antigen presentation, apoptosis, and autophagy. In the pathological context, cathepsin has been positively correlated with tumor invasion and angiogenesis^[45]. Indeed, B and L cathepsins are prognostic markers of different cancers, among them breast, where their increased expression is associated with a poor prognosis^[46].

Among the different cathepsins, the K isoform has been identified as a good therapeutic target in bone metastasis treatment, due to its pivotal role in bone resorption. Recently, inhibition of cathepsin B was also proven to be effective at inhibiting bone and lung metastases in a mouse model of BrCa^[47]. The crucial role of cathepsin K in osteoclast functions has been clarified by the evidence that a human mutant form of this gene determines a rare genetic disease called pycnodysostosis, characterized by an impairment of bone resorption. Preclinical data from animal models of BrCa bone metastases showed the ability of the cathepsin K inhibitor AFG-495 to reduce osteolytic lesions as well as local tumor growth^[48]. Another recent compound is odanacatib (MK-0822), a promising drug already used for osteoporosis treatment. A phase II clinical trial involving BrCa patients with bone metastases showed a reduction of bone resorption markers after

4 wk of treatment^[49].

Metalloproteases

Due to their function of degrading extracellular matrix, MMPs are obviously involved in the general process of invasion and metastasis. Indeed, some of them play a specific role in the onset of bone metastases, such as MMP-7, which is able to cut membrane-bound RANKL, thus increasing its local activity and favoring bone metastasis development^[50]. MMP-1, along with a disintegrin-like and metalloproteinase with thrombospondin motifs 1 (ADAMTS1), promotes proteolytic cleavage of epidermal growth factors (EGFs), which in turn inhibit osteoblast production of OPG, thus favoring osteoclastogenesis^[51]. Nannuru *et al.*^[52] showed that treatment of mice with Cl66 mammary tumors with MMP13 antisense oligonucleotides led to a significant reduction in bone destruction and in the number of activated osteoclasts at the tumor-bone interface, likely by reducing MMP-9 and RANKL expression.

Vascular cell adhesion proteins

This family is also mainly involved in carcinogenesis and metastasis. Among them, vascular cell adhesion proteins (VCAMs) seem to give a specific contribution to the development of bone metastases, as demonstrated by a recent study. Indeed, by interacting with its integrin receptor $\alpha 4 \beta 1$, VCAM-1 promotes the recruitment of osteoclast precursors. Moreover, treatment of metastatic mice with VCAM-1-blocking antibody significantly reduced bone and lung metastases^[53].

Mammalian target of rapamycin

Rapamycin is an immunosuppressive and antitumoral drug previously used to prevent graft rejection. Rapamycin inhibits mammalian target of rapamycin (mTOR), which is a serine-threonine kinase that stimulates cell survival and proliferation and whose deregulation is associated with the development of several tumors. What makes rapamycin an interesting target for bone metastases treatment is the evidence that the signal triggered by mTOR is important for the survival of osteoclasts^[54]. Moreover, a recent study showed that treatment with rapamycin reduced both the incidence and the area of the osteolytic lesion, through the inhibition of osteoclast formation^[55].

Integrins

Integrins include a large family of surface receptors that mediate cell-extracellular matrix interactions. Osteoclasts mainly express $\alpha v \beta 3$ integrin, which plays a crucial role in osteoclast adhesion, a process that is mandatory for correct bone resorption^[56]. Preclinical evidence shows that $\alpha v \beta 3$ inhibition blocks osteolysis and tumor growth in animal models of bone metastases. These studies were the starting point for ongoing clinical trials that are testing the effectiveness of various $\alpha v \beta 3$ antagonists in different bone metastatic cancers^[57].

Erythroblastic leukemia viral oncogene homolog (ErbB) receptors

The ErbB family of receptor tyrosine kinases represents an attractive therapeutic target in carcinomas. In fact, ErbB kinases are frequently overexpressed in these cancers and regulate important aspects of cancer progression by activating several key intracellular signaling intermediates, including PhosphoInositide 3 Kinase (PI3K), Ras-Raf Mitogen Activated Protein Kinase (MAPK), c-Jun N-terminal Kinase (JNK) and PhosphoLipase C (PLC)^[58]. The ErbB family comprises four members: ErbB1 [alias epidermal growth factor receptor (EGFR) and HER1]; ErbB2 (alias HER2 and Neu); ErbB3 (alias HER3); and ErbB4 (alias HER4). For EGFR and HER2, sufficient data have been collected to support their use as two of the five protein surrogate markers for assessing BrCa subtypes [ER, progesterone receptor (PgR), HER2, EGFR, and cytokeratin-5 (CK5)]^[59]. The role of the other ErbB kinases in BrCa progression remains controversial. In addition, only HER2 is found to be amplified in about 25% of primary BrCa cases. Although the data indicate that EGFR is not overexpressed in primary BrCa with respect to the normal breast epithelia^[60], recent studies have suggested a significant association of EGFR expression with the aggressive basal-type BrCa^[61] and with circulating BrCa cells^[62,63].

Current evidence does not support the hypothesis that EGFR or HER2 expression can predict bone metastasis^[17]. In contrast, ErbB-expressing BrCa shows the predilection to metastasize to visceral sites, including the brain, liver, and lung^[64]. Nevertheless, numerous preclinical studies have proposed a leading role for ErbB kinases in the progression of bone metastases^[65]. In particular, ErbB receptors may participate in the positive feedback underlying the vicious cycle. In fact, EGFR ligands present in the bone microenvironment are able to directly stimulate bone cells and osteolysis. Among the recognised ligands of the ErbB family, EGF, TGF- β , and amphiregulin (AREG) have been proposed as the main players in the bone microenvironment. EGF and TGF- β stimulation of osteoclastogenesis and bone resorption is accompanied by decreased OPG expression and sustained production of RANKL by bone stromal cells^[56,66]. In addition, EGFR is expressed by osteoblasts, and its activation stimulates osteoblast proliferation and decreases mineralization^[67]. EGFR ligands may also function by an autocrine loop, stimulating the release of cytokines by BrCa cells that directly influence osteoclastogenesis or indirectly stimulating EGFR signaling within bone cells. The key cytokine produced by BrCa cells in this way is PTHrP, which is able to stimulate RANKL expression and inhibit OPG expression in cells of the osteoblast lineage^[68]. In addition, PTHrP induces the expression and shedding of AREG and TGF- β , increasing the availability of ErbB ligands in the bone microenvironment. In turn, osteoblasts release EGFR ligands and perpetuate the cycle of osteoclast activation *via* RANKL and thus bone destruction^[65].

Src

In the last few years, Src, a nonreceptor tyrosine kinase, has attracted increasing interest due to its involvement in both tumorigenesis and bone metabolism. Src activity appears to be significantly associated with the development of bone metastases and late-onset relapse to bone. Using a bioinformatics approach, it was demonstrated that the role of Src was independent of the distinct molecular subtypes of human BrCa and also ER status^[69].

In BrCa cells, Src is frequently overexpressed and overactivated, allowing the transduction of signaling pathways associated with proliferation, adhesion, invasion, and angiogenesis^[70]. In particular, Src is a key mediator for several cell surface receptors, including EGFR and HER2^[71]. Src-overexpressing tumors may have a specific survival advantage in the bone microenvironment. In fact, activated Src is required for Chemokine (C-X-C) motif Ligand 12 (CXCL12)/Stromal cell-Derived Factor (SDF)-mediated cell survival and abrogates TNF-Related Apoptosis-Inducing Ligand (TRAIL)-mediated apoptosis^[69]. Preclinical studies confirmed that treatment with Src inhibitors effectively reduced BrCa growth compared with untreated cells^[72]. In several mouse models of BrCa, inhibition of Src activity decreased metastases and improved survival^[73,74]. Other *in vivo* studies have found inhibition of bone metastasis but not of metastases at visceral sites, confirming the specific role for Src in bone turnover^[69]. Moreover, Src activity has been linked to resistance to anti-hormonal therapy^[75]. That BrCa bone metastases are more frequent in ER+ than ER- tumors suggests the possibility that Src inhibition could overcome resistance to endocrine therapy and block metastatic growth. The results from a clinical trial recruiting ER+ and HER2+ BrCa seemed to confirm a better potential activity of an inhibitor of Src in ER+ tumors with respect to HER2+ cases^[76].

At the same time, Src is a key signaling molecule in the physiology of bone, since osteoclast function appears to be dependent on the activation of Src^[77]. Indeed, src-deficient mice develop osteopetrosis, with a significant reduction of bone resorption, although the number of osteoclasts was equal to that of wild-type (WT) littermates, thus indicating that Src is mandatory for osteoclast activity^[78]. Significantly, when Src inhibitors were used in *in vivo* models of BrCa bone metastasis, it was reported that bone resorption as well as BrCa growth at the metastatic site were significantly inhibited^[74]. There are currently several Src inhibitors in preclinical development or in clinical trials for the treatment of solid tumors^[79]. One of these compounds, dasatinib, a dual Src/Abl inhibitor, approved for the treatment of leukemia, was successfully investigated in phase I / II clinical trials in patients with prostate and BrCa bone metastases. Dasatinib treatment was associated with a substantial decrease in bone resorption markers and with a lack of disease progression in a significant percentage of breast and prostate cancer patients^[80,81]. However, although recent data of the Randomized Study Comparing Docetaxel Plus Dasatinib to

Docetaxel Plus Placebo in Castration Resistant Prostate Cancer (READY) trial confirmed the reduction in bone resorption, they showed no overall survival improvement, suggesting the need for appropriate predictive biomarkers^[82].

Chemokines

Chemokines constitute a family of small secreted cytokines. Their name comes from their ability to induce chemotaxis in adjacent responsive cells. Chemokines are generally divided into four classes according to the number and position of cysteine residues: CC, in which the residues are adjacent; CXC, in which the residues are separated by one amino acid; the family XC, which has only one cysteine residue; and CX₃C, in which the cysteine residues are separated by three amino acids^[83]. Some chemokines are pro-inflammatory, being able to induce an immune response consisting of attraction of immune cells at the site of infection, while others are involved in homeostasis, controlling the process of cell migration. These proteins exert their effect by interacting with specific transmembrane receptors called chemokine receptors, present on many different cell types^[84]. The chemokine receptors belong to the family of G protein-coupled receptors (GPCRs), which have an extracellular region that binds to chemokines, seven transmembrane α -helices, and a cytoplasmic side associated with a G protein. Several studies highlighted the importance of CXCL12 (SDF-1) and CXCR4 in BrCa. The first studies on the role of CXCR4 in BrCa date back to 2001^[85], and its specific involvement in the migration of BrCa cells from the primary site through the basement membrane was reported by Yagi *et al.*^[86] in 2011. Mutation at the COOH-terminal domain of CXCR4 also plays a role in receptor regulation during the process of epithelial-to-mesenchymal transition^[87]. It has also been shown that CXCR4 levels are high in bone metastasis, suggesting that the CXCL12/CXCR4 axis plays an important role in its pathogenesis. CXCL12 is a homeostatic chemokine constitutively expressed also in those organs that are the most common metastatic sites of BrCa, including bone marrow, but its secretion by damaged tissues is particularly abundant. CXCR4 expression is low or absent in normal breast tissue, while it is upregulated in neoplastic tissue; moreover, CXCR4 levels are related to the degree of tumor malignancy. However, additional studies are needed to confirm that the increase of CXCR4 expression can be a predictive marker for metastatic diffusion. Shim *et al.*^[88] showed that, in cultured cells, the binding of CXCL12 to CXCR4 induced CXCR4 translocation from the cytoplasm to the nucleus. After its translocation to the nucleus, CXCR4 works as a transcription factor^[89], leading to upregulation of cytokines such as MCP-1 and IL-8 and metalloproteases such as MMP-2 and MMP-9. The importance of CXCR4 in BrCa progression was confirmed by the use of anti-CXCR4 antibody or specific siRNA, *in vitro* and *in vivo*, showing its ability to block the formation and the dissemination of metastasis.

Given the involvement of chemokines and chemokine receptors in tumor progression, many molecules have been developed to counteract their biological activity. These molecules belong to two categories: peptides and small molecules. Bicyclams are low-molecular-weight agents that have been shown to act as potent and selective CXCR4 antagonists. Bicyclam AMD 3100 (Plerixafor) is currently being investigated in clinical trials alone or in combination with bisphosphonates^[83]. The 14-residue polypeptide 4F-benzoyl-TN14003 (BKT140) is a highly selective and unique CXCR4 antagonist^[90]. Besides its ability to inhibit BrCa metastases by impairing migration, it could also be used as a diagnostic tool to identify CXCR4 receptor-positive tumor cells in culture as well as in paraffin-embedded clinical tumor samples^[91]. Finally, *in vivo* studies have demonstrated that the synthetic peptide antagonist CTCE-9908 is able to reduce the incidence and extent of bone metastases. Unfortunately, results from the first clinical trials conducted in patients with advanced metastatic disease showed good tolerance but low response^[92].

Transcription factors

The osteolytic phenotype is associated with the activity of a specific pattern of transcription factors, including glioma-associated oncogene family zinc finger 2 (GLI2), RUNX2, and hypoxia-inducible factor-1 (HIF-1). In particular, GLI2 stimulates PTHrP expression by tumor cells and is mainly involved in the development of melanoma-induced bone metastases^[93,94]. With regard to RUNX2, it has been demonstrated that inactivating mutations of its gene in BrCa cells significantly inhibit bone metastasis development in animal models^[95]. Finally, HIF-1, besides its pivotal role in hypoxia-associated tumor progression, also stimulates tumor-driven osteolysis (Figure 3). Transcriptional activity of HIF-1 is induced by the reduction in oxygen (O₂) availability. HIF-1 is a heterodimeric protein composed of an O₂-regulated HIF-1 α subunit and a constitutively expressed HIF-1 β subunit^[96]. HIF proteins, when stabilized, trigger the transcription of numerous target genes involved in tumor growth and in promoting the feed-forward of the metastatic cycle^[97]. Hypoxia is a frequent consequence of the growth of solid tumors. It is well known that many hypoxia-response genes regulated by HIF-1 α are genes involved in controlling energy metabolism. These include VEGF, which permits increased O₂ delivery to cells by stimulating angiogenesis, and glycolytic enzymes, which allow cells to survive O₂ deprivation^[98]. Paradoxically, therapeutic inhibition of tumor-induced angiogenesis could stimulate autocrine growth factor secretion downstream of HIF-1, thus producing a more aggressive phenotype in BrCa cell lines^[99].

Concerning the role of hypoxia in pre-metastatic niche formation, clinical studies have shown that expression levels of HIF-1 α in BrCa patients increase proportionally with the severity of the pathologic stage^[100]. As reported by Le *et al*^[101] in 13 different types of human cancers, HIF-1 α was overexpressed in two-thirds of the

all regional lymph nodes and bone metastasis examined. Furthermore, increased levels of HIF-1 α were associated with a poor prognosis^[102,103]. Several data have indicated that factors stimulated by HIF-1 α are associated with bone metastasis through their capacity to modify extracellular bone matrix and stimulate metastatic homing of cancer cells^[85,104,105].

Some of the most well-known target genes of hypoxia, which are able to promote metastatic progression, include lysyl oxidase (LOX), LOX-like (LOXL) family proteins such as LOXL2 and LOXL4, TGF- β , MMP2, MMP9, CXCR4, SDF-1, and VEGF^[106]. It was demonstrated that HIF-1, in hypoxic BrCa cells, could promote BrCa metastasis by inducing the expression of LOX proteins. This phenomenon is probably due to increased VEGF secretion by endothelial cells (ECs) and the modification of collagen molecules in the extracellular matrix (ECM)^[107]. While the role of LOX in metastases was initially attributed to its capacity to remodel the ECM in the proximity of the primary tumor, subsequent studies revealed that LOX could remodel the ECM at distant sites and recruit bone marrow-derived cells to the metastatic niche^[108].

In the bone microenvironment, overactivated HIF-1 α can increase the development of osteolytic bone metastases *via* dysregulation of factors involved in the vicious cycle. Several findings have suggested that acidosis, which is caused by glycolytic metabolism of hypoxic cancer cells, has a negative effect on osteoblast differentiation as well as on osteoblast functions^[109,110]. In contrast to the effect on osteoblast differentiation, hypoxia seems to stimulate osteoclast-like cell formation. Frick *et al*^[111] demonstrated higher RANKL mRNA expression in bone cells caused by metabolic acidosis with respect to control. In addition, osteoblasts express components of the HIF-1 pathway, and hypoxia can upregulate the expression of VEGF-A, the major inducer of tumor angiogenesis^[112].

Although HIF-1 is an attractive therapeutic target and several different strategies have been developed to directly target HIF-1, none of these inhibitors have been translated to the clinical setting. However, different chemical compounds and chemotherapeutic drugs that indirectly target HIF-1 α , such as EGFR inhibitors, digoxin and other cardiac glycosides, anthracyclines, geldanamycin and other heat shock protein 90 (HSP90) inhibitors and, recently, topotecan and topoisomerase I inhibitors, have been shown to counteract primary cancer progression, angiogenesis, and metastasis in mouse models^[113-117]. Among these inhibitors, promising results were obtained with the HIF-1 α inhibitor 2-methoxyestradiol (2ME2), which was able to decrease osteolytic lesion area and tumor burden in an *in vivo* model of bone metastasis^[118,119].

CONCLUSION

Bone represents a peculiar distant site for dissemination of BrCa cells: it is actively colonized and frequently becomes a fertile “soil” for tumor growth after the develop-

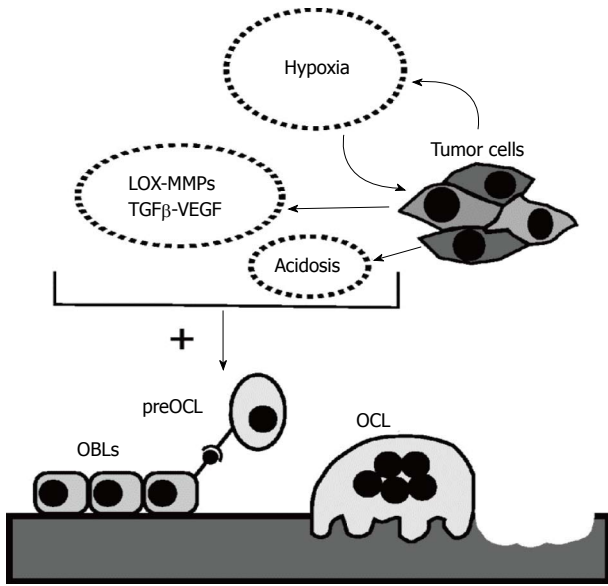


Figure 3 Potential pro-metastatic role of hypoxia in bone. Hypoxia stimulates targeting of cancer cells to bone and facilitates their survival within the bone microenvironment. The presence of a hypoxic environment, further sustained by cancer growth, induces the secretion of hypoxia-inducible factors by cancer cells. These factors, in conjunction with parallel acidification of the microenvironment, are associated with osteoclastogenesis. TGF- β : Transforming growth factor- β .

ment of self-reinforcing crosstalk between tumor and bone cells. This interaction determines osteolysis and the consequent extensive morbidity, but it is also fundamental for cancer progression. Several preclinical and clinical data have demonstrated that the therapeutic strategy directed at interrupting the crosstalk between cancer and bone cells is effective in ameliorating prognosis. Therefore, understanding the effects of the factors implicated in physiopathology of bone remodeling may help to identify future targets for a curative therapy of bone metastasis.

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WJCO 5th Anniversary Special Issues (2): Breast cancer**Effects of psoralens as anti-tumoral agents in breast cancer cells**

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Abstract

This review examines the biological properties of coumarins, widely distributed at the highest levels in the fruit, followed by the roots, stems and leaves, by considering their beneficial effects in the prevention of some diseases and as anti-cancer agents. These compounds are well known photosensitizing drugs which have been used as pharmaceuticals for a broad number of therapeutic applications requiring cell division inhibitors. Despite this, even in the absence of ultraviolet rays they are active. The current paper mainly focuses on the effects of psoralens on human breast cancer as they are able to influence many aspects of cell behavior, such as cell growth, survival and apoptosis. In addition, analytical and pharmacological data have demonstrated that psoralens antagonize some metabolizing enzymes, affect estrogen receptor stability and counteract cell invasiveness as well as cancer drug resistance. The scientific findings summarized highlight the pleiotropic functions of phytochemical drugs, given that recently their target signals and how these are modified in the

cells have been identified. The encouraging results in this field suggest that multiple modulating strategies based on coumarin drugs in combination with canonical chemotherapeutic agents or radiotherapy could be a useful approach to address the treatment of many types of cancer.

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Key words: Psoralens; Breast tumor; Bergapten; Growth factors; Estrogens

Core tip: This review examines the biological properties of coumarins by considering their beneficial effects in the prevention of some diseases and as anti-cancer agents. The attention is mainly focused on the effects of psoralens on human breast cancer as they are able to influence many aspects of cell behavior. More recently, it has been reported that these drugs in breast cancer cells are capable of antagonizing some metabolizing enzymes, to affect estrogen receptor stability and to counteract cell invasiveness as well as cancer drug resistance.

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INTRODUCTION

Coumarins are classified as members of the benzopyrone family of compounds, all which consist of a benzene ring joined to a pyrone ring^[1]. There are four main coumarin sub-types: the simple coumarins (*e.g.*, coumarin, 7-hydroxycoumarin and 6,7-dihydroxycoumarin); the furanocoumarins (*e.g.*, psoralen, angelicin) that consist of a five-

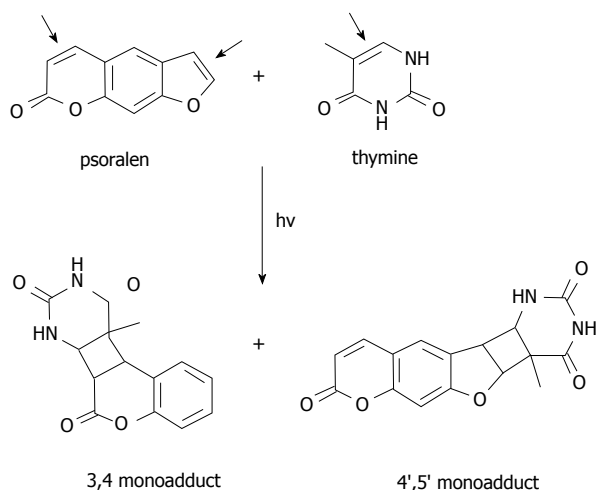


Figure 1 Interaction between psoralen and DNA. Upon absorption of ultra-violet radiation A (UVA) photons, the psoralen excited state can react with a thymine and covalently attach the DNA. The initial photoadduct can absorb a second UVA photon and react with a second thymine on the opposing strand of DNA helix to crosslink the two strands.

membered furan ring attached to the coumarin nucleus, divided into linear or angular types with substituents at one or both of the remaining benzoid positions; the pyranocoumarins that are analogous to the furanocoumarins but contain a six-membered ring (*e.g.*, seselin, xanthyletin); and the coumarins substituted in the pyrone ring, often at 3-C or 4-C positions (*e.g.*, warfarin)^[2].

Coumarins comprise a very large class of compounds found throughout the plant kingdom^[3-5]. They are present in fruit, roots, stems and leaves, but some essential oils, particularly cinnamon bark, lavender oil and cassia leaf oil, are also a good source. Coumarin members have also been isolated from microorganisms. For example, coumarin group antibiotics, such as novobiocin, coumermycin A1 and clorobiocin, come from various *Streptomyces* species. These antibiotics are potent inhibitors of DNA gyrase^[6,7]. Aflatoxins, isolated from the *Aspergillus* species, are a group of highly toxic fungal metabolites.

In the 1970s, furanocoumarins attracted scientific attention when they were introduced in clinical practice^[8]. Two of the most important and well known furanocoumarins are psoralen and angelicin, which have been demonstrated to influence cell division and differentiation. In addition, anticancer^[9,10], immunomodulatory^[11], antibacterial^[12], antioxidant^[13] and neuroprotective functions^[14] have also been shown.

Psoralens, extremely toxic to a wide variety of prokaryotic and eukaryotic organisms, are mainly extracted from the ripe fruit of *Psoralea corylifolia* (Leguminosae), an erect annual herb widely used in Ayurvedic medicine and traditional Chinese medicine. 8-methoxypsoralen (8-MOP) and 5-methoxypsoralen (5-MOP), or bergapten, are the psoralen compounds that occur in nature but several analogues have also been described^[15-20].

These molecules cause cell damage by covalent binding to DNA after ultraviolet radiation A (UVA) irradiation. They have a planar tricyclic structure with two pho-

toactive sites (3,4-pyrone and 4,5-furan double bonds). The initial intercalation and interaction with double-stranded DNA occur after absorption of a photon of UVA and afterwards a pyrimidine residue of the DNA covalently binds to the first photoreactive site with a 5,6-double bond. The psoralen monoadducts formed in the DNA can further react photochemically with a pyrimidine base on the complementary strand of the DNA. In fact, it is precisely the planar structure of psoralens which allows them to intercalate between DNA bases^[21], thus preventing cell mitosis (Figure 1). This feature is clinically relevant and the combination of psoralen and UVA irradiation (PUVA therapy) has been employed in autoimmune or hyper-proliferative skin diseases, including psoriasis and vitiligo^[8]. However, extensive studies have pointed to other biological and clinical applications.

PSORALENS IN NORMAL AND TRANSFORMED CELL PHENOTYPES

Effects on normal cells

Diets rich in fruit and vegetables are often a predominant source of phytochemical compounds with defensive health effects. Initially, natural products were used as concentrated herbal extracts. However, the identification of the biological activity of each single component became a very complex matter due to a mixture of other constituents. In fact, herbal drugs or extracts contain a combination of active constituents, which are well known in popular medicine for the treatment of various kinds of disorders, such as asthma, coughs, nephritis, vitiligo, cavities and gastroenteric diseases. In Asian countries such as Mongolia, when people were infected with *Helicobacter pylori*, an alternative non-antibiotic method based on green tea catechins was found to strongly inhibit *H. pylori* urease activity *in vitro* and to suppress the bacterial-induced gastritis^[22]. A few years later, in a cohort study of gastric carcinomas after screening a total of 25 food phytochemicals, bergamottin was designed as the most promising agent^[12]. Grapefruit and grapefruit-based products are rich in flavonoids, coumarins and carotenoids, which in the long run have been shown to have anti-inflammatory^[23], anticarcinogenic^[9,10,24], antibacterial^[12] activities and significant protective effects on cardiovascular diseases^[9,13]. Furthermore, these bioactive compounds possess antioxidant properties because they act as free radical scavengers, therefore protecting cellular structures and functions in many stressful conditions.

A rich source of coumarins and coumarin containing compounds are the *Psoralea corylifolia* L. seeds, well known in traditional Chinese medicine as "Buguzhi". The plant has long been used for its *magical effects* to cure various skin diseases but, over the years, many other properties have been discovered^[3,10]. The first historical notes on the biological effects of furanocoumarins are related to their photoactivation ability. As previously mentioned, PUVA has been suggested as a potential therapeutic to treat psoriatic lesions and other dermatological condi-

tions^[25,26]. Studies reproduced in the 1980-90s on PUVA therapy for psoriasis have reported the comparison of oral and bathwater delivery of 8-MOP^[27]. Compared with systemic administration, selectively bathing the epidermis with concentrated psoralen leads to a more complete reversal of the pathological epidermal alterations^[11].

Psoriatic keratinocytes inappropriately synthesize a number of immune-related molecules and express a higher amount of epidermal growth factor receptors and insulin-like growth factor receptors that can well support the cellular hyperplasia of the psoriatic lesions. In fact, one of the first studies on PUVA demonstrated how this therapy strongly suppressed the mitogenic stimuli on keratinocytes^[11].

A number of conditions with an autoimmune basis other than psoriasis, such as vitiligo, cutaneous T-cell lymphoma, pemphigus vulgaris, systemic sclerosis and rheumatoid arthritis, have benefited from the above treatment^[28-30].

The compounds best known and widely used for these applications are 4,5',8-trimethylpsoralen (TMP), 8-MOP and 5-MOP. All these assessed in human cell line cultures *in vitro* as well as in *in vivo* studies showed anti-proliferative activity and apoptotic effects. Along with other derivatives, two angular furanocoumarins angelicin and 4,6,4'-trimethyl angelin (TMA) in human keratinocytes photoinduce cellular death and cell cycle arrest in G1 phase. The molecular responses involve up-regulation of p21^{waf/Cip} and p53 activation, with mitochondrial-induced cytochrome *c* release and the consequent apoptotic reaction^[31].

Effects on tumoral cells

In addition to these uses, coumarins display anticancer activities. Interest in this field stemmed from reports by Thornes who evidenced the immunomodulatory activity of coumarin and its utility in malignant melanoma^[32]. The photoactivated coumarins are effective in preventing proliferation of bladder^[33] and mucoepidermoid carcinoma^[34], mammary cancer cell^[35] and human melanoma cell line^[36], with potential for their use in clinical treatments. Despite their photoactivity even in the absence of UV radiation, they have biological properties.

In fact, the native coumarins have been shown to affect adhesion and motility of neoplastic cells. This aspect was well elucidated in the highly invasive murine melanoma cell line B16-F10 by Velasco-Velazquez MA (2003).

In the latter cell type, compared to the non-malignant fibroblastic cells, the authors reported that 4-hydroxycoumarin (4-HC) was able to affect the assembly of actin filaments, thus decreasing the cellular adhesion to extracellular matrix proteins and motility only in the tumoral cell type.

Since adhesion of tumor cells to extracellular matrix is required during the metastatic process, 4-HC might be useful to prevent metastasis and could be used as an adjuvant therapy for melanoma^[37].

The chemopreventive role of coumarin 5-MOP, in

the absence of photoactivation, was investigated in human hepatocellular carcinoma (HCC) cell line by studying apoptotic and cytotoxic responses^[38].

This study suggested that the suppressive effect of 5-MOP includes at least three modes of action: (1) it first kills cells directly; (2) induces apoptosis by arresting cells at the G₂/M phase of cell cycle; and (3) induces apoptosis through an independent pathway with cell-cycle arrest. The authors concluded that the inhibition of cyclin B1 by 5-MOP may play an important role in mitotic arrest and provide an additional way to prevent cells from entering the M phase and undergoing apoptosis.

Antitumoral activity of the methanolic seed extract of *P. corylifolia* L was evaluated in two human cancer cell lines: oral carcinoma cell line and erythroleukemia cells and their corresponding multidrug-resistant cell lines. Both psoralen and isopsoralen constituents were able to inhibit the growth of these cells in a dose-dependent manner. They can also inhibit the growth of normal human primary cells but the IC₅₀ values were higher than those of tumoral cells, suggesting that these two active components had potential selective cytotoxicity^[10].

The resistance aspect of cancer cells to chemotherapeutic agents is one of the major obstacles in achieving an effective treatment for cancer. This results from a variety of factors, including individual variations in patients and somatic genetic differences in tumors. The most common reason for cancer drug resistance involves overexpression of membrane drug efflux pumps, such as P-glycoprotein, but other mechanisms might be implicated. Various Chinese herbal drugs have been evaluated for their specific actions against multi drug resistant (MDR) cancer cells. In an experimental study by Wu JYC of the University of Hong Kong^[39], a bioassay-guided fractionation of extracts from *Radix Peucedani* (also known as "Baihua Qianhu" in Chinese medicine) led to the isolation of the pyranocoumarin compounds, (±)-3'-angeloyl-4'-acetoxy-cis-khellactone, a good candidate as a MDR reversing agent for tumoral cells. Strong synergistic interactions were demonstrated when pyranocoumarins were combined with common anti-tumor drugs, including doxorubicin, paclitaxel, puromycin or vincristine, in multidrug resistant human oral epidermoid carcinoma cell line (MDR KB-V1) compared to its drug-sensitive cell line, KB-3-1. Pyranocoumarins increased doxorubicin accumulation in KB-V1 cells and the same treatment down-regulated the expression of P-glycoprotein.

BREAST CANCER CELLS

Estrogen receptor status and estrogen/antiestrogen responsiveness

Estrogenic hormones are essential for mammary gland development but the same microenvironment plays a pivotal role in the initiation and progression of breast tumorigenesis. In addition, the weight of genetic factors that contribute to the development of breast cancer must be also taken into account.

Estrogen signaling pathways are mediated by two nuclear estrogen receptor (ER) proteins, ER-alpha and ER-beta, with different roles. ER-alpha transduces proliferative responses, thus determining cellular growth and tumor progression, while ER-beta has inhibitory actions^[40-43].

Besides these nuclear receptors, the GPR30/GPER, a member of the seven-transmembrane G protein-coupled receptor family, has been implicated in mediating the effects of estrogens in various normal and cancer cells. In particular, GPER is able to trigger gene expression and proliferative responses induced by estrogens and even ER antagonists in hormone-sensitive tumor cells^[44,45].

Indeed, a whole series of intracellular events, such as the rapid phosphorylation of mitogen-activated protein kinases (MAPK) ERK1/2, the activation of PI3-kinase (PI3K) and phospholipase C (PLC), the increase in cAMP concentrations and intracellular calcium mobilization, was shown to follow GPER activation by both estrogens and anti-estrogens^[45].

Approximately 70% of breast cancers are ER-alpha positive and estrogen-dependent. However, the majority of these tumors will transit from an estrogen-dependent to an estrogen independent state that is usually associated with an aggressive form of the disease. During the initial stages of the disease, depletion of ER-alpha from breast cancer cells is a potent approach to prevent estrogen-dependent growth. For this reason, an anti-estrogen such as tamoxifen, acting as a competitive inhibitor of the receptor, has been implemented in the therapeutical protocols for breast cancer. However, the anti-hormonal long-term use of tamoxifen for most treated women leads to the development of drug resistance and an increase of endometrial cell proliferation with risk of endometrial carcinogenesis^[46-48].

Therefore, the goal of minimizing the negative side effects of estrogens on breast tumor has stimulated the search for a new molecule able to block the agonistic effects of both estrogen and tamoxifen. The pure anti-estrogen, Fulvestrant (ICI 182780), which competes with estrogen for binding to ER with a higher affinity, fulfils these properties very well. This drug is particularly effective as a second-line treatment when tumor cells develop resistance to tamoxifen^[49].

In addition to blocking ER activity, scientific research has recently focused attention on the possible use of the aromatase inhibitors (AIs) since breast cancer cell growth is closely supported by estrogen production. Steroidal (exemestane) and non-steroidal (anastrozole, letrozole) aromatase inhibitors are an additional strategy to counteract estrogen function and signaling. These compounds either bind and inactivate aromatase or compete with endogenous substrates to reduce estrogen synthesis. They are approved for use in endocrine treatment of postmenopausal breast cancer and have demonstrated efficacy in patients that develop resistance to anti-hormonal therapy^[50-52].

Several models have been proposed to explain the

transition of breast tumor from an estrogen-dependent to an estrogen-independent status, including expression of variant or mutated ER-alpha, altered expression of co-factors or downstream estrogen target genes, post-receptor and pharmacological alterations^[53] as well as ligand-independent activation of ER-alpha by other signaling pathways.

ER and growth factors

Accumulated evidence has indicated that a constitutive expression of growth factor or growth factor receptors in breast cancer cells plays an important role in pharmacological resistance. In many cases, an over-production of polypeptide growth factor with an increased activation of the corresponding signaling pathways can bypass the requirement of mitogenic estrogen signaling during progression of breast cancer^[54].

Intricate interactions between ER-alpha and polypeptide growth factors, such as IGF-I, epidermal growth factor (EGF), transforming growth factor (TGF)-alpha and TGF-beta, are involved in the maintenance of proliferation and survival signals. In ER positive breast cancer cells, estrogens increase the mitogenic potential of IGF-I, sensitizing the cells to IGF-I action through the amplification of IGF-I signal^[55-59].

The mechanisms of ER-alpha/IGF-I crosstalk is bidirectional and includes the ligand-independent activation of ER-alpha by IGF-I as well as the regulation of the IGF-I system by ER-alpha^[60,61]. Other than this type of interaction, the crosstalk between ER-alpha and members of the EGFR family is well known. This receptor was found to be amplified in a human breast cancer cell line and named human epidermal growth factor receptor 2 (HER2)^[62].

Amplification of HER2 in human mammary epithelial cells induces proliferative advantages, transformed characteristics, tumorigenic growth and in 3D models induces proliferative and anti-apoptotic changes that mimic early stages of epithelial cell transformation^[63,64]. Overexpression of the HER2 protein, either through gene amplification or transcriptional deregulation, is seen in approximately 25%-30% of breast and ovarian cancers and confers worse biological behavior^[65].

Patients with these characteristics have lower ER levels and are modestly less responsive to anti-estrogens; therefore, they develop the hormone-resistant phenotype. Moreover, high levels of HER-2/*neu* expression constitutively activate survival signals involving PI3K/Akt, which is closely related to MAPK hyperactivity^[62,66].

Tyrosine kinase associated receptors control most of the fundamental cellular processes, including cell proliferation, differentiation, metabolism, migration and survival. The over-expression of these signals facilitates the emergence of anti-hormone resistance in breast cancer. In such cases, potential interventions with anti-growth factor agents, either alone or in combination with anti-estrogen agents, have been reported and have shown promising results^[67].

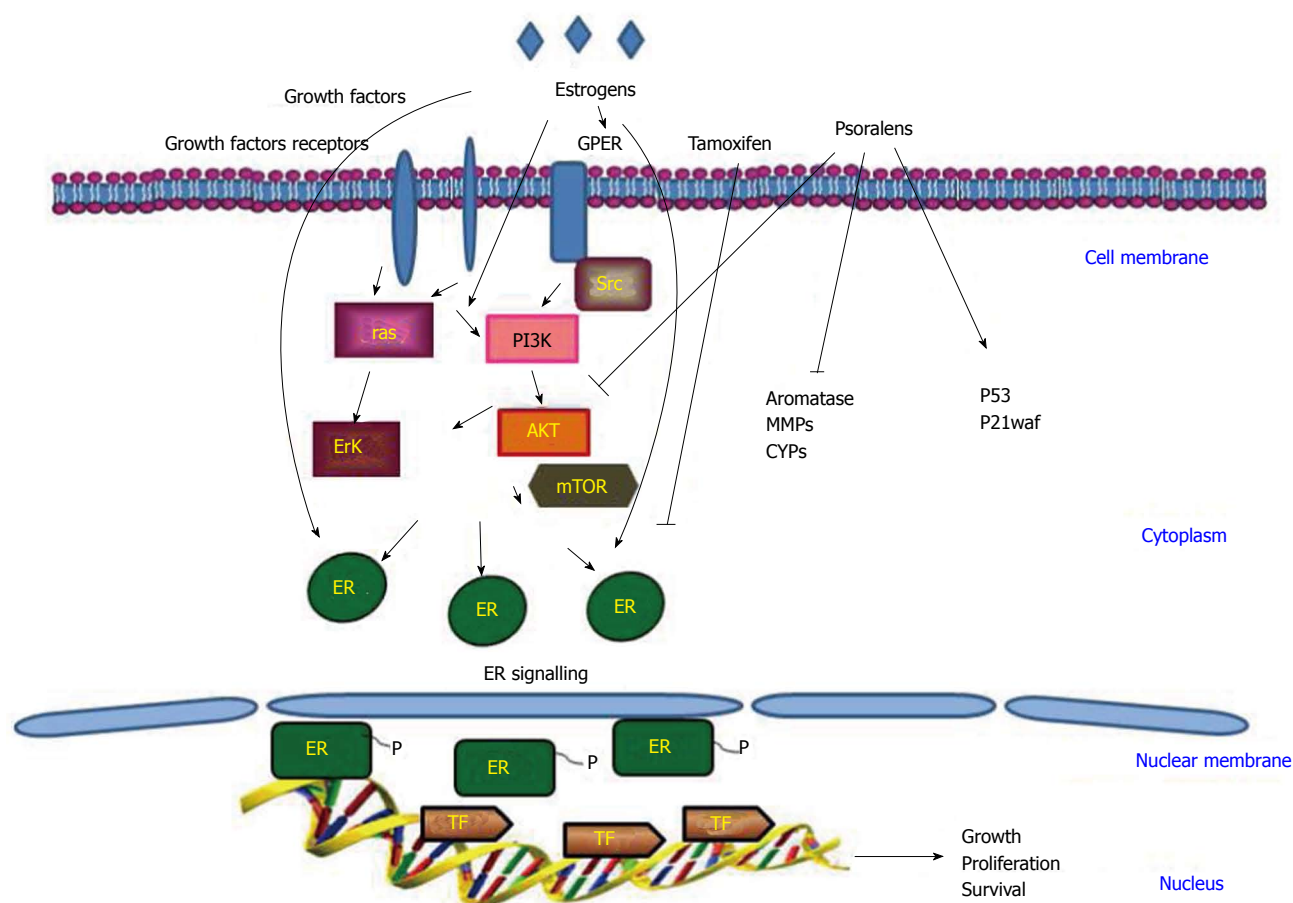


Figure 2 Estrogens and growth factor signaling in breast cancer. Mitogenic signals from estrogens and growth factors activate PI3K/AKT-Ras-Erk pathway to target estrogen receptor (ER). The phosphorylation cascade promotes ER activation. The receptor is recruited to a transcription factor (TF) that binds to responsive elements on DNA for the corresponding transcriptional responses. Antagonistic actions of tamoxifen on ER are shown. Psoralens may act by inhibiting aromatase enzyme, metalloproteinases (MMPs) and CYP enzymes. They address antagonistic effects on PI3K/AKT survival signals and apoptotic response with the involvement of p53 and p21 waf. Estrogens also bind the GPER, a member of the seven-transmembrane G protein-coupled receptor family, to trigger proliferative responses.

Targeting multiple pathways simultaneously may help to kill cancer cells and onset of slow drug resistance. In addition, the association of chemotherapy or radiotherapy with pure or synthetic analogs of phytochemical drugs may take advantage of the synergic effects of the combined protocols, resulting in the possibility of lower doses, consequently reducing toxicity. An overall view of the main signal transductions active in breast cancer is shown in Figure 2.

PSORALENS AND BREAST CANCER

Breast cancer signals and psoralen influence

A large number of epidemiological studies suggest that a daily intake of phytochemicals can reduce the incidence of several types of cancers, including breast tumors^[68-71]. Moreover, genetic variation in pathways affecting absorption, metabolism and distribution of these natural substances can influence exposure at the tissue level, thus modifying disease risk in individuals^[72,73].

The increasing research on this has revealed that the antiproliferative action of psoralens in many tumoral cells, as well as in breast carcinoma, is not only due to

their photoactivation, but that these molecules exert their responses even in the absence of radiation. The biological activities in target tissues have been related to the binding of psoralens with specific receptor proteins identified in cytoplasmic and membrane fractions of responsive cells. Binding of psoralens to these proteins is of high affinity and reversible^[74]. Coumarins and coumarin-related compounds have been reported to possess significant growth inhibitory activities in *in vivo* models and against a panel of breast cancer cell lines, in which the structure-activity relationships has also been evaluated^[75-77].

Until a few years ago, the transductional pathways activated by psoralens in target cells were not well known; however, the growing interest on this aspect led to the identification of main signals by which the anti-tumoral action is exerted. Moreover, in our first study^[78] it was demonstrated that bergapten "*per se*" without photoactivation was able to influence transductional pathways mainly involved in the regulation of cell survival in two hormone-dependent and hormone-independent human mammary tumoral cell lines, expression of the two biological variants of breast cancer respectively, MCF-7 and SKBR-3. The psoralen induced growth inhibition and

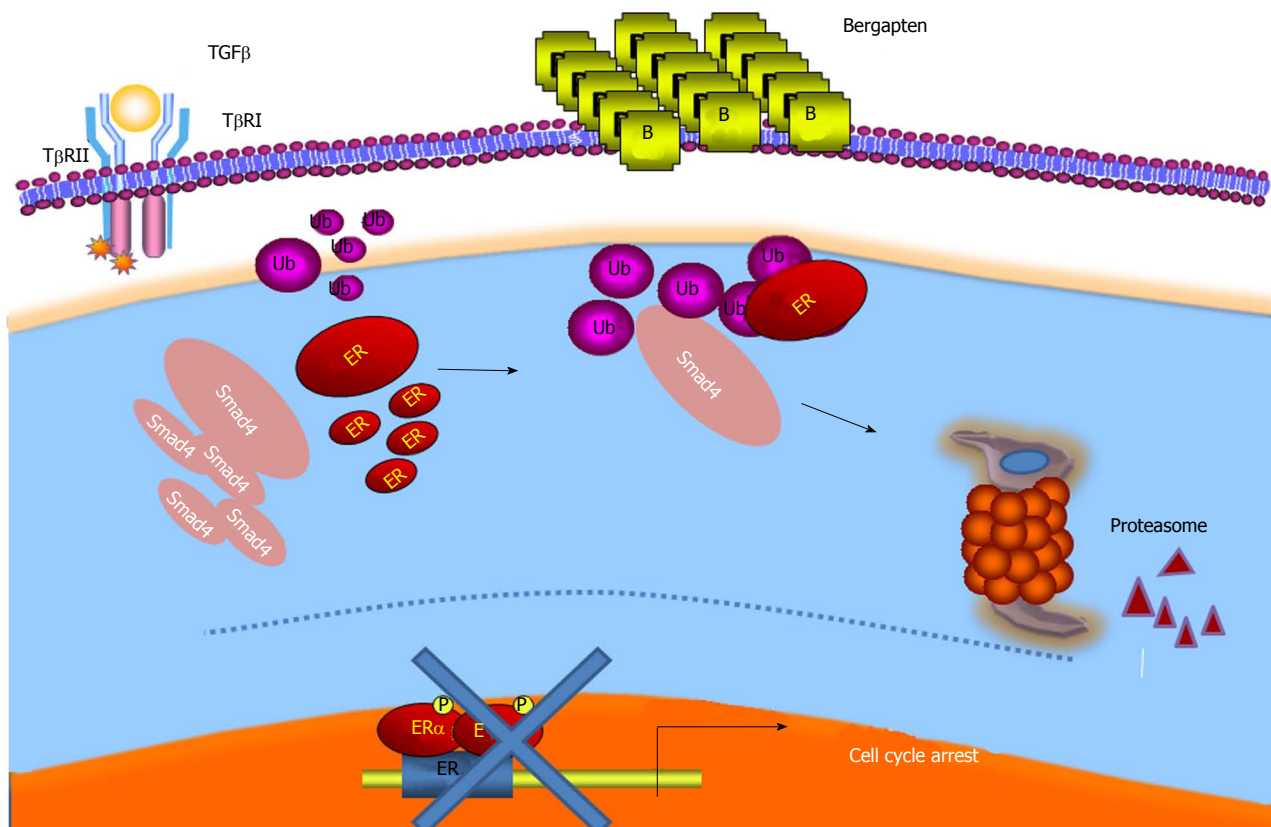


Figure 3 Bergapten affects estrogen receptor stability in breast cancer cells. Mechanism through bergapten (B) in breast cancer cells induces the polyubiquitination (Ub) of estrogen receptor (ER) with the involvement of transforming growth factor- β /SMAD4 protein. Immunoprecipitation assay performed in MCF-7 cells revealed that ER/SMAD4 and polyubiquitin are co-associated. The psoralen enhances the amount of SMAD4 and Poly-Ub complexed to ER, thereby regulating breast cancer cell progression^[87].

apoptosis through the up-regulation of the cyclin inhibitor p21 waf and p53 mRNA and proteins. The molecular study addressed how bergapten transactivated p53 gene promoter through the involvement of the NF- κ B transcriptional factor and the p38 MAPK activation.

Besides this, in hormone-dependent MCF-7 cells, the psoralen counteracted the stimulatory effects of two important mitogenic factors, estradiol and IGF-I on the PI3 kinase/Akt survival pathway.

Similarly, in the presence of photoactivation, bergapten preferentially addressed apoptosis at lower doses than those reported in the previous paper, as revealed by the increase of p53, caspase activation and DNA ladder, while in the absence of UV, the psoralen significantly reduced the p-Akt survival signal^[79]. In estrogen-receptor positive breast cancer cells, as previously mentioned, estradiol exerts its main role supporting the proliferation and growth of mammary tissue.

Aromatase is the enzyme responsible for the conversion of androgens into estrogens and synthetic aromatase inhibitors, such as letrozole, anastrozole and exemestane, have proven to be effective in endocrine regimens for ER-positive breast cancer. Together with these molecules, several flavones have also been demonstrated to be effective inhibitors of aromatase and NADPH-quinone reduc-

tase 1 and 2 both play an important role on breast carcinogenesis. Among the coumarin-derived compounds, the 4-benzyl-3-(4'-chlorophenyl)-7-methoxycoumarin has been shown to be a potent aromatase inhibitor. The structure-activity studies have evidenced that the three functional groups of the coumarin [the 3-(4'-chlorophenyl), 4-benzyl, and 7-methoxyl groups] are important for its ability to inhibit aromatase^[72,80] and not only this (Figure 2). The realization that, in addition to the formation of estrogens by the aromatase pathway, steroids with estrogenic properties could also be formed *via* a sulfatase route has stimulated the interest of other authors in developing potent steroid sulfatase (STS) inhibitors. The furanocoumarins have proved to be successful steroid sulfatase inhibitors once tested in breast cancer cells and for this they may be useful in suppressing estrogen-dependent breast tumors^[72,81,82]. The development and testing of both aromatase and sulfatase inhibitors are in progress and should resolve the question as to whether inhibition of only aromatase or sulfatase is superior to inhibition of only aromatase or STS activity when used for the treatment of hormone-dependent breast cancer.

Several observations have also documented the interplay between E2/ER and growth factor signals such as the TGF- β -dependent pathway. Indeed, it has been

evidenced that ER- α is able to physically interact with components of the latter pathway, SMAD2, SMAD3 and SMAD4, and to abrogate TGF- β signaling cascade^[83,84]. On the other hand, while TGF- β signaling has been demonstrated to stimulate ER- α transcriptional activity, the complex of SMAD3 and SMAD4 inhibits its activity^[85,86]. Analogously, treatment of breast cancer-sensitive and tamoxifen-resistant cells with bergapten induced a depletion of ER protein through a degradative process that sees the involvement of the SMAD4 protein^[87] (Figure 3).

This study once again draws attention to the anti-tumoral properties of psoralen and highlights a new molecular mechanism through which bergapten may prevent the crosstalk between the receptor and growth factor mitogenic signaling by affecting ER- α stability in breast cancer tamoxifen-sensitive and resistant cells. However, in a recent paper^[88], it was demonstrated well that psoralen can also affect the Erb2 receptor tyrosine kinase whose over-expression, as previously reported, characterizes the most aggressive forms of breast cancer.

Independently of interstrand DNA crosslinks, the photo-activated 8-MOP interacts with the Erb2 catalytic autokinase domain, blocking its activity, and furthermore, it can reverse therapeutic resistance by triggering tumor cell apoptosis.

Psoralen action on pro-metastatic and detoxification enzymes

A further negative aspect of estrogens is to promote the migration and motility of breast cancer cells, as demonstrated in an “*in vitro*” assay, and in fact, they increase the closure of wounded confluent culture. This phenomenon is dependent on the expression of ER, because antiestrogens completely abolish the migratory potency of estrogens^[89]. A number of proteolytic enzymes participate in the degradation of environmental barriers, such as the cell-extracellular matrix (ECM) and basement membrane. Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases, play an important role in the proteolysis of ECM components, thus supporting metastatic and angiogenetic processes^[90-92].

Agents able to suppress MMP signaling are useful to target cancer metastasis. Some natural products have been demonstrated to play a remarkable role in inhibiting MMP enzymes in breast and other type of cancers^[93-97].

The antimetastatic activity of furanocoumarin bergamottin has been described in human fibrosarcoma HT-1080 cells and in MCF-7 breast cancer cells where the compound suppresses MMP-9 gene expression by repressing the transcriptional activation in the MMP-9 promoter. The results obtained in this study evidenced that the furanocoumarin inhibited PMA or TNF- α -induced activation of MMP-9 by suppressing NF- κ B activation in tumoral cells^[98].

Considering that psoralen influences bone metabolism and that breast cancer frequently metastasizes to the skeleton, one study^[99] investigated whether it can affect this process *in vivo*. Histological, molecular, biological

and imaging analyses revealed that psoralen inhibits bone metastasis; in fact, it regulated the function of osteoblasts and osteoclasts in tumor-bearing mice. Accordingly, the authors suggest the possible therapeutic role of the drug for metastatic breast cancer.

In addition to the above mentioned effects, the furanocoumarin bergamottin has also been described to inhibit members of the family of CYP enzymes (cytochrome P450s) involved in the metabolism of many xenobiotics and drugs. It is well established that tumorigenesis is closely linked to the metabolism of pro-carcinogenic substances, which when subjected to biotransformation into the cells become dangerous for the body. Certain linear furanocoumarins (*e.g.*, bergamottin, imperatorin, isopimpinellin) and a simple coumarin (osthuthin) were found to inhibit cytochrome P450 and to reduce the formation of DNA adducts induced by benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene^[24,100-103]. This evidence has encouraged more studies in which five new tetracyclic benzofurocoumarins were synthesized and then tested in three different human tumor cell lines: MDA MB 231 (breast adenocarcinoma), HeLa (cervix adenocarcinoma) and TCC-SUP (bladder transitional cell carcinoma). All of them significantly inhibited cell proliferation and this was mainly linked with the inhibition of CYP2A6 enzyme, belonging to the family of CYPs. The effectiveness of the drugs is related to the chelation of the oxygen from the furan ring with the iron from the heme, possibly resulting in the inactivation of the enzyme, and this might be one of the main causes that preclude tumor cell proliferation^[104].

CONCLUSION

Currently, there is a great scientific interest towards natural anticancer drugs due to their multiple target activities on tumoral cells. As reported here, from the early studies on the activity of psoralens, much has now been documented, especially with regards to their mechanism of action at the molecular level.

The anti-tumoral activity of these molecules against breast cancer has been the main point reported in this review. Many intracellular signals that maintain high survival of breast cancer cells are selectively affected by these drugs. Starting from the latest experimental investigations, it appears that even the most aggressive and resistant cell phenotypes are responsive to psoralens since they antagonize metabolic pathways, protease enzymes, cell cycle progression and even interfere in the crosstalk between receptors and growth factor mitogenic signaling. The combination of natural products with the traditional chemotherapeutic agents, with the purpose of using low doses, can be well addressed and may be a new opportunity for the treatment of breast tumors, thereby decreasing the side effects at the systemic level.

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WJCO 5th Anniversary Special Issues (2): Breast Cancer

Main controversies in breast cancer

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Abstract

In this article, we have reviewed available evidence for diagnosis, treatment, and follow-up in female breast cancer (BC). Into daily clinical practice some controversies are occurred. Especially, in the diagnosis field, despite the fact that the optimal age in which screening mammography should start is a subject of intense controversy, there is a shift toward the beginning at the age of 40 although it is suggested that the net benefit is small for women aged 40 to 49 years. In addition, a promising tool in BC screening seems to be breast tomosynthesis. Other tools such as 3D ultrasound and shear wave elastography (SWE) are full of optimism in BC screening although ultrasonography is not yet a first-line screening method and there is insufficient evidence to recommend the systemic use of the SWE for BC screening. As for breast magnetic resonance imaging (MRI), even if it is useful in BC detection in women who have a strong family history of BC, it is not generally recommended as a screening tool. Moreover, based on the lack of randomized clinical trials showing a benefit of presurgical breast MRI in overall survival, it's integration into breast surgical operations remains

debatable. Interestingly, in contrast to fine needle aspiration, core biopsy has gained popularity in presurgical diagnosis. Furthermore, after conservative surgery in patients with positive sentinel lymph nodes, the recent tendency is the shift from axillary dissection to axillary conserving strategies. While the accuracy of sentinel lymph node after neoadjuvant chemotherapy and second BC surgery remains controversial, more time is needed for evaluation and for determining the optimal interval between the two surgeries. Additionally, in the decision between immediate or delayed breast reconstruction, there is a tendency in the immediate use. In the prevention of BC, the controversial issue between tamoxifen and raloxifene becomes clear with raloxifene be more profitable through the toxicities of tamoxifen. However, the prevention of bone metastasis with bisphosphonates is still conflicting. Last but not least, in the follow-up of BC survivors, mammography, history and physical examination are the means of an early detection of BC recurrence.

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Key words: Breast cancer; Controversies; Diagnosis; Treatment; Follow-up

Core tip: Taking into consideration the progress in diagnosis and treatment in the female breast cancer, it is inevitable that some controversies will come up in daily clinical practice. The aim of this review is to illustrate some of these conflicting issues and make them less "ambiguous". Thus, this has been achieved in the issues of mammography, magnetic resonance imaging, fine needle aspiration and core biopsy, axillary dissection, internal mammary node sampling, accelerated partial breast irradiation, the sequence of chemoradiotherapy, negative margin width, while controversial are still remain the themes of tomosynthesis, 3D ultrasound, shear wave elastography, positron emission tomography-computed tomography (PET-CT), CT-scan and bone scintigraphy, hormoneotherapy, bisphosphonates and sentinel lymph node biopsy.

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INTRODUCTION

Globally, breast cancer (BC) is one of the most frequent diagnosed cancers^[1]. More than 1.6 million new cases of BC are identified among women, according to the recent worldwide available data^[2]. Especially, in North America, in western and in northern Europe the incidence rate is higher than in Asia and socioeconomical development seems to be the leading cause^[1,3,4]. In addition, the cumulative incidence of BC raised by more than a quarter between 1980 and 2010 among 187 countries^[2]. This raise has been succeeded thanks to BC awareness and early detection of breast malignancy.

Taking into consideration the progress in diagnosis and treatment, it is inevitable that some controversies will come up in daily clinical practice^[5]. The aim of this review is to illustrate some of these conflicting issues and make them less “ambiguous”. Especially, in the diagnosis field, the subjects which are discussed below are mammography, breast tomosynthesis, 3D ultrasound, shear wave elastography, magnetic resonance imaging, fine needle aspiration and core biopsy, computed tomography, positron emission tomography-computed tomography (PET-CT), axillary node dissection, sentinel lymph node biopsy, internal mammary node sampling and negative margin widths. As for the controversial issues based on treatment, these are partial breast radiotherapy, breast reconstruction, sequence of radiotherapy and chemotherapy, hormotherapy and biphosphonates. However, the follow-up of BC survivors has not been overlooked.

IS MAMMOGRAPHY NECESSARY IN WOMEN BEFORE THE AGE OF 50?

According to prevailing belief, early detection is a vital first step in defense against BC. Undoubtedly, mammography is the gold standard in BC screening and is widely used in order to reduce BC deaths. The optimal age in which mammography screening should start is a subject of intense controversy. Specifically, while there is a consensus for routine screening in women among 50-69 years, it is still under debate whether women aged 40-49 years could benefit from screening with mammography^[6]. As a result, there are different recommendations among organizations and by extension among countries concerning screening. The United States Preventive Services Task Force (USPSTF) recommended toward biennial screening at age of 50 and against screening in women aged 40-49 years^[7], “overlooking” that a mammography screening reduces BC mortality by 15% for women aged

39 to 49 years^[8] and sparking a controversy in the medical world. However, as it is shown by a recent study, the effect of those guidelines on mammography rates in women older than 40 years was negligible^[9]. Conversely, some organizations such as American Cancer Society (ACS) and American College of Radiology (ACR) have different position than that of USPSTF, recommending annual mammography screening beginning at age 40^[10,11]. It is noteworthy that a new study with 7301 patients argued in favor of screening before age 50 years, because it is proved that most deaths from BC occurred in women who were unscreened^[12]. Additionally, a meta-analysis which conducted by Greek scientists indicated a significant reduction in BC mortality, as a result of screening mammography in women younger than 50^[13]. Similar effectiveness is confirmed by a Sweden study^[14]. Taking into account all the above and the fact that BC occurs in many cases in women under age 50, there is a tendency toward offering screening mammography before 50 years. As an example, in the United Kingdom in 2010, by the age of 50 around 10000 women were diagnosed with BC and 80% of all diagnoses were in the over 50s, concluding that about 1 in 5 women were diagnosed with BC by the age of 50^[15]. It is worthwhile to note that guidelines vary between countries, depending on socioeconomic development of each one.

CAN BREAST TOMOSYNTHESIS BE PROPOSED AS A SCREENING TOOL?

Great scientific interest has been focused on breast tomosynthesis (BT), which is a relatively new three dimensional imaging technology for the fight against BC nowadays. BT uses a digital detector and an X-ray source, which moves in an arc around breast and takes multiple images^[16]. Then, BT's information is sent to a computer, where it is reconstructed in order to produce a 3D image of breast tissue thickness 1 mm. It seems that BT solves the problem of tissue overlap, which encountered in 2D mammography^[16]. Despite, BT approved by the US Food and Drug Administration^[17], it is a controversial issue whether it could be the standard care in BC screening. Although, it was found that BT has a marginally greater sensitivity and greater specificity, compared to digital mammography^[18], there were conflicting findings regarding BT's sensitivity from other data. Some investigators found that traditional mammography was slightly superior to BT in sensitivity^[19] and that BT potentially has worse performance in the detection of microcalcifications^[20]. On the other hand, it was recently demonstrated that the usage of BT in combination with digital mammography (“adjunctive BT”) has as a result an increase in BC detection rates^[21]. Similarly, a recent study concluded that adjunctive BT could improve the diagnostic performance in mammography and, summarizing older data, mentioned that BT has probably a higher sensitivity when compared with 2D mammography and reduce recall rates^[22], a similar conclusion of Haas *et al*^[23] especially

in women under the age of 50 and in women with dense breast tissue. According to all aforementioned reasons, BT is a promising revolutionary tool in BC screening. At present, BT is used only as an adjunct to conventional mammography. Consequently, clinical trials are necessary in order to justify its routine use in screening population.

SHOULD HIGH RESOLUTION 3D ULTRASOUND BE USED AS A SCREENING MODALITY IN YOUNG PATIENTS WITH DENSE BREASTS?

Although mammography is the gold standard in BC screening, it may not be effective in all patients, such as young women with dense breasts^[24]. Also, it is noteworthy that women with dense breast tissue have a 3 to 5 fold increase in BC risk, in contrast to those women with a lack of dense breast tissue^[25]. Owing to all aforementioned reasons, new tools for BC screening such as breast ultrasound, are needed. Remarkably, the United States Food and Drug Administration (FDA) approved in 2012 an automated breast ultrasound system (ABUS), as an adjunct to mammography, especially in women with dense breasts^[26]. As a screening tool, the method could be proposed for the imaging evaluation of non-palpable masses in women under 30 years of age who are not at high risk for development of BC, and in lactating and pregnant women^[27]. 3D breast ultrasound is a special advanced examination, which provides information of the coronal plane^[28]. Recent available data are full of optimism about the utility of 3D breast ultrasound in young women with mammographically dense breasts. Specifically, a study indicated that the extra usage of 3D breast ultrasound was more efficient than mammography alone^[27]. However, there is no evidence that 3D ultrasound decreases mortality rates^[29]. Thus, 3D breast ultrasound is a promising tool and may be used in screening in women with dense breasts widely. Nevertheless, there are no guidelines for its use as screening, instead of mammography until now^[30]. Summing up the discussion above, this issue remains a subject of intense controversy and randomized clinical trials are required.

IS SHEAR WAVE ELASTOGRAPHY A VALUABLE TOOL?

Elastography is a technique of breast imaging tissue stiffness which has been introduced into ultrasound in order to contribute to lesion differentiation^[31,32]. Namely, shear wave elastography (SWE) uses the acoustic radiation force provided by the ultrasound beam itself. Although, the predictive significance of this method remains to be elucidated, most recent studies pointed out that SWE improves the specificity of B-mode ultrasound^[33-36] and provides a good diagnostic performance during breast ultrasound^[32,34,36,37]. Interestingly, SWE increased the speci-

ficity of breast mass assessment from 61.1% to 78.5% and the positive predictive value from 52.6% to 67.1% in a multicenter study with 939 breast masses, while the improvement in sensitivity was insignificant^[36]. Moreover, it is noteworthy that several studies demonstrated that SWE may have an important role in reducing the number of unnecessary breast biopsies^[34] and that could be useful to assess the cystic content of a breast lesion^[35] and axillary lymph node status^[38].

WHO SHOULD HAVE BREAST MRI FOR SCREENING?

Potential use of breast magnetic resonance imaging (MRI), a specialized non-invasive test is extensively studied nowadays. This method uses radio waves and strong magnets in order to determine the morphology of the inner breast. Latest studies, indicated that breast MRI is a valuable screening modality in women with a family history suspicious for inherited predisposition to BC^[39,40]. In fact, from these women, annual MRI in accordance with mammography is the current recommendation of several organizations such as American Cancer Society^[41], National Institute for Health and Care Excellence^[42] and European Society of Mastology-EUSOMA^[43]. Specifically, according to the recent guidelines, the main indication for annual MRI screening is the existence of BRCA1 or BRCA2 gene mutation. Moreover, there is some suggestion that women who have a first-degree relative (parent, brother, sister or child) with a BRCA1 or BRCA2 gene mutation, but personally have not been genetic tested, ought to be screened by MRI once a year^[41]. Similar recommendation applies for women who have a strong family history of BC^[42]. The prevalent age for starting breast MRI screening ranges from 25 to 30 years^[41,43]. However, several organizations recommend to women with family history of BC, MRI starting 10 years earlier than the age of diagnosis of the youngest affected relative^[11]. According to all aforementioned reasons and the limitation of evidence about the best age in which to start screening^[41], this decision should be tailored to women's unique situation. As an example, in women with Li-Fraumeni syndrome [an autosomal dominant disorder associated with abnormalities in the tumor protein p53 gene (TP53)], breast surveillance with breast MRI should be considered beginning at 20 years of age^[44]. Similarly, consensus recommendations for BC surveillance in women with Cowden syndrome [an autosomal dominant disorder associated with abnormalities in the phosphatase and tensin homolog (PTEN) gene] include annual mammogram and/(or) breast MRI starting at age 30 to 35 or 5 to 10 years before the earliest known BC in the family^[45]. Nowadays, another main debate is about the possibility of moving from the old recommendation of "MRI as an adjunct" to the new one "MRI alone"^[46]. Currently, MRI is not generally recommended as screening tool by itself, despite the fact that it has better sensitivity than mammography (especially in young women), it still has more false positive recalls^[39,41].

Furthermore, MRI is a quite expensive procedure^[47] and has no evidence on reducing BC mortality^[48].

DOES PRESURGICAL BREAST MRI INFLUENCE OVERALL SURVIVAL?

According to general belief, breast MRI is an extremely sensitive imaging assessment tool, which is able to detect BC^[40]. However, the integration of MRI into breast surgical operations remains debatable. Specifically, whether presurgical breast MRI has some impact on overall survival is a controversial and complex subject^[49]. Some investigators who support the use of breast MRI pre-operatively argue that it may have an influence in overall survival rates^[50]. This view is supported because of the potential benefits of MRI in decrease of recurrence rates^[51,52]. Conversely, recent available data has shown that this approach does not improve patient's outcomes^[53]. Interestingly, a meta-analysis which conducted in 2013 pointed out that MRI leads to overtreatment with probably unnecessary mastectomies^[54], a different conclusion than that of Killelea *et al*^[52]. Furthermore, a United Kingdom randomized trial (COMICE) indicated that preoperative MRI did not change the re-operation rates^[55]. In conclusion, there is a lack of randomized clinical trials showing a benefit of presurgical breast MRI in overall survival. Thus, in order to exist a definitive answer to this issue, additional studies are required.

PRESURGICAL DIAGNOSIS: FNA OR CORE BIOPSY?

Both fine-needle aspiration (FNA) and core biopsy (CNB) are the current procedures of choice for the detection of BC. FNA is executed with the use of a 10 or 20 mL plastic syringe and a 23 to 27 gauge needle. The syringe can adapted to a special device, which brought negative pressure. As keeping negative pressure, syringe makes reciprocating movements into the mass, while rotating physician's wrist^[56]. Also, in order to succeed nipple aspiration, a specially constructed syringe can be applied by Zervoudi's technique^[57]. On the other hand, CNB is a method that removes small solid samples of tissue using a needle with wide lumen. Both of these aforementioned procedures have advantages and disadvantages, as it is shown in Table 1^[58-61]. In recent years, there is a shift toward the use of CNB. However, whether FNA or CNB is better remains contentious and there is a lack of consensus among different BC centers. Specifically, some investigators summarized that FNA has superiority over CNB and that may be useful and reliable as a first diagnostic step for the detection of palpable breast lesions^[62,63]. Moreover, they found that FNA had a same predictive value with CNB^[64]. Conversely, other researchers demonstrated that CNB offers a more definitive histologic diagnosis in contrast to FNA, which has limitations in diagnostic accuracy, sensitivity and specificity^[56,58].

A main disadvantage of FNA cytology is the "inability" to distinguish between *in situ* and invasive cancer^[65]. On the contrary, CNB may permit the distinction between *in situ* and invasive cancer. As a result, CNB has gained popularity widely, but the final decision on whether to use one or another is based on a number of factors, such as the clinical features of the lesion, the likelihood of achieving an indicative diagnosis and the experience of the operator^[58].

FOLLOW-UP TO DETECT METASTASIS: CT SCAN AND BONE SCINTIGRAPHY OR PET/CT?

Currently, if computed tomography (CT) scan and bone scintigraphy could be used as a standard practice in BC follow up or whether PET/CT is more efficient, is controversial. Most published scientific studies, indicated that whole body PET/CT has greater sensitivity and specificity in detecting metastasis, compared to other approaches^[66]. In other words, recent available data revealed that PET/CT is superior to CT scan and bone scan and provides better accuracy in bone metastases detection, in patients with BC^[67-69]. However, an individual multicenter study concluded that bone scintigraphy, which is inexpensive^[68], is more effective in bone metastases determination than PET/CT^[70]. Moreover, PET/CT is related with low sensitivity in identification of tumors, smaller than 1 cm^[71]. Furthermore, in asymptomatic patients, it is noteworthy that none of the imaging tests, including CT scan, bone scintigraphy and PET/CT provides survival improvement^[72]. According to the above, imaging studies (apart of mammography and breast MRI in special occasions) are not recommended as a routine practice in people with no symptoms of metastases^[72-74]. However, in symptomatic patients, there is not enough evidence whether PET/CT could be replaced CT scan plus bone scintigraphy.

AFTER CONSERVATIVE SURGERY, IN PATIENTS WITH POSITIVE SENTINEL LYMPH NODES, SHOULD AXILLARY DISSECTION BE PERFORMED OR NOT?

Axillary dissection was considered as the gold standard practice for many years in patients with a positive sentinel lymph node. Nowadays, in accordance with the counterintuitive results of many studies, there is a key controversy on whether this approach is always necessary after a positive sentinel lymph node^[75]. In fact, both the ACOSOG Z0011 randomized trial and the IBCSG 23-01 controlled trial indicated that the routine use of axillary dissection could be safely omitted in women with early BC who have only one or two positive sentinel nodes^[76,77]. Interestingly, they showed that there is no statistical difference in overall survival and in disease free

Table 1 Comparison between fine needle aspiration and core biopsy

	FNA	CNB
Ability to distinguish invasive from <i>in situ</i> lesions	No	Yes
Accurate for palpable lesions	Yes	Yes
Accurate for non palpable lesions	No	Yes
Useful for hypocellular and sclerotic lesions	No	Yes
Diagnosis of papillary lesions	Low	Moderate
Distinction of low grade lesions	Very difficult	Difficult
Suitable for difficult or superficial sites	Yes	No
Appropriate for patients with coagulation abnormalities	Yes	No
Complication rate	Very low	Low
Minimal invasiveness	Yes	No
Special experience required	Yes	No
Rapid (initial) diagnosis	Yes	No
Patient discomfort	No	Yes
Long tissue processing time	No	Yes
Cost	Inexpensive	More expensive than FNA
Requirement of anesthesia	No	Yes

FNA: Fine needle aspiration; CNB: Core biopsy.

survival between patients who underwent axillary dissection and those that did not, but who received systemic therapy and radiation therapy (RT). These results were also confirmed by AMAROS study, which found that radiotherapy may be sufficient for most patients with a positive sentinel node^[78]. Indeed, the 2013 St. Gallen Consensus Conference recommended that in patients with macrometastasis in 1-2 sentinel lymph nodes, completion of axillary dissection can be avoided in patients who receive RT^[79]. On the other hand, individual studies pointed out that the omission of axillary dissection in women with sentinel node micrometastases is related to an increased 5-year recurrence rate^[80]. Summarizing all the above data and taking into account a recent review, a complete axillary node dissection is suggested in patients with positive sentinel node undergoing a mastectomy without RT^[81]. Furthermore, for patients with micrometastases (> 0.2 mm and no greater than 2.0 mm) or macrometastases in three or more nodes, after sentinel lymph node dissection, completion of axillary dissection is recommended for staging purposes and to ensure local control^[82]. In conclusion, according to the above data, the recent tendency is the shift from axillary dissection to axillary conserving strategies in selected patients with positive sentinel lymph nodes.

WHICH IS THE IMPACT OF MICROMETASTASIS IN SENTINEL NODE ON DFS AND OS?

The presence of micrometastasis in sentinel lymph nodes has raised the issue on whether has some impact on disease free survival (DFS) and overall survival (OS). Several

studies indicated that women with micrometastasis in sentinel lymph node did not have significant difference in DFS and OS *vs* node negative patients^[83]. Remarkably, in a study published by Hansen *et al.*^[84], patients with micrometastasis, pN0(i+) [regional lymph node(s) with (≤ 200) malignant cells in an area ≤ 0.2 mm] and pN1mi [regional lymph node(s) with malignant cells in an area > 0.2 mm but ≤ 2.0 mm (and/or with > 200 cells in an area ≤ 2.0 mm)] did not appear to have a worse 8-year DFS or OS in comparison with patients who were sentinel node negative^[85]. The latter was also confirmed by another population based study in which has been proved that there is hardly any impact on OS during the first years after diagnosis in patients with sentinel node micrometastasis. In contrast to all aforementioned studies, other studies concluded that the appearance of sentinel node micrometastasis has been associated with shorter positive DFS and OS rates^[86,87]. Summarizing, the influence of micrometastasis on BC outcomes remains uncertain, enhancing plenty of controversy among investigators.

IS SENTINEL NODE AFTER NEOADJUVANT CHEMOTHERAPY ACCURATE?

Patients who are candidates for neoadjuvant chemotherapy (NACT) and have a clinically negative axillary examination at presentation (cN0) may have a sentinel lymph node biopsy (SLNB) either prior to or after neoadjuvant chemotherapy. The timing is often determined by preferences of the treating physician, and in the absence of data suggesting a preferred strategy, either is reasonable. It is suggested that if the SLNB is negative (pN0), before or after NACT, no further axillary evaluation is required^[88]. Candidates for nodal evaluation who are about to undergo NACT are initially either clinically node-negative or clinically node-positive patients. However, the application of SLN surgery for staging the axilla, following NACT, for women who initially had clinically node-positive (cN1) BC [and, after NACT, clinically node-negative (cN0) BC] is unclear because of high false-negative rates (FNR) of SLNB reported in previous studies. Actually, considering that FNR is $> 10\%$, changes in approach and patient selection that result in greater sensitivity would be necessary to support the use of SLN surgery, after NACT, as an alternative to axillary lymph node dissection (ALND)^[89]. In addition, it seems “rationale” that SNDB is a more reliable diagnostic method before NACT and that after NACT, SLNB has a lower detection rate and a higher FNR compared with SLNB done before NACT. However, based on the results of the American College of Surgeons Oncology Group (ACOSOG) Z1071 trial and the SENTINA (SENTinel NeoAdjuvant) study, a prospective, multicenter cohort study, a clear relationship was found between the number of SLNs and false negative rates^[90]. Clearly, as much SLNs are removed, as low the false-negative rate is^[89-91]. For patients initially presenting with clinically node positive disease who then

received NACT, it was convincingly demonstrated that only when ≥ 3 nodes were harvested during SLNB, the FNR was comparable to that of patients initially presenting with clinically node negative disease^[91]. Furthermore, it seems that the false-negative rate of SLNB after NACT is roughly comparable to the one of SLN biopsy in general (10.5%)^[92,93], albeit it was suggested that there is insufficient evidence to recommend SLNB after NACT as a standard procedure^[92]. As for the “accuracy”, there are studies which confirm that SLN remains an accurate tool after NACT in selected patients with operable BC^[94,95] while in contrary others conclude that the diagnostic reliability is better before the systematic treatment^[90]. In conclusion, the accuracy of SLN after NACT remains a conflict through the published studies and further evaluation is needed.

IS SENTINEL NODE IN SECOND BC SURGERY (PRIOR CONSERVATIVE SURGERY) ACCURATE?

Approximately 10 to 15 percent of the patients with early BC, who had undergone breast-conserving surgery (BCS), will develop loco-regional recurrence disease within 10 years^[96,97]. Axillary staging in these patients is important for obtaining locoregional control and predicting prognosis^[98]. Nowadays, the concept of repeating sentinel node biopsy (SNB) is a potential clinical scenario. Inquiring into published data, the dominant aspect is that SNB is technically feasible and accurate and can be successfully performed^[99-101]. In similar assumptions ended a recent systematic review and meta-analysis of the literature published by Maaskant-Braat *et al.*^[99] after taking into account all studies on repeat SNB in locally recurrent BC. The main conclusions of the above review were that repeat SNB has a low false-negative rate, spares patients an unnecessary axillary lymph node dissection and its information can lead to a change in adjuvant treatment strategy. Nonetheless, more studies are required to determine the optimal interval before repeat SNB^[82].

INTERNAL MAMMARY NODE SAMPLING IN CENTRAL AND INTERNAL QUADRANT BC: USEFUL OR NOT?

Even if axillary sentinel lymph node (SLN) biopsy is a standard procedure for staging clinically node negative patients with BC, the value of sentinel lymph node biopsy for the internal mammary chain (IMC) remains marginally controversial^[102]. As for the tumor location and the internal mammary node (IMN) involvement, Paredes *et al.*^[103] reported that the predictive factor for the IMC involvement was location of the tumors in the inner quadrants ($P < 0.001$), while Cserni and Szekeres pointed out that data from extended radical mastectomy series cannot be extrapolated to patients suitable for SLN^[103,104]. Indeed, SLN biopsy does not reliably identify IMN involvement

because of interference from radioactivity at the primary tumor site and there is a high rate of technical failure^[82]. In addition, the axillary lymph node (ALN) involvement has been noticed as a predictive factor for IMN involvement^[105]. It is rarely found IMN metastasis without ALN metastasis according to Ramsay *et al.*^[106]. Prognosis for patients with axillary and IM involvement is worst while axillary node negative patients will be found to have regional metastasis to the IMN in 8 to 10 percent of cases^[82,104]. In case of positive diagnosis of IMN involvement, the treatment decisions may be affected regarding adjuvant systemic therapy and regional irradiation^[82,105]. However, randomized trials show no evidence that IMN resection through extended mastectomy compared with radical or modified radical mastectomy improves survival^[107,108]. Thus, the IMN dissection was abandoned. All these boils down to the fact that IM SLN biopsy is not routinely recommended (considered investigational) and further studies need to be undertaken^[82,102,109,110].

CAN PARTIAL BREAST RADIOTHERAPY BE SELECTED IN BIFOCAL CANCERS?

Accelerated partial breast irradiation (APBI) is used as an alternative technique to conventional whole breast irradiation (WBI) in selected patients with early BC after breast conserving surgery (BCS)^[111]. Recommendations for the selection of patients have been published from the American Society of Breast Surgeons (ASBS), the American Brachytherapy Society (ABS), the American Society for Radiation Oncology (ASTRO) and the European Society for therapeutic Radiology and Oncology (ESTRO)^[112-115]. The criteria for the selection conducted according to the published clinical evidence so as the APBI be effective. Polgár *et al.*^[115] argued that the relatively poorer results of early APBI studies with high local recurrence rates exceeding 1% per year could be attributed to inadequate patient selection criteria and/or suboptimal treatment technique and lack of appropriate QA procedures^[116]. Particularly, APBI should be limited to patients between 45 (ABS ≥ 50) and 70 years of age, with small (≤ 3 cm), unifocal, unicentric and lymph node negative tumors resected with negative margins and without adverse histologic features (including lobular carcinoma, *in situ* ductal carcinoma and extensive intraductal carcinoma). Consequently, a patient with a bifocal tumor cannot be selected for partial breast irradiation.

WHICH IS THE IMPACT ON RECURRENCE IN IMMEDIATE OR DELAYED RECONSTRUCTION AFTER MASTECTOMY?

Immediate (IBR) or delayed breast reconstruction (DBR) stipulates the time of reconstructive surgery after mastectomy. Even if the impact of loco-regional recurrence comparing IBR and DBR has not been evaluated, nu-

merous studies compare the recurrence ratio of IMR and DBR with mastectomy alone. Particularly, a published meta-analysis in 2012 demonstrates no evidence for increased frequency of local breast recurrence with IBR compared to mastectomy alone (OR: 0.98; 95%CI: 0.62-1.54) while another study reports that IBR had an acceptable 5-year local recurrence rate of 2.9% (95%CI: 0.1-5.7)^[117,118]. In case of DBR, Lindford *et al*^[119] (2013) concluded that delayed autologous reconstruction after mastectomy doesn't appear to adversely influence disease progression when compared to patients treated with mastectomy only. The appropriate time should be settled on minimizing the potential complications and optimizing the postoperative outcome. Nonetheless, in case of women who require postmastectomy radiotherapy (RT) the best option of reconstruction is controversial although need for postoperative RT is considered a relative contraindication to IBR^[120]. On one hand, based in a retrospective study, DBR should be proposed to these women as the loco-regional recurrence rate is lower when RT is given before reconstruction and patient demise may be increased when radiation therapy is performed following breast reconstruction^[121]. On the other hand, this was not proven by other data showing that mastectomy with immediate expander-implant reconstruction was associated with acceptable 5-year locoregional control, distant metastasis-free survival and overall survival^[122]. All these boils down to the fact that there is no evidence cancelling IBR or DBR based on recurrence. There is a tendency more and more using IBR over DBR considering, among others, that several studies revealed that women undergoing IBR experienced significant psychosocial benefits^[120].

WHICH IS THE OPTIMAL TIME TO START CHEMOTHERAPY AFTER BC SURGERY AND WHICH SEQUENCE OF RADIOTHERAPY AND CHEMOTHERAPY SHOULD BE ADMINISTERED?

Radiotherapy (RT) and chemotherapy (CT) are used to improve local control and reduce the risk of dying from BC. Nevertheless, for women with early stage BC who have been treated surgically, it remains marginally uncertain whether both treatments should be given at the same time (concurrently) or one after the other (sequentially) and in which order^[123]. Four schemes of sequencing RT and CT have been tried or adopted: administering CT before RT (more frequently used), administering CT and RT concurrently with an overlap of at least 21 d^[124], using a "sandwich" treatment schedule by administering three cycles of CT followed by RT and then administering three more cycles of CT^[125] and administering RT before CT. Adjuvant chemotherapy can be administered within 4-6 wk after the surgery while a delay of more than 12 wk could be detrimental^[126]. Abbas *et al*^[127], in a total of 267 patients divided into 3 groups, found that disease free

survival (DFS) at 2.5 years was 83.5%, 82.3% and 80% for patients receiving radiation before chemotherapy, sandwich and after finishing chemotherapy respectively concluding that DFS is not altered by treatment sequence. Pooling data of three randomized trials in women with early stage BC, Hickey concluded that local control and overall survival was similar comparing concurrent CT and RT, RT followed by CT and CT followed by RT when RT was commenced within seven months after surgery (as this was the maximum delay in the included studies). However, RT followed by CT was associated with an increased risk of neutropenic sepsis compared with CT followed by RT and concurrent chemoradiation increased anaemia, telangiectasia and pigmentation^[123]. Similarly, a randomized trial including 2396 women with early stage BC who received CMF with or without an anthracycline and who were treated with concomitant or sequential radiation therapy, concluded that in concomitant therapy there was a significant increase in acute skin toxicity (25 *vs* 16 percent)^[128]. It seems that concurrent chemoradiation is more toxic than sequential therapies. Taking everything into account, the concomitant use of RT and CT hasn't gain universal acceptance while the clinical practice uses CT before RT^[129].

HORMONOTHERAPY IN POSTMENOPAUSAL WOMEN: WHICH ONE AND FOR HOW LONG?

Undoubtedly, hormonotherapy constitutes a principal component in treatment of hormonal positive BC. Selective estrogen receptor modulators (*e.g.*, tamoxifen and toremifene), estrogen receptor downregulators (*e.g.*, fulvestrant) and aromatase inhibitors (*e.g.*, anastrozole, exemestane and letrozole) are all different types of hormonal therapy medicines^[41]. It is noteworthy that despite tamoxifen was the previous established therapy, it was replaced by the usage of aromatase inhibitors (Als) in postmenopausal women. This shift is based on the positive findings in studies which compared Als to tamoxifen^[130]. Notably, a meta-analysis indicated that Als significantly decrease the risk of recurrence and improve outcomes^[131]. However, tamoxifen remains an option of therapy, particularly in women with contraindication to Als, but not as a first choice^[132]. For many years, the ideal duration of tamoxifen treatment was 5 years. Nevertheless, recent randomized trials, such as ATLAS study demonstrated that the use of tamoxifen over 10 years has superiority in recurrence and mortality, compared to a 5-year therapy^[133]. On the other hand, there are many unanswered questions that generate plenty of controversy regarding the use of Als, which is the initial treatment in postmenopausal women. Firstly, it is unclear which of aromatase inhibitor is better. However, all Als appear to have similar efficacy, as it is shown by MA.27 study^[134]. Secondly, it is still uncertain which is the ideal duration of Als therapy. The standard treatment lasts 5 years, but

more studies are required in order to prove whether therapy with an AI could be efficient for more than 5 years. Thus, until now, for postmenopausal women, it is recommended by several organizations to begin treatment with an AI for 5 years, or with tamoxifen for 5 years followed by an AI for 5 years, or treatment with tamoxifen for 2 to 3 years followed by an AI in order to complete a 5-year therapy^[41,72,132]. In conclusion, the choice of suitable hormone therapy should be depend on patient's unique situation.

TAMOXIFEN-RALOXIFENE: WHICH IS BETTER FOR BC PREVENTION?

Focusing on tamoxifen and raloxifene, many trials have been conducted in order to point out which one is the most effective for BC prevention. The STAR trial (Study of Tamoxifen and Raloxifene) compared tamoxifen with raloxifene in 19490 high-risk postmenopausal women for a 5-year period. The initial results were almost equal with both drugs reducing the risk of BC approximately 50%. In the long-term follow-up, tamoxifen had a greater chemoprevention effect than raloxifene [1.24, 95%confidence interval (CI), 1.05-1.47]. Actually, long-term raloxifene retained 76% of the effectiveness of tamoxifen in preventing invasive BC^[135]. However, there are other trials which compared tamoxifen and raloxifene with placebo. In MORE (Multiple Outcomes of Raloxifene Evaluation) trial, 13 cases of BC were confirmed among the 5129 women assigned to raloxifene *vs* 27 among the 2576 women assigned to placebo [relative risk (RR), 0.24; 95%CI: 0.13-0.44]^[136]. In CORE (Continuing Outcomes Relevant to Evista) trial, the 4-year incidences of invasive BC and estrogen receptor (ER)-positive invasive BC were reduced by 59% [hazard ratio (HR) = 0.41; 95%CI = 0.24 to 0.71] and 66% (HR = 0.34; 95%CI = 0.18 to 0.66), respectively, in the raloxifene group compared with the placebo group^[137]. Finally, in Raloxifene Use for the Heart (RUTH) trial, in 10101 postmenopausal women with coronary heart disease or multiple risk factors for this disease, raloxifene reduced the incidence of invasive BC by 44% (HR = 0.56; 95%CI = 0.38-0.83)^[138]. Similarly, after 7 years of follow-up, the cumulative rate of invasive BC was reduced from 42.5 per 1000 women in the placebo group to 24.8 per 1000 women in the tamoxifen group (RR = 0.57, 95%CI = 0.46 to 0.70) in the National Surgical Adjuvant Breast and Bowel Project P-1 (NSABP-1) study. Furthermore, in the International Breast Cancer Intervention Study (IBIS-I), after a median follow-up of 96 mo after randomization, 142 BCs were diagnosed in the 3579 women in the tamoxifen group and 195 in the 3575 women in the placebo group (4.97 *vs* 6.82 per 1000 woman-years, respectively; RR = 0.73, 95%CI = 0.58 to 0.91). Although this study showed a somewhat "smaller" reduction of the risk of BC, the risk-reducing effect of tamoxifen appeared to persist for at least 10 years and, equally important, most side effects of tamoxifen did not continue after the 5-year treatment period^[139]. The

rates of BC were much lower in the tamoxifen group among women at high risk for BC (placebo, 6.26 per 1000 women-years, tamoxifen, 1.50 per 1000 women-years; RR = 0.24, 95%CI = 0.10 to 0.59) in the Italian Randomized Tamoxifen Prevention Trial^[140]. On the contrary, an interim analysis of the Royal Marsden Hospital tamoxifen randomised chemoprevention trial, with 2494 healthy women, the overall frequency of BC was the same for women on tamoxifen or placebo [tamoxifen 34, placebo 36, RR=1.06 (95%CI = 0.7-1.7)]^[141]. Summarizing the facts (not all included above) for the comparison of tamoxifene *vs* raloxifene, it can be concluded that postmenopausal women can choose the most effective tamoxifen (accepting its toxicities), or they can choose the (slightly) less effective (but more tolerable) raloxifene^[142]. Furthermore, according to recent data, anastrozole effectively reduces incidence of BC in high risk postmenopausal women^[143]. Finally, it must be emphasized that United States Preventive Services Task Force (USPSTF) recommends against the routine use of medications for risk reduction of primary BC in women who are not at increased risk for BC^[144].

DO BISPHOSPHONATES DECREASE THE RISK OF BONE METASTASIS?

Bone metastasis is the most common metastasis in women with BC^[145]. The effects of bisphosphonates in women with early-stage BC (EBC) have been evaluated after several meta-analyses as an adjuvant therapy with aromatase inhibitors (AI). In 2010, a meta-analysis, which included data from 13 eligible trials involving 6886 patients randomized to treatment with bisphosphonates or either placebo or no treatment, concluded that there is no significant reduction in bone metastasis (BM) and overall disease recurrence. Only in a subgroup analysis, use of zoledronic acid (ZOL) was associated with a statistically significant lower risk for disease recurrence (OR, 0.675; 95%CI: 0.479-0.952, *P* = 0.025)^[146]. Furthermore, another meta-analysis and systematic review published in 2012, reports that the use of bisphosphonates did not reduce the incidence of BM when compared with placebo^[147]. In contrast, a recent meta-analysis demonstrates that the use of ZOL improves overall survival (OS) compared with placebo (HR,0.81, 95%CI: 0.70-0.94)^[148]. Moreover, there are three international randomized studies, Z-FAST, ZO-FAST, and E-ZO-FAST, which were performed to evaluate the bone-protective effects of ZOL^[149]. After the analyses of the potential disease recurrence effects of ZOL, a significant activity in preventing bone loss during adjuvant AI therapy in postmenopausal women with EBC was noticed. However, heterogeneity between the trials for disease-free survival (DFS) and OS parameters resulted in statistically significant interaction *P* value, meaning that pooling of the data between studies would not be statistically valid^[149]. Last but not least, according to recent presented studies in the San Antonio Breast Cancer Symposium, the use of bisphosphonates

remains controversial. Coleman *et al.*^[150], after selecting 11036 postmenopausal women, demonstrated that those who were on bisphosphonate therapy experienced distant recurrences in 18.4% while women who were not in 21.9% with high statistically significant difference ($P = 0.0003$) and distant bone metastases in 8.8% and 5.9% ($P < 0.0001$) respectively. In addition, Susman *et al.*^[151] reported that BC specific mortality was reduced by 3.1% from 18.3% in women who were not treated with bisphosphonates to 15.2% for those who were on treatment ($P = 0.004$)^[150,151]. In contrast, in the other related to bisphosphonates presentation based on Neo-Adjuvant Trial Add-On (NATAN), von Minckwitz concluded that there was no difference in DFS and OS^[152]. However, even if bisphosphonates are used as an adjuvant therapy, they should be in addition with nutritional (calcium 1000 mg and 400 international units vitamin D), physical and lifestyle modifications^[153].

OPTIMAL FOLLOW-UP IN BC SURVIVORS: WHAT SHOULD BE DONE, UNTIL WHEN?

The number of BC survivors has improved within the last decades due to earlier diagnosis and effective treatments in order to prevent recurrence^[154,155]. In follow up guidelines, routine physical examination with a careful taking history has been the most valuable means of detecting BC recurrence^[156,157]. The European Society for Medical Oncology (ESMO) recommends regular visits every 3 to 4 mo in the first 2 years, every 6 mo from years 3 to 5 and annually thereafter^[72]. "In contrast", the American Society of Clinical Oncology (ASCO) recommendation for physical examinations is every 3 to 6 mo for the first 3 years, every 6 to 12 mo for years 4 and 5 and annually thereafter^[73]. Based on the evidence, mammographic surveillance remains the principal examination in detecting curable recurrences and improving survival^[158]. ESMO suggests ipsilateral (after breast-conservation surgery) and contralateral mammography every 1 or 2 years and ASCO recommends a post-treatment mammogram 1 year after the initial mammogram and at least 6 mo after completion of radiation therapy. According to ESMO, in the follow-up of patients on endocrine therapy, routine blood tests are usually indicated due to the potential side-effects of these drugs namely in the lipid profile. Furthermore, for patients on tamoxifen, an annual gynaecological examination (by an experienced gynaecologist) is recommended^[72]. However, routine ultrasound assessment of endometrial thickness is not suggested^[65]. Finally, for patients on aromatase inhibitors (AIs), regular bone density evaluation is advised^[72]. According to ASCO, in asymptomatic patients, other laboratory or imaging tests (*e.g.*, blood counts, chemistry tests, chest X-rays, bone scans, magnetic resonance imaging, liver ultrasound exams, CT scans or any tumor markers) are not recommended for routine BC follow-up^[73]. Last

but not least, the follow up should not only focus in cancer surveillance but also in late-treatment complications such as psychosocial issues^[157].

ARE MARGINS FOR DCIS AS IMPORTANT AS WE THOUGHT?

Since the treatment decision for patients with DCIS was difficult, a prognostic tool has been created. In 1996, Silverstein *et al.*^[159] have been developed the Van Nuys Prognostic Index (VNPI) by combining three significant parameters (tumor size, margin width, pathologic classification). However, in 2003 the University of Southern California added the patient age as a fourth parameter. The score ranges for 4 to 12 and the final goal was the prediction of local recurrence^[160]. The scores which were given to the four parameters range from 1 to 3. In case of margins, the score 1 is given for margin width ≥ 10 mm, the score 2 for 1 to 9 mm and the score 3 to less than 1 mm.

Even if this classification includes margins, it remains controversial the specific margin width which eliminates the risk of ipsilateral breast tumor recurrence (IBTR). Thus, the Society of Surgical Oncology (SSO) and the American Society for Radiation Oncology (ASTRO) examined the relationship between margin width and IBTR after taking into consideration systematic review and metaanalysis of the literature by including 28162 patients^[161]. They concluded that wider margin widths do not lower the risk for IBTR and consecutively wider negative margins, known as no ink on tumor, are not required. As a result, this new clinical recommendation has changed the way of thinking about negative margin widths. Even if, this was something new in clinical practice, for some others this was just a vindication^[162]. An updated version of VNPI could be proposed without the margins as a graded parameter and/or the substitution of margins with the hormone receptors status [anecdotal proposal of profs G Iatrakis and S Zervoudis (co-researchers A. Bothou and E Tomara)].

CONCLUSION

Clearly, there is much more evidence needed to clarify which answer is the correct one in the twenty-one aforementioned issues. The lack of recommended guidelines and reliable studies which include enough patients and give the possibility to generalize the results, are the main reasons why clinicians still have not consensus in clinical practice. Thus, multicentric studies and meta-analyses are required in order to clear up the less "acceptable" interventions and established the more "approved".

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WJCO 5th Anniversary Special Issues (2): Breast cancer

Impact of CYP2D6*6 in the adjuvant treatment of breast cancer patients with tamoxifen

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particularly in terms of response to tamoxifen therapy and breast cancer outcome.

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Key words: CYP2D6; Tamoxifen; Breast cancer; Adjuvant treatment

Core tip: Currently, routine cytochrome P450 (CYP)2D6 testing among women with breast cancer is not recommended and the significance of CYP2D6 phenotype in decision making regarding the administration of tamoxifen is unclear. The present study summarizes current literature regarding clinical studies on CYP2D6*4, particularly in terms of response to tamoxifen therapy and breast cancer outcome.

Abstract

Biotransformation of tamoxifen to the potent antiestrogen endoxifen is performed by cytochrome P450 (CYP) enzymes, in particular the CYP2D6 isoform. CYP2D6*4 is one of the most frequent alleles associated with loss of enzymatic activity. The incidence of CYP2D6*4 among Caucasians is estimated up to 27%, while it is present in up to 90% of all poor metabolizers within the Caucasian population. The hypothesis under question is whether the presence of one or two non-functioning (null) alleles predicts an inferior outcome in postmenopausal women with breast cancer receiving adjuvant treatment with tamoxifen. The numerous existing studies investigating the association of CYP2D6 with treatment failure in breast cancer are inconsistent and give rather conflicting results. Currently, routine CYP2D6 testing among women with breast cancer is not recommended and the significance of CYP2D6 phenotype in decision making regarding the administration of tamoxifen is unclear. The present study summarizes current literature regarding clinical studies on CYP2D6*4, par-

Markopoulos C, Kykalos S, Mantas D. Impact of CYP2D6*6 in the adjuvant treatment of breast cancer patients with tamoxifen. *World J Clin Oncol* 2014; 5(3): 374-381 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i3/374.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i3.374>

INTRODUCTION

Tamoxifen (TAM) is a selective estrogen receptor modulator (SERM) and 5 years of Tamoxifen treatment is considered as “standard of care” in the adjuvant setting in most cases of premenopausal patients with estrogen receptor positive (ER+) breast cancer, as well as in postmenopausal patients in different treatment strategies, alone or in combination with aromatase inhibitors. Recently, it was shown that, for women with ER-positive disease, continuing Tamoxifen to 10 years rather than stopping at 5 years produces a further reduction in recurrence and mortality, particularly after year 10. These results, taken together with results from previous trials

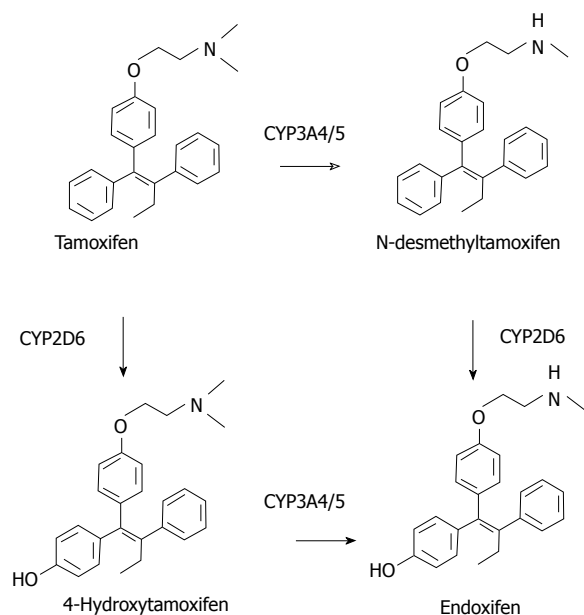


Figure 1 Tamoxifen metabolism pathways.

of 5 years of Tamoxifen treatment *vs* none, suggest that 10 years of tamoxifen treatment can approximately halve breast cancer mortality during the second decade after diagnosis^[1].

Tamoxifen has also been used as prophylactic treatment for those women considered being in high risk for developing breast cancer due to positive family history or the presence of atypia or lobular neoplasia *in situ* in a breast biopsy. Major side effects associated with tamoxifen use are hot flashes, as well as increased incidence of endometrial cancer and deep venous thrombosis. Besides acting as SERMs, it has been found that some of tamoxifen's metabolites also act as aromatase inhibitors *in vitro*^[2].

Norendoxifen consists the main metabolite of tamoxifen which is the most potent aromatase inhibitor of the tamoxifen metabolites. It causes the same decrease *in vitro* in aromatase activity as letrozole, an exclusive aromatase inhibitor.

Tamoxifen is actually a pro-drug with a weak affinity to ER that exerts its therapeutic action after been transformed in the liver. Tamoxifen metabolism mostly occurs *via* two pathways, 4-hydroxylation and N-demethylation, both of which result in the very potent secondary metabolite, endoxifen (Figure 1).

CYP2D6, a member of the cytochrome P450 mixed-function oxidase system, is one of the most important enzymes involved in the metabolism of xenobiotics in the body. CYP2D6 is highly polymorphic, as 93 alleles with varying functions and distribution among the nations have been reported. In particular, CYP2D6 is responsible for the metabolism and elimination of approximately 25% of clinically used drugs^[3]. This enzyme also metabolizes several endogenous substances such as hydroxytryptamines and neurosteroids^[3].

There is considerable variation in the efficiency and amount of CYP2D6 enzyme produced between individu-

als. Hence for drugs that are metabolized by CYP2D6 (that is CYP2D6 substrates), certain individuals will eliminate these drugs quickly (extensive metabolizers) whilst others will do that slowly (poor metabolizers). If a drug is metabolized too quickly then drug's efficacy might be reduced, while if the drug is metabolized too slowly, toxicity may result^[4]. Therefore, the dose of the drug might have to be adjusted in order to balance the speed at which it is metabolized by CYP2D6^[5].

CYP2D6*4 is the most frequent allele associated with loss of enzymatic activity within the Caucasian population (incidence reaching 27%); meaning that its significance in decision making regarding the administration of tamoxifen in breast cancer patients across Europe might be crucial. The clinical efficiency could be even stronger in small countries with a more consistent and homogeneous population.

The numerous existing studies investigating the relevance of CYP2D6 in the treatment of breast cancer are not unique and give conflicting results. The purpose of the present article is to focus especially on studies involving the CYP2D6*4, as this allele is present in up to 90% of all PMs in Caucasians^[6].

LITERATURE SEARCH

We performed a preliminary data search in PubMed which revealed 307 abstracts under the general terms "CYP2D6" and "tamoxifen". Consequently, we tried to narrow our research by excluding abstracts that were outside of clinical trials and the new approach revealed 23 abstracts that met the last criteria. We choose those with exact match in our review, especially involving CYP2D6*4 genotype, while we also selected original articles focused in the treatment of breast cancer with tamoxifen correlated with the CYP2D6 genotype in retrospective series among ER+ patients. We finally selected and took under consideration 31 abstracts as they appear to our references including general articles in order to explain the enzyme that correlates with our review along with tamoxifen usage.

RESEARCH

Clinical impact of CYP2D6*4 on breast cancer outcome was the main subject of our review. There are numerous studies performed that provide conflict results to support or not this assumption. Goetz *et al*^[7] performed a retrospective randomised phase III study in postmenopausal women with ER+ breast cancer. They concluded that tamoxifen- treated patients being homozygous for CYP2D6*4 had shorter relapse-free time and decreased DFS but not OS compared to *4/wt and wt/wt carries ($P = 0.030$, $P = 0.020$ and $P = 0.360$ respectively). The same conclusions could be extracted in a follow study for the same cohort evaluating the role of co-administration of CYP2D6 inhibitors^[8].

A further retrospective study by Schroth *et al*^[9], com-

pared 206 patients receiving adjuvant tamoxifen and 280 patients not receiving tamoxifen. They tried to extend the assessment of patients with poor metabolic status and assigned more CYP2D6 alleles with impaired activity (*4, *5, *10 and *41). They showed a significant association between CYP2D6-PM and -IM and an unfavourable outcome (more recurrences, shorter relapse-free time and reduced DFS) in patients receiving adjuvant tamoxifen therapy. The incidence of allele *4 has not been reported by the authors. A larger study from the same author refers to results from retrospective analysis of German and US cohorts of 1325 total patients treated with adjuvant tamoxifen. All women were genotyped for CYP2D6 alleles associated with reduced (*10, *41) or absent (*3, *4, *5) activity. Although no difference in overall survival could be observed heterozygous EM/IM and PM had significant higher recurrence rates and worse event-free and disease-free survival. CYP2D6*4 was present in 20% of study cases^[10]. A small series of 84 patients under adjuvant treatment with tamoxifen from Spain focused exclusively in allele *4. The authors concluded that combined genotype wt/*4 plus *4/*4 was significant associated with higher risk of disease relapse. This patient group had clearly lower benefit of tamoxifen treatment^[11]. Another retrospective study from Spain with 91 patients receiving tamoxifen in the adjuvant setting gave similar results. The genotyping was performed for 11 common CYP2D6 alleles. CYP2D6*4 was present in 22 cases, in 12 of which there was heterozygous in combination with a functional allele (*1, *2 or *35). Poor metabolizers were found to have worse DFS^[12]. A further population-based study from the Netherlands examined 85 patients for the clinical affection of allele *4 in breast cancer survival among tamoxifen users. The breast cancer mortality was also found to be significantly increased in patients with *4/*4 genotype^[13]. The report of Thompson *et al*^[14], is also of great interest. They genotyped 33 CYP2D6 alleles (including *4) in 618 tamoxifen-treated patients, using a pool of two cohorts from United Kingdom. Patients with at least one reduced function CYP2D6 allele showed a tendency for worse DFS, although not statistically significant. However, analysis restricted to four common variant alleles *4, *5, *10 and *41 showed no significant differences regarding DFS. In a sub-group analysis of post-menopausal women only, decreased metabolizers had a clear higher risk of disease recurrence. The influence of CYP2D6*4 on chemoprevention with tamoxifen was evaluated in a small subset of 46 patients with breast cancer and 136 controls pooled from a large Italian trial. The authors concluded that patients homozygous for allele *4 may be less likely to benefit from tamoxifen in the course of chemoprevention, as the cancer events were significantly higher in this study arm^[15]. The effect of variation in CYP2D6 activity on the clinical course of patients with familial breast cancer was approached in another study from United Kingdom. The 115 enrolled patients were genotyped for the CYP2D6 *wt, *3, *4, *5 and *41 alleles. Poor metabolizers showed a significantly

worse overall survival, a condition that was also observed in the subset analysis considering only CYP2D6*4, especially in BRCA2 carriers^[16]. Karle *et al*^[17] analysed the effect of the CYP2D6 genotyping in a palliative setting, enrolling 88 patients with ER positive advanced breast cancer. Co-medication with known CYP2D6 inhibitors was an exclusion criterion. The overall survival was significantly shorter for the poor or intermediate metabolizers when compared to an extensive group. Allele *4 was the most frequent non-functional variant, being detected in 37 cases. The authors concluded that CYP2D6 testing in advanced breast cancer is reasonable, especially if administration of tamoxifen is considered^[17]. A retrospective genetic analysis of patients pooled from the Austrian prospective TIGER study, incorporated 493 women with ER positive tumours and focused exclusively in allele *4. The overall frequency of allele*4 was as high as 31%, with 5.7% of all patients being genotyped as CYP2D6*4/*4. Overall, no significant difference in tumour free survival was reported; however, a subgroup analysis of patients treated with concomitant chemotherapy showed that CYP2D6*4 poor metabolizers had a shorter mean time interval to disease progression. However, It has to be reported that information on co-medication were not available^[18]. The impact of CYP2D6 in the outcome of breast cancer was further studied in a population-based cohort trial of 313 women that were adherent to tamoxifen therapy for at least one year (Table 1). It should be mentioned that 22.4% of enrolled patients were premenopausal, while administration of adjuvant chemotherapy was reported in 19,5% of the cases. Ninety seven women were heterozygous and 8 were homozygous for CYP2D6*4 respectively. This incidence was in accordance to the Hardy-Weinberg equilibrium. DNA for genotyping was extracted exclusively from patients' peripheral blood. CYP2D6 was shown to be an independent predictor of outcome, as its reduced activity was associated with recurrence and breast cancer specific survival. These results were more prevalent in the premenopausal subgroup of this cohort. As a possible explanation for this relationship, the authors proposed the higher endogenous estrogen levels in premenopausal ages that require efficient transformation into anti-estrogenic metabolites^[19].

In contrast to the studies mentioned above, Abraham *et al*^[20] published results in the opposite direction. In a large cohort of 3155 patients with confirmed treatment with tamoxifen from United Kingdom, the metabolic status of CYP2D6 was associated with breast cancer survival. In 587 of these patients additional chemotherapy had been administered, while for 1041 patients no data concerning concomitant chemotherapy were available. The authors could prove the absence of relevance between survival and variable CYP2D6 variants and argue therefore against CYP2D6 genetic testing in a clinical setting. A separate subset analysis for allele *4 showed also no statistically significant association regarding breast cancer specific- or overall-survival^[20].

Table 1 The impact of CYP2D6*6 genotype in clinical parameters

Ref.	Parameter	Extensive metabolisers	Intermediate metabolisers	Poor metabolisers
Goetz <i>et al</i> ^[8]	RFS		NS (<i>P</i> = 0.075)	Worse (<i>P</i> = 0.005)
	DFS		NS (<i>P</i> = 0.097)	Worse (<i>P</i> = 0.008)
	OS		NS	NS
Scroth <i>et al</i> ^[9,10]	RR	14.90%	20.90%	29.00%
	MR	16.70%	18.00%	22.80%
Ramóny Cajal <i>et al</i> ^[12]	DFS	118 mo	114 mo	98 mo
Bijl <i>et al</i> ^[13]	RBCM		NS	Increased (<i>P</i> = 0.041)
Newman <i>et al</i> ^[16]	OS			Worse
Karle <i>et al</i> ^[17]	PFS	14 mo		9 mo
Abraham <i>et al</i> ^[20]	BCSS			NS
Nowell ^[23]	BCR, OS		NS	NS
Rae <i>et al</i> ^[25]	RR		NS	NS
Regan <i>et al</i> ^[26]	BCE		NS	NS

RFS: Relapse free survival; DFS: Disease free survival; OS: Overall survival; RR: Recurrence rate; MR: Mortality rate; RBCM: Risk of breast cancer mortality; PFS: Progression free survival; BCSS: Breast cancer specific survival; BCR: Breast cancer relapse; BCE: Breast cancer events; NS: Non-significant difference.

On the other hand, some published studies report a protective effect of CYP2D6*4. The relation between CYP2D6*4 genotype and tamoxifen therapy was validated in a cohort of 226 postmenopausal patients participating in the Stockholm Breast Cancer Group clinical trial. Nine patients were genotyped to be homozygous carriers of allele *4, while further 55 were wt/*4. Comparison was made among arms with and without tamoxifen therapy. Tamoxifen-treated patients with at least one CYP2D6*4 allele had a significantly longer survival time than those being homozygous for wildtype genotype^[21]. In a different and larger study by the same authors, data of 677 patients from Sweden, all treated with tamoxifen for stage II and III breast cancer, have been analysed. Patients homozygous for allele *4 showed a significant survival advantage when compared to the other two genotype patterns (wt/*4 or wt/wt)^[22]. Nowell *et al*^[23] investigated 337 breast cancer patients registered in the Arkansas Cancer Research Center of the USA, performing a CYP2D6*4 genotyping. 162 patients were under tamoxifen treatment, while the remaining 175 received no hormonal therapy. The number of patients with either one or two alleles *4 in the tamoxifen and the non-tamoxifen arm was 48 and 49 respectively. No remarkable impact of CYP2D6*4 on recurrence or overall survival in both study arms could be detected. The authors did actually mention that in all subgroups, the CYP2D6*4 variant seemed to have a slight beneficial role as it was associated with decreased risk of recurrence or death^[23]. A case-controlled study in a subgroup of patients enrolled in the Women's Environment Cancer and Radiation Epidemiology (WECARE) study investigated the association between CYP2D6 polymorphisms and risk for contralateral breast cancer among patients receiving tamoxifen. The authors failed to detect such an association in a cohort of 119 and 312 women with contra- and unilateral disease respectively. It should be mentioned that 39 patients had ER negative tumour status, while a small proportion of patients with ER positive cancers was treated with ad-

ditional chemotherapy. This observation was also issued among CYP2D6*4 carriers^[24]. The Arimidex, Tamoxifen Alone or in Combination (ATAC) study was a double-blind randomized clinical trial in which postmenopausal women with early-stage breast cancer were randomly assigned to receive anastrozole alone, tamoxifen alone, or a combination of both agents in a double-blind fashion. Rae *et al*^[25], designed a genetic substudy including 588 patients who enrolled in the tamoxifen group of the ATAC trial only in the United Kingdom. All these women were genotyped for CYP2D6, while 544 of them had hormone receptor-positive cancers. The investigators could not detect any statistically significant association between CYP2D6 activity (EM, IM or PM) and breast cancer outcomes for patients with ER+ early-stage breast cancer treated with adjuvant tamoxifen. They reported that their results could not be ascribed to any clinical pathological factors such as tumor size, grade, nodal status, and age. The Breast International Group (BIG) 1-98 study was a randomized, phase 3, double-blind trial that compared five years of treatment with various adjuvant endocrine therapy regimens in postmenopausal women with ER+ breast cancer. Regan *et al*^[26] investigated the clinical relevance of CYP2D6 phenotype by obtaining tissue samples from 4861 of 8010 postmenopausal women who enrolled in this prospective trial. A specific correlation for allele *4 has been also performed. The CYP2D6 results are interestingly very similar to these from ATAC. Patients who were homozygous (PM phenotype) or heterozygous (IM phenotype) for CYP2D6*4 variant had risk of breast cancer events that were not statistically significantly different from patients who were homozygous for wild-type alleles. CYP2D6*4 status did not influence the outcome in patients under tamoxifen therapy. According to these findings, CYP2D6 genetic testing is not recommended prior to adjuvant administration of tamoxifen. The results of the BIG 1-98 trial have been presented as recommendation at the 33rd annual San Antonio Breast Cancer Symposium, 2010. The results of another large

prospective multicenter randomised trial (Austrian Breast and Colorectal Study 8 - ABCSG trial 8) have been newly published. In this cohort 3901 postmenopausal patients with resected ER+ early cancer were randomised to receive either five years tamoxifen or two years tamoxifen followed by anastrozole administration over three more years. The incidence of negative events between carriers of one or two poor alleles (*3, *4, *6, *10, *41) vs patients with two extensive alleles was compared. The possibility of such an event was significantly higher when poor alleles were present and only during the period of tamoxifen administration. It should be noted that *4 was present in 58.45% of all non-EM cases^[27].

DISCUSSION

The advisory Committee for Pharmaceutical Science (Clinical Pharmacology Subcommittee) recommended in 2006 that CYP2D6 variations influence the levels of endoxifen and that patients with ER+ breast cancer who are poor metabolizers (due to genotype or drugs co-administration) have an increased risk for breast cancer recurrence. Contrary to this statement and due to lack of strong evidence, the American Society of Clinical Oncology (ASCO)^[28] and the St. Gallen's expert consensus (2011) do not recommend the routine use of CYP2D6 genotyping in the setting of decision making about administration of tamoxifen.

The analysis of existing literature regarding the clinical importance of CYP2D6 polymorphism in the outcome of breast cancer actually leads to conflicting results. Notably, the contradictory findings on this topic might also be due to the heterogeneity across the studies in terms of data collection, analysis and interpretation.

Interestingly and contrary to the main hypothesis, there are three reports that support improved outcome in patients who are carriers of CYP2D6*4^[21-23]. Although the interpretation of these findings remains unclear, these studies do actually support the effectiveness of tamoxifen in the group of intermediate and poor metabolizers. Since then, no other large study could confirm such a favourable impact of allele*4 in the clinical course of ER+ breast cancer.

A large number of published studies show a clear negative relationship between CYP2D6*4 genotype and breast cancer outcome. In five of them the investigation focused exclusively on allele*4, while in the study of Newmann *et al*^[16] performed in patients with familial breast cancer, a subanalysis for *4 alone has also been performed^[7,8,11,13,15]. In six other, the analysis included a larger pool of intermediate/poor metabolizers based on a combined basis of common non-functional alleles, including allele*4^[6,7,9,11,14,16]. All of them were unique regarding the worse clinical course of ER+ breast cancer under tamoxifen therapy in non-extensive metabolizers. In the study of Thompson, a higher recurrence risk could be proven, in a sub-group analysis of only post-menopausal women, while Margolin *et al*^[19] described this association

mainly in premenopausal subjects.

Furthermore, there are numerous studies supporting the strong relation of CYP2D6 genotype on the circulating serum levels of tamoxifen metabolites. A recent work of Zafra-Ceres *et al*^[29] studied 90 Spanish women with ER positive breast cancer concluded that the CYP2D6*4/*4 genotype was associated with statistically significant lower 4-OH tamoxifen and endoxifen levels. Irvin *et al*^[30] examined a brighter combination of CYP2D6 alleles and reported similar results. It could be additionally demonstrated that a daily dose adjustment from 20 to 40 mg could sufficiently increase the endoxifen concentration in patients with impaired CYP2D6 function. Although this data indirectly support the main hypothesis, there is no strong evidence about the influence of endoxifen levels on the clinical course of breast cancer^[30].

On the other hand, a large population based case-cohort study from the United Kingdom showed no association between any CYP2D6 variants and breast cancer specific survival in the setting of tamoxifen therapy. A sub-group analysis regarding allele*4 alone gave consistent results. However, it should be mentioned that a large proportion of included cases had received concomitant chemotherapy^[20]. Furthermore, Brooks *et al* investigated a cohort of breast cancer patients, pooled from the WECARE study, and showed that the CYP2D6*4 variant is not associated with higher risk of contralateral breast cancer, in the setting of tamoxifen treatment.

All these reported studies implicating allele*4 are retrospective and partially refer to limited cohort numbers. It is reasonable to assume that due to the small number of subjects, the proportion of poor metabolizers is unavoidably limited, a fact that influences the level of evidence of any extracted results regarding this group.

The comparison groups within the existing studies varies, as the heterozygous genotypes wt/*4 (IM) are not consistently stratified in one comparison group. Although most published studies classify them together with the homozygous *4 genotype, in three studies they are incorporated in the homozygous wt arm^[7,13,22].

The function of CYP2D6 can be altered or inhibited by several pharmaceutical substances. Agents such as fluoxetine, paroxetine, bupropion, quinidine and cinalcact act as strong inhibitors of CYP2D6 and should therefore be cautiously prescribed in combination with tamoxifen^[31]. Unfortunately, there is little and inconsistent data within the majority of available studies, regarding the co-administration of a CYP2D6-inhibitor. This fact strongly insults the reliability and the evidence level of any statistically significant result.

Another important aspect is the adherence of examined population to therapy. It is known that the long lasting administration of tamoxifen is associated with various adverse effects, such as hot flashes, that strongly impair the quality of life of the treated patients. Due to possible side effects, incompliance or therapy discontinuation, especially between extensive metabolizers or non-trial women, remains relatively high^[32]. Although data over the

adherence to tamoxifen therapy should be known in order to safely validate the existing results, this information is absent in nearly all relevant studies^[22].

The estrogen-receptor status of breast malignancies clearly affects the outcome of the administered hormonal therapy. Many of all existing studies analyse cohorts that include a number of patients with ER-negative disease^[15,16,20,23,24], while two other provide no sufficient data regarding the hormonal status of the enclosed population^[11,13]. This condition represents a strong limitation in the interpretation of the published results.

It is important to mention that the three latest large studies refer exclusively to postmenopausal women with ER positive breast cancer disease^[25-27]. Goetz *et al*^[27], have proven a statistically important incidence of negative events within carriers of poor CYP2D6 variants, while the two other studies failed to confirm such an association. Although they favour against a CYP2D6 genetic testing prior to tamoxifen therapy, it should be mentioned that both studies showed a substantial departure from the Hardy-Weinberg equilibrium regarding the frequency of allele*4^[25,26].

An important bias of most published trials is the followed genotyping procedure, as the estimation of CYP2D6 variant was performed at paraffin-fixed cancer tissue and not at hosts' blood-or buccal-derived DNA. However, tumor DNA may show significant differences from germline DNA, considering the frequent occurrence of "loss of heterozygosity" in cancer biology. A possible misclassification leads to a smaller arm of intermediate metabolizers (IM) in favour of extensive metabolizers (EM), a fact that strongly insults the final results and conclusions.

Furthermore and in order to safely evaluate the influence of CYP2D6 genotype on tamoxifen therapy, any performed study should actually enclose patients treated with tamoxifen monotherapy.

These two last conditions are unfortunately not satisfied in all three large prospective trials. It should be mentioned that the examined DNA was extracted from formalin-fixed paraffin-embedded tumor tissue, while concomitant chemotherapy is reported in a large proportion of the cohorts. These strong limitations set the reliability of the various results in doubt.

As a final comment, it remains questionable whether the possible ethnic heterogeneity of the examined population within the existing cohorts has an influence on any extracted results. This assumption makes the evaluation of all this data extremely difficult.

CONCLUSION

CYP2D6*4 consists the most frequent allele with poor metabolizing function among Caucasians, that possibly affects the clinical course of breast cancer. At present there is an ongoing debate, whether a dose or duration adjustment of administered tamoxifen could guarantee a better outcome in carriers of CYP2D6*4.

The current recommendations regarding breast cancer treatment do not include the routine use of CYP2D6 genotyping in the adjuvant setting of tamoxifen. On the other hand, there is strong evidence supporting a negative impact of allele*4 in the outcome of the disease, that should not be ignored. Large prospective trials, limited exclusively to postmenopausal women with ER positive breast cancer, have been newly completed and offer a huge bank of data eligible to further assessment. These three latest studies lead to conflicting results and are all amenable to criticism due to numerous limitations. In European countries with consistent and homogeneous population the frequency of CYP2D6*4 is expected to be quite high. For this group of patients, the question about the clinical importance of CYP2D6*4 in the course of breast cancer remains practically unanswered. Future research should explore prospectively the role of CYP2D6 activity in patients receiving tamoxifen, in particular in premenopausal patients for which tamoxifen represents the standard of care in the adjuvant and metastatic setting.

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WJCO 5th Anniversary Special Issues (2): Breast cancer**Significance of immunohistochemistry in breast cancer**

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Abstract

The biological characteristics of the tumour are used to estimate prognosis and select appropriate systemic therapy for patients with (breast) cancer. The advent of molecular technology has incorporated new biomarkers along with immunohistochemical and serum biomarkers. Immunohistochemical markers are often used to guide treatment decisions, to classify breast cancer into subtypes that are biologically distinct and behave differently, and both as prognostic and predictive factors. Steroid hormone receptors, markers of tumour proliferation, and factors involved in angiogenesis and apoptosis are of scientific interest. In this review we will provide information on the immunohistochemical markers used in the management of breast cancer patients using available data from the literature. We consider the utility of established immunohistochemical markers, and discuss the challenges involved in integrating novel molecular markers into clinical practice.

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Key words: Breast cancer; Immunohistochemistry; Markers

Core tip: Immunohistochemistry has an important role in the pathology of breast disease, as well as in other benign or malignant tumours. There is a growing list of avail-

able products (antibodies) or antigen retrieval techniques, which all contribute to the broader utility of immunohistochemistry for solving diagnostic problems or for determining prognosis and response to therapy in breast pathology. Myoepithelial markers are useful in helping to distinguish benign lesions from malignant lesions. The most common immunohistochemical breast cancer prognostic and therapeutic markers used include: estrogen receptor, human epidermal growth factor receptor-2, Ki-67, progesterone receptor, and p53. In addition, markers of angiogenesis and apoptosis are also important.

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INTRODUCTION

Immunohistochemistry (IHC) is used to characterize intracellular proteins or various cell surfaces in all tissues. Individual markers or more often panels of various marker proteins can be used to characterize various tumour subtypes, confirm tissue of origin, distinguish metastatic from primary tumour and provide additional information which may be important for prognosis, predicting response to therapy or evaluating residual tumour post-treatment. There is a growing list of available products (antibodies) or antigen retrieval techniques, which all contribute to the broader utility of immunohistochemistry for solving diagnostic problems or for determining prognosis and response to therapy in breast pathology. Diagnostic and prognostic markers are described although some of them can be included in both.

DIAGNOSTIC MARKERS

The most important diagnostic problems that occur in

Table 1 Diagnostic markers

Marker	Staining pattern	Useful for
Smooth muscle actin	Cytoplasmic staining	Myoepithelial differentiation
Calponin	Cytoplasmic staining	Myoepithelial differentiation
p63	Nuclear	Myoepithelial differentiation
Smooth muscle myosin heavy chain	Cytoplasmic	Myoepithelial differentiation
CD10	Membranous staining	Myoepithelial differentiation
S100	Cytoplasmic	Myoepithelial differentiation
High-molecular-weight cytokeratins (14 and 5/6)	Cytoplasmic	To distinguish invasive carcinoma from benign proliferations; expressed by lobular carcinomas
Cytokeratin 8	Peripheral cytoplasmic	Ductal carcinoma cells
Cytokeratin 8	Perinuclear staining	Lobular carcinoma cells
CK 7 and 20		Mammary origin of a metastatic carcinoma
E-cadherin	Membranous staining	Usual ductal carcinomas
Hormone receptors estrogen receptor, progesterone receptor	Nuclear	Identified subtypes, mammary origin
HER2 neu	Membranous staining	Identified subtypes
Gross cystic disease fluid protein 15	Cytoplasmic	Mammary origin of a metastatic carcinoma
Mammaglobin A	Cytoplasmic	Mammary origin of a metastatic carcinoma
Carcinoembryonic antigen, CEAD-14 clone	Cytoplasmic	Evaluation of metastatic mammary carcinoma

mammary gland tumor pathology are: the differential diagnosis of various types of benign lesions and carcinoma; differentiating between carcinoma *in situ* and invasive carcinoma, diagnosis and differentiation of microinvasion and its imitating lesions and confirming the breast as the primary site in metastatic carcinoma. In the absence of advanced molecular biological techniques, IHC can be used to identify histologic subtype or molecular phenotype. Some of these problems can be solved using IHC markers (Table 1). It is well known that normal glandular breast tissue is composed of three cell types which express different subsets of proteins: luminal, basal and myoepithelial. The luminal cells express cytokeratins (CK 7, 8, 18, 19), epithelial membrane antigen (EMA), milk fat globule membrane antigen (MFGM), α -lactalbumin, estrogen receptor (ER), and progesterone receptor (PR). Myoepithelial cells express basal cell type CKs and specific markers: smooth muscle actin, calponin, S100 and p63, while basal cell types express different cytokeratins (5/6, 14, 17)^[1-3].

Myoepithelial markers: SMA, Calponin, p63, SMMHC

Myoepithelial markers are useful in helping to distinguish invasive carcinoma from benign proliferations with a similar morphological appearance, benign proliferative lesions and most preinvasive lesions with an intact myoepithelium. Invasive carcinomas lack the myoepithelial cell layer that normally surrounds benign breast glands. There is an exception, microglandular adenosis, a benign proliferative lesion which lacks the myoepithelial cell layer^[3-5]. In the same context, to assess intraductal proliferative lesions, high-molecular-weight cytokeratins (cytokeratin 14 and cytokeratin 5/6) can be helpful in distinguishing ductal hyperplasia from low-grade ductal carcinoma *in situ* (DCIS). Atypical ductal hyperplasia or *in situ* carcinoma can arise in otherwise benign papillary lesions and is defined as a type of ductal hyperplasia that morphologically simulates DCIS. Characteristically, atypical ductal hyperplasia has a uniform population of cells and most lesions

are small and focal, involving only a portion of a duct or only a few small ducts measuring less than 2 mm. Using IHC, positive myoepithelial staining is seen in the benign area with attenuated or absent staining in areas of atypia or *in situ* carcinoma. It is possible that the area of atypia or *in situ* carcinoma may not even be represented in the limited sample from a core needle biopsy.

Smooth muscle actin (SMA) has long been used as a myoepithelial marker in breast pathology diagnosis as a sensitive marker of myoepithelial differentiation, even if it is not specific, because any cell with substantial expression of actin is positive for SMA (myofibroblasts and blood vessels are positive for SMA).

This becomes problematic in lesions where there are either myofibroblasts or blood vessels in close proximity to the epithelial lesion. One pitfall is the presence of myofibroblasts within desmoplastic stroma adjacent to nests/glands of invasive carcinoma being misinterpreted as myoepithelial cells, resulting in a false-negative diagnosis. This is why the use of a panel of markers (p63, calponin, smooth muscle myosin, CD10, S100) or a more specific marker such as p63 are recommended.

One option is calponin, a protein belonging to the contractile apparatus in smooth muscle cells, which is considered to have the same sensitivity as SMA, however, similar to SMA, staining of myofibroblasts and smooth muscle in blood vessels can be obtained. As with SMA, cytoplasmic staining of myoepithelial cells tends to encircle the nucleus as opposed to the staining pattern of myofibroblasts. Compared to other markers (p63 or smooth muscle myosin heavy chain (SMMHC)), it tends to show more complete staining of the myoepithelial layer.

p63 is a homolog of p53, and has been shown to be expressed exclusively in myoepithelial cells in normal breast and can be very useful in differential diagnosis involving benign lesions such as sclerosing adenosis, radial scars and papillary lesions. The advantage of using p63 is its nuclear localization and absence of staining in smooth

muscle cells, such as myofibroblasts and blood vessels. Thus, it provides almost 100% specificity, however, its sensitivity has been reported to be approximately 90%. This is demonstrated by the so-called “focal gaps” in staining in the myoepithelial layer, partly due to the plane of section. In addition, it has now been shown that about 10% to 15% of invasive tumors, particularly high-grade and metaplastic carcinomas, express p63, although the staining is usually weaker than that seen in myoepithelial cells. Similarly, foci of squamous differentiation stain positively.

Like other smooth muscle markers, SMMHC is associated with contractile elements and is present in all cells with such properties. It is expressed primarily in myoepithelial cells, but is also expressed in blood vessels. An advantage of SMMHC is that it demonstrates less cross-reactivity in myofibroblasts than calponin and SMA. Overall, the studies so far suggest that among smooth muscle markers, SMMHC provides the best results, in terms of both sensitivity and specificity.

When inflammation or reactive fibrosis obscure the interface between involved ducts and adjacent stroma in some cases of DCIS, IHC can help to clarify the integrity of the duct wall. Usually ductal carcinoma cells are negative for myoepithelial cells markers: S100, SMA, SMMHC, calponin, CK5, CK14, CK17, CD10, and p63^[3,6-14]. The specific markers among these are SMMHC, calponin, and p63, these as well as some basal CKs have an advantage in that they do not stain myofibroblasts. Is this correct?

In most laboratories, however, the choice between these markers depends on individual experience, preference or financial resources. A combined approach, a nuclear and a cytoplasmic myoepithelial marker is the best option to increase the diagnostic utility of these markers. Pitfalls in the use of myoepithelial markers include those related to interpretation, fixation and technical aspects, and possible biological effects. Interpretative issues include the possibility of mistaking myofibroblasts for myoepithelial cells due to cross-reactivity of cytoplasmic epitopes (in particular, SMA and calponin). Fixation and technical issues include underfixed tissue not immunostaining optimally; in such situations, entrapped benign glands may be mistaken for invasive carcinoma due to lack of staining of myoepithelial cells, resulting in a false-positive diagnosis of carcinoma. The key to solving these issues is to include adequate internal controls.

Lobular or ductal carcinoma: E-cadherin, CK8

Determining whether an *in situ* lesion is lobular carcinoma or ductal carcinoma has clinical management implications and is another situation in which IHC proves its worth. Generally, ductal and lobular carcinomas, either invasive or *in situ* can be distinguished in hematoxylin-eosin-stained sections. In cases with non-specific morphologic characteristics, categorization can be performed through IHC, and E-cadherin is currently used to differentiate

between the two. The majority of ductal carcinomas express cytoplasmic E-cadherin, whereas most lobular carcinomas lack expression of E-cadherin^[3,15,16]. In addition, the differences in CKs expression may be used: high-molecular-weight CK (clone 34βE12) is usually expressed by lobular carcinomas, but is absent or expressed at low levels in most cases of DCIS^[3,17,18]. In the same context, CK 8 is stained in ductal carcinoma cells in the peripheral cytoplasm, while perinuclear staining is characteristic of lobular carcinoma^[19].

Identification of subtypes of breast cancer

Analysis of both adjuvant and neoadjuvant trials has shown that not all chemotherapeutics have equal effects on breast cancer patients, therefore, further individualization of chemotherapy may be required. Data on differences in chemotherapy sensitivity to taxanes and anthracyclines suggest that there are significant differences across disease subtypes, which if further validated, could be used to guide the best decision-making in patient treatment^[20]. The St Gallen expert panel which met at the 12th International Breast Cancer Conference held at St Gallen (Switzerland) in March 2011, identified four subtypes of breast cancer according to oestrogen and progesterone receptors, and overexpression and/or amplification of the human epidermal growth factor receptor 2 (HER2) oncogene. The four subtypes were luminal A, luminal B, Erb-B2 overexpression and basal-like. The expert panel provided systemic treatment recommendations for the subtypes including endocrine therapy alone for luminal A, endocrine ± cytotoxic therapy for luminal B (HER2 negative); cytotoxics + anti-HER2 + endocrine therapy for luminal B (HER2 positive); cytotoxics + anti-HER2 for HER2 positive (non luminal); and cytotoxics for triple negative.

Markers for mammary origin in metastatic carcinoma: GCDFP15, mamaglobin, CEA

In the case of small metastasis of infiltrating lobular carcinomas, false negative results are far more frequent than those in infiltrating ductal carcinoma^[21]. Medullary carcinoma metastasis or other subtypes of mammary carcinoma (lobular, sarcomatoid) can often be mistaken for malignant lymphoma (with “signet ring” cells, clear cells, with carcinoma pattern, sarcomatoids). In these situations, a positive reaction for CK and lack of reactivity for lymph markers suggest a diagnosis of metastasis. In as many as 24% of lymph nodes reported metastasis-free by standard histological examination, various authors found metastasis when multiple or serial sections were cut. Immunohistochemical markers also improve the specificity and accuracy of cell detection; therefore it is important to evaluate their utility in improving standard histological procedures. In the case of large metastasis in the axillary lymph nodes, IHC can demonstrate by a positive reaction for epithelial markers the carcinomatous nature of cells, difficult to appreciate as epithelial, in particular, in

the case of axillary metastasis of infiltrating lobular carcinoma (relatively uniform appearance of tumor cells and low mitotic activity). For small metastasis of infiltrating lobular carcinoma, false negative results are much more common than in infiltrating ductal carcinoma^[21]. In addition, medullary carcinoma metastasis or other subtypes of breast carcinoma can sometimes be confused with malignant lymphoma (cells in the so-called “signet ring”, clear cell); in these situations, a positive reaction for CK and lack of reactivity for lymphoma markers suggest a diagnosis of metastasis.

The identification of metastatic carcinoma of the breast may be difficult in the absence of a previous history of breast cancer. Various immunophenotypic markers have been introduced to aid in this process. Markers for mammary origin include receptors for hormones, such as androgen receptors (ARs) and gross cystic disease fluid protein 15 (GCDFP-15)^[3]. GCDFP-15 is present in the liquid of breast cysts and any apocrine cells: mammary glands, salivary glands, sweat, Paget’s disease, *etc.* Therefore, carcinoma of the breast and others show reactivity to GCDFP-15. Even in these conditions, the positive predictive value and specificity for the detection of breast cancer is 98%-99%^[22], but moderately sensitive (50%-74% for breast carcinoma), which is why it is important to add other markers to the diagnostic panel such as ER, PR, AR, and HER-2/*neu*, mammaglobin, and CKs (7 and 20). In this context, ARs and/or HER-2/*neu* are given additional value in a great number of ER-negative high-grade ductal carcinomas^[3].

Lately, mammaglobin has been described as a breast cancer-specific gene, and its utility as a novel breast cancer marker has been confirmed^[3,23,24]. Mammaglobin A and B identified in breast cells are overexpressed in breast cancer. Mammaglobin A is more specific for breast and gynecologic organs, while mammaglobin B may be found in a number of other tumors, especially gastrointestinal malignancies. Many studies have suggested that elevated mammaglobin levels in breast cancer are associated with clinical and biological features defining a less aggressive tumor phenotype. Mammaglobin expression is not changed at the metastatic or lymph node site. It can help, in combination with other markers, to establish the correct diagnosis of metastatic breast carcinoma. Although many carcinomas would not be included in the differential diagnosis of breast carcinoma, the specificity of this marker was 92%^[25].

In the same study, when the immunohistochemical staining pattern of mammaglobin was compared with GCDFP-15 in the breast carcinomas, mammaglobin had higher sensitivity than GCDFP-15. In addition, the mammaglobin antibody cocktail stained deeper than GCDFP-15, and among positive cases, the number of cells stained with mammaglobin was higher than with GCDFP-15. Despite some non-specificity of the mammaglobin antibody, these data provide convincing evidence for the inclusion of mammaglobin in a panel for the workup of carcinoma of an unknown primary site.

During the diagnosis of breast carcinoma, it should be taken into consideration that the sensitivity of mammaglobin is better than that of GCDFP-15^[25-27].

Carcinoembryonic antigen (CEA) is a well-known tumour marker glycoprotein of 180 kDa. The polyclonal antibody reacts strongly and diffusely with ductal mammary carcinomas, lung and large intestine carcinomas; CEAD-14 clone reacts with a small subset of mammary carcinomas, usually high grade, which is useful in the evaluation of metastatic mammary carcinoma in the lung, liver, brain and lymph nodes; 13% of breast carcinomas are positive for CEAD-14, with a focal reaction, but diffuse in high-grade carcinomas. A negative CEAD-14 pulmonary tumour is more likely to be a metastasis and not a primitive lung tumour, which is positive for other specific markers (such as thyroid transcription factor-1, TTF1)^[28].

MARKERS OF PROGNOSIS AND RESPONSE TO THERAPY

The most common immunohistochemical breast cancer prognostic and therapeutic markers used include: ER, HER2, Ki-67, PR, and p53. In addition markers of angiogenesis and apoptosis are used.

Hormone receptors

Nowadays, immunohistochemical detection of ER and PR is part of the routine work-up of breast cancer, and in some cases of DCIS the presence of ERs is an indication for tamoxifen therapy. There are many scoring systems and many studies have compared their ability to predict treatment response and correlations with outcome. The first scoring system counted the percentage of positive cells and ignored staining intensity^[29,30]. When we determine the proportion of positive stained cells, at least 1% is considered a hormonally treatable state. According to the International Breast Cancer Study Group scheme which is the basis of the most recent St Gallen treatment guidelines, breast cancer is divided into three groups based on the percentage of positive cells: responsive (10%), response uncertain (1%-9%), and nonresponsive (0%). In other words, a threshold of 1% positive cells indicates the option for hormonal therapy. These guidelines are widely followed in many countries from Europe and the United States, but they seem to be insufficient.

Many users report results as an Allred score, which comprises both the percentage of positive cells and staining intensity^[31]. A total score of 3 or more, corresponding to 1% to 10% positive cells, characterises the lowest positive result and corresponds to the St Gallen endocrine response uncertain category in which case adjuvant hormone treatment can be recommended, but has an uncertain benefit^[3,32,33]. Immunohistochemical staining for ER in DCIS, without associated invasive lesions has a role in estimating the potential positive effect of tamoxifen. The National Surgical Adjuvant Breast and Bowel Project

Protocol B-24, in patients with DCIS treated with partial mastectomy and then irradiation, who received placebo or tamoxifen for five years showed a conclusive reduction in both ipsilateral and contralateral breast cancer in the adjuvant tamoxifen group^[3,34,35].

HER-2/Neu expression

HER-2/neu was one of the first oncogenes studied in samples of invasive breast cancer and it is identified in 10%-20% of breast cancer patients. It is a marker for sensitivity to Herceptin (trastuzumab), and resistance to tamoxifen^[36]. Although Her-2/neu can be detected using many methods, only two are currently approved and recommended for its detection: IHC and fluorescence *in situ* hybridization (FISH). Standardized immunohistochemical techniques exhibit a very good correlation with FISH methods. Such standardization requires the use of 10% neutral-buffered formalin as a fixative allowing at least 6-8 h of tissue fixation, and not more than 48 h. IHC evaluates overexpression of the receptor protein at the surface of the cells, while FISH evaluates the status of the *HER2* gene in the nucleus. In the majority of *HER2*-positive cancers, *HER2* protein overexpression is the result of gene amplification, thus both methods should be highly correlated.

Immunohistochemistry reactions for HER-2 are scored by HercepTest where 0 and 1+ scores are negative, 2+ is weakly positive and 3+ is positive. A positive HER-2 result consists of a uniform and intense membrane staining of more than 30% of tumour cells and further evaluation is unnecessary for invasive cancers that stain definitely positive or negative. Weakly positive or equivocal or 2+ cases should be tested for gene amplification by FISH. A positive result using this method indicates more than 6 HER-2 gene copies per tumour cell nucleus or a HER-2 gene to chromosome 17 ratio of more than 2.2^[3,36-38].

Selection of the best treatment, especially if the patient is a candidate for HER2-targeted therapy, depends on accurate laboratory results of the assessment of HER2 status. All aspects of the test are performed in a highly standardized fashion with good quality control and the quality controls must be continuously monitored. The standardization includes aspects of pre-analytical sample tissue handling, the type and duration of fixation, tissue processing, assay performance, interpretation, and reporting^[37].

Due to the intrinsic subjectivity of the results of the two techniques (IHC and FISH), the identification of new methods with less subjectivity is necessary. Recently, the chemiluminescent technique has been used as a quantitative assay^[39,40]. It has many benefits such as high sensitivity and accuracy, stability of reagents, easy-to-use protocols, non-photodegradable products, and a good correlation between IHC and immunohistochemiluminescence results.

Markers of apoptosis and cell proliferation: Ki-67 proliferation index, BCI-2, p53

Ki-67, a non-histone protein, involved in the early steps of polymerase I-dependent ribosomal RNA synthesis is a predictive and prognostic marker in cancers and has been extensively studied. When Ki-67 level is above 10%-14%, breast cancer patients are defined as high-risk^[41,42]. According to the St. Gallen Consensus (2009), the Ki-67 index is useful for selecting patients with hormone receptor-positive breast cancers for the addition of chemotherapy to endocrine therapy. Thus, breast tumours are classified as low, intermediate, and highly proliferating according to a Ki-67 labelling index of under 15%, 16%-30%, and over 30%, respectively. Data from the Clinical Cancer Registry Regensburg showed that Ki-67 expression was associated with common histopathological parameters, especially grading and survival, but is an additional independent prognostic parameter for disease-free survival and overall survival in breast cancer patients^[43].

The neoadjuvant setting is useful for analyzing the value of Ki67 as a predictive and prognostic tool. The majority of studies investigating complete pathological response have identified a high Ki67 proliferation rate as a predictive factor for a higher rate of complete pathological response^[44,45]. However, it was found that patients in whom progression occurred had a higher proliferation rate than those who responded to neoadjuvant chemotherapy. This suggests a nonlinear effect of Ki67 on treatment response and probably on prognosis as well^[44,45].

Ki-67 expression has been used to determine the effects of different doses of tamoxifen on breast cancer proliferation^[42,46]. The change in Ki-67 expression induced by lower doses of tamoxifen was comparable to that achieved with the standard dose, indicating that tamoxifen retains antiproliferative activity at low doses^[46,47]. Dowsett *et al*^[48], in a small study, showed that a higher Ki-67 labelling index after two weeks of neoadjuvant therapy with tamoxifen was associated with shorter recurrence-free survival, whereas higher Ki67 expression at baseline was not. According to Ellis *et al*^[49], a significant reduction in the Ki-67 index after a short period (a few weeks) of hormonal treatment may be a simple and affordable way to select patients with ER-positive breast cancer who may not benefit from adjuvant chemotherapy.

Another proliferation marker in tumour tissue is the Ki-S2 antibody. This antibody recognises a proliferation-specific nuclear protein expressed exclusively in the cell cycle phase S, G₂, and M. Therefore, actively proliferating cells that constitute a subset of the population recognised by Ki-67 were specifically labelled. The cycling ratio was defined as the ratio of the Ki-S2 labelling index to the Ki-67 labelling index and represents the relative fraction of cells in proliferation. Alterations in cell cycle regulation at the G₁-S transition strongly influence breast can-

cer progression^[42,50]. Prognosis is probably indicated by the percentage of cells in S through M phases of the cell cycle and measurement of the Ki-S2 index may also improve to allow an accurate prognosis and to identify patients with a low risk of recurrence who may not require adjuvant therapy^[42,51].

With regard to the molecular breast cancers, high Ki-67 proliferation index can be used to classify triple negative breast cancer into subtypes with different prognoses or responses to treatment. For this purpose, the number of Ki-67 positive cells among the total number of counted tumour cells was determined and the high expression of Ki-67 was defined as $\geq 10\%$. It is known that patients with triple negative breast cancer have a poor survival, despite their high response rate to neoadjuvant chemotherapy, and those with high Ki-67 have more aggressive clinical features^[52].

The use of adjuvant chemotherapy in early breast carcinoma is controversial, with many advocating its use in high-risk patients as defined by specific pathologic parameters. Both BCL2 and p53, which are involved in apoptosis and cell proliferation, play an important role in determining tumour growth and may help to define high-risk patients more accurately. In breast cancer patients, BCL2 expression is significantly associated with hormone receptor status and p53 is an important prognostic marker in early breast cancer^[53].

BCL2 belongs to a group of protein key regulators of apoptosis or programmed cell death. The tumorigenic potential of inappropriate BCL2 protein expression associated with an adverse outcome was first described in subsets of non-Hodgkin's lymphoma as a result of the chromosomal translocation $[t(14,18)]$ ^[54,63].

Overexpression of BCL2 protein has been identified in a variety of solid organ malignancies, but in contrast to non-Hodgkin's lymphoma, BCL2 protein expression in breast cancer is associated with a nonaggressive phenotype of low-grade, slowly proliferative ER+ breast tumours^[55-57]. This favourable prognostic effect of BCL2 in breast cancer is explained by its non-apoptotic functions^[55,58]. BCL2 is expressed in normal breast glandular epithelium and is upregulated by oestrogen, possibly as a direct result of transcriptional induction^[55,59]. Its amplification or copy number gain is a rare condition and correlation between transcript and protein levels in breast cancer is nonlinear, involving post-transcriptional regulation^[55,60]. Moreover, many studies have demonstrated that expression of BCL2 is associated with improved survival in breast cancer, however, this was attributed to its correlation with ER status^[55,61,62]. If we compare patients with ER+/BCL2- disease to those with ER-/BCL2+ disease, the former have been found to have a worse prognosis than the latter^[61,62].

The prognostic value of BCL2 is present across molecular subtypes, and is independent of parameters such as stage, grade and tumour size. BCL2 can be used to prevent patients undergoing unnecessary cytotoxic therapy and provides additional prognostic information^[55].

The other marker, p53 is well studied in cancers, but its value in predicting clinical outcome in breast cancer is debatable. The p53 gene is located on the short arm of chromosome 17 and encodes a 375 amino acid nuclear phosphoprotein that prevents propagation of genetically modified cells^[64]. Wild-type p53 is a tumour suppressor protein and plays an essential role in regulating genomic stability by controlling the cell cycle and inducing apoptosis when cell damage cannot be repaired^[65-67]. In normal cells, p53 has a very short half-life due to ubiquitylation and proteasome degradation^[68,69]. IHC can be used, as wild-type p53 protein is rapidly degraded, while TP53 mutations (18%-25% of primary breast carcinomas) are often associated with the production of a stable protein. In addition, sequencing of the p53 gene in all breast cancers would be expensive and time-consuming for routine practice^[70,71,73]. A higher tumour grade, negative ER and PR status, and the more aggressive basal subtype were associated with abnormal p53 immunohistochemical expression or p53-positive status^[70,72,73]. With regard to early breast cancers, some scientists have reported that a p53 mutation has no influence on the outcome and therefore, the value of p53 status is too weak to be recommended as a routine marker in clinical practice^[74].

Angiogenesis markers

Tumor growth and metastasis are dependent on tumour angiogenesis and this complex process involves a delicate balance between angiogenic and antiangiogenic factors. Numerous studies have investigated the relationship between tumour angiogenesis, prognosis and response to antiangiogenic drugs. Analysis of these factors in tumour or serum of breast cancer patients by IHC or multiplex protein assay (FASTQuant® Microspot Assays) can improve diagnosis and prognosis of the disease. There is a large list regarding angiogenesis markers: Angiogenin, Ang2, keratinocyte growth factor (KGF), fibroblast growth factor basic, intercellular adhesion molecule (ICAM)-1, platelet-derived growth factor-BB and the vascular endothelial growth factor family. With regard to these markers, it has been observed that patients with breast cancer exhibited high levels, as well as high serum levels when compared to patients with benign breast diseases. When some of these markers were evaluated either in tumour or serum in breast cancer patients, they showed an association with standard clinical parameters, ER status and intratumoural microvessel density of tumours^[75].

The commonly used method to determine angiogenesis is counting intratumoral blood vessels (MVD) stained with factor VIII related antigen or anti CD31 or CD34 using light microscopy. The main difficulty is the significant variability in density between different areas of tumor and among observers. Counting newly formed stained microvessels is a useful tool in the early detection of metastatic potential and in the selection of patients for whom anti-angiogenesis drugs might be beneficial. The reactivity level of CD34 antigen was assessed by IHC

in all types of invasive ductal breast cancer and its level seems to be a useful predictor for the development of local lymph node metastasis and can indicate the benefit of antiangiogenic treatment^[76,77].

Anti-angiogenic drugs have been approved recently for the therapy of advanced cancers, including breast cancers. These drugs, alone or in combination with chemotherapy, are able to improve overall or progression-free survival in cancer patients. Unfortunately, the lack of validated biomarkers to allow the selection of patients who are most likely to benefit from targeted drugs such as bevacizumab, sunitinib, sorafenib and pazopanib, limits the rational use of these drugs and the ability to determine optimal dose and scheduling of these drugs^[78].

Most of the biological and clinical activity of the anti-angiogenic drugs currently approved for cancer therapy is against the VEGF-related pathways. The VEGF system is part of the platelet-derived growth factor gene family, and interacts with its specific receptors; VEGFR-1 (flt-1) and VEGFR-2 (flt-2) for VEGF-A, a very potent angiogenic growth factor. VEGF-B, interacting with VEGFR-1, seems to have an important role in the maintenance of existing vessels, but this protein is not well studied. VEGF A and B, their receptors VEGFR-1 and 2 are expressed in a variety of normal cells, and overexpression has been described in malignant tumors^[79-82].

There are different techniques used to assess VEGF-A, IHC being the most convenient in routine diagnosis as well as research, as it allows single cell analysis combined with morphology. The results are currently based on visual examination of IHC-stained tissue slides and several different scoring systems have been used^[79,83,84]. Some of these scoring systems evaluate the intensity of immunoreactivity, while others combine the examination of intensity score with the percentage of cells stained resulting in a semiquantitative scoring system. Unfortunately, such scoring is defined by subjectivity and debatable intra- and interobserver reproducibility. Therefore, using the same tumour sections, some laboratories also introduced an automated method for analyzing VEGF expression and obtained the AI score. Methods for computer-assisted image analysis of VEGF-A improved reproducibility by reducing some of the variation between measurements^[79,85,86].

The prognostic importance of VEGF in invasive breast cancer is associated with tumour stage and ER status, and inversely correlated with tumour grade and measurement of tumour VEGF, as an indicator of angiogenesis, which is more reliable prognostically than measurement of microvessel density or serum VEGF^[87,88]. In addition, tamoxifen treatment was associated with higher circulating and platelet-derived VEGF levels^[88].

CONCLUSION

IHC has become an integral part of the pathology laboratory. It is a more mature technology and accessible to the majority of pathology laboratories. IHC can be used

for diagnostic issues, estimating prognosis or predicting response to therapy. The best approach in the use of immunohistochemical markers is to combine them with the examination of standard hematoxylin-eosin slides and use panels of markers. Once the potential value of a new immunohistochemical method is appreciated, the burden will be to ensure standardization of the test protocol to maintain conformity and minimize interlaboratory variation. Results may vary widely depending on the choice of fixative, choice of antibody manufacturer, and the type of immunostaining methods. A scoring system for test results should be regularly adopted and properly reported. IHC testing is cost-effective and can be carried out in parallel with other tests. In the event of equivocal results, a back-up test using multigene assays or others methods should be made available. Once these parameters are standardized, IHC will assume a better and well-defined role in the management of patients with cancer.

It is clear that the role of IHC in detecting biomarker expression in pathology largely depends on research studies which demonstrate differential immunohistochemical expression and other studies that show good correlation between positive expression and response to new therapy. Although a gene study is a sensitive technique, it lacks specificity in distinguishing different cells, and may be contaminated by other cells. In addition, gene profile analysis is complex and inconvenient for routine clinical use.

With regard to subtypes, while the majority of scientists were against the requirement of multi-gene expression array profiling for subtype definition, approximately half the panel of St. Gallen 2013 opted for the use of a clinic-pathologic definition as sufficient for subtype definition. In conclusion, only ER, PR, Ki-67 and Her-2/neu are recommended for clinical use.

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WJCO 5th Anniversary Special Issues (2): Breast Cancer

Toremifene in the treatment of breast cancer

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it has a more positive effect on serum lipids than does tamoxifen. Toremifene is therefore effective and safe in the treatment of breast cancer. It provides not only a useful therapeutic alternative to tamoxifen, but may bring specific benefits.

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Key words: Breast cancer; Toremifene; tamoxifen; Adjuvant treatment; Advanced breast cancer

Core tip: Toremifene is safe and effective in the treatment of breast cancer. Toremifene and tamoxifen are equivalently effective in the treatment of breast cancer, although some studies show an advantage for toremifene. Safety and tolerability is also broadly equivalent, although toremifene may cause fewer uterine neoplasms, serious vascular adverse events and has a more beneficial effect on plasma lipids than does tamoxifen.

Abstract

Although more widespread screening and routine adjuvant therapy has improved the outcome for breast cancer patients in recent years, there remains considerable scope for improving the efficacy, safety and tolerability of adjuvant therapy in the early stage disease and the treatment of advanced disease. Toremifene is a selective estrogen receptor modifier (SERM) that has been widely used for decades in hormone receptor positive breast cancer both in early and late stage disease. Its efficacy has been well established in nine prospective randomized phase III trials compared to tamoxifen involving more than 5500 patients, as well as in several large uncontrolled and non-randomized studies. Although most studies show therapeutic equivalence between the two SERMs, some show an advantage for toremifene. Several meta-analyses have also confirmed that the efficacy of toremifene is at least as good as that of tamoxifen. In terms of safety and tolerability toremifene is broadly similar to tamoxifen although there is some evidence that toremifene is less likely to cause uterine neoplasms, serious vascular events and

Mustonen MVJ, Pyrhönen S, Kellokumpu-Lehtinen PL. Toremifene in the treatment of breast cancer. *World J Clin Oncol* 2014; 5(3): 393-405 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i3/393.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i3.393>

INTRODUCTION

Despite improvements in screening and treatment, breast cancer remains a significant cause of morbidity and mortality; accounting for almost one-third of all cancer diagnoses in the US and is second only to lung cancer as a cause of cancer mortality^[1]. Over a million women are diagnosed each year worldwide and almost half a million deaths due to breast cancer are recorded each year^[2]. Whilst incidence rates are considerably higher in developed than in developing countries, survival rates are low in developing countries, probably due to the lack

of screening and systematic use of adjuvant therapy^[2]. Historically low rates of breast cancer in, for example, the Eastern World have shown a rapid increase in recent years^[3,4]. Although typically a disease of later life, breast cancer among younger, pre-menopausal women appears to be more common than in the Western World^[5,6]. Breast cancer among young women, although comprising only around 7% of diagnosed cases, is associated with later presentation, high grade tumors, hormone receptor negativity and human epidermal growth factor receptor 2 (HER2) overexpression; factors that lead to a poorer overall prognosis^[7].

The place of estrogen receptor modifiers (SERMs) in the treatment of breast cancer is now well established. Survival in breast cancer patients in the developed world has increased considerably over the past several decades, due principally to more widespread screening and the systematic use of adjuvant therapy^[8,9]. Whilst adjuvant therapy with SERMs, aromatase inhibitors, cytotoxic agents, monoclonal antibodies and other agents has transformed the outlook for breast cancer patients, there remains considerable unmet need for improvements in both efficacy and tolerability.

Toremifene

The first SERM to be introduced, tamoxifen, provided a revolutionary new treatment option for patients with hormone receptor positive breast cancer. With more than 30 years of experience now available this treatment modality has shown itself to be effective and physicians have learned how to manage its side effects. The mixed agonist/antagonist properties of SERMs define both their therapeutic effects and their undesirable effects. The different structural properties of different SERMs appears to influence their oncogenicity in both laboratory^[10,11] and clinical studies^[12-19].

The structure of toremifene differs from that of tamoxifen in that a chlorine atom replaces one of the hydrogen atoms in the ethyl side chain. This difference may modify the metabolism of toremifene such that the production of DNA adducts may be prevented or reduced compared with tamoxifen^[20,21].

Almost 25 years have elapsed since the first marketing authorization of toremifene and during that time considerable clinical experience has accumulated indicating its efficacy, safety and good tolerability. Indeed, so good was the safety and tolerability that the initially registered dose (60 mg per day) has been widely increased to 240 mg per day.

Toremifene dosage

As shown in Tables 1 and 2 a variety of doses of toremifene are, or have been, in common use. Although some early dose finding studies failed to distinguish between a wide range of doses from 20 mg to 200 mg^[22], 60 mg was chosen as the most appropriate balance between anti-estrogenic effects and tolerability in Phase II studies; significant side effects being observed at the highest

doses tested (680 mg)^[23]. Dose finding and other studies suggested that 60 mg was safe and effective in breast cancer^[24-26] and more effective than 20 mg^[27,28]. However, extrapolation from animal studies suggested that even higher doses would be well tolerated; phase I studies showed that 460 mg daily for five consecutive days was the highest fully tolerated dose^[23]. A conservative approach led to 240 mg being chosen for the high dose formulation. Subsequent phase III clinical trials appear to bear out the enhanced efficacy of the higher dose formulations of toremifene^[29-31].

Randomized studies with toremifene

There have been ten randomized controlled studies comparing toremifene with tamoxifen^[29-38]. Collectively, these studies, which include a total of more than 5500 patients show rather clearly that toremifene is not less effective than tamoxifen. These studies are summarized in Table 1.

Toremifene in advanced breast cancer

An early double-blind, Japanese study in 114 women with advanced or recurrent breast cancer found toremifene 40 mg and tamoxifen 20 mg resulted in similar response rates (26.3% *vs* 28.1%) and duration of response (155.0 *vs* 154.5 d)^[39]. However, the time to onset of complete response was significantly shorter with toremifene than with tamoxifen (91 *vs* 169 d; $P < 0.05$).

A Nordic study compared toremifene 60 mg with tamoxifen 40 mg in 415 postmenopausal women with advanced breast cancer in a double-blind randomized manner^[32]. Response rates were similar in toremifene and tamoxifen groups (31.5% *vs* 37.3%). Time to treatment failure and median overall survival were also similar.

An open study conducted in Eastern Europe compared toremifene 60 and 240 mg, with tamoxifen 40 mg in 463 postmenopausal women with advanced breast cancer^[31]. Response rates of 20.4% and 20.8% were achieved with toremifene 60 mg and tamoxifen, respectively. Although the response rate with the higher dose of toremifene was slightly higher (28.7%), it did not differ significantly from the other treatments. The findings were similar for time to progression and overall survival, quality of life, as assessed by changes in the Eastern Cooperative Oncology Group (ECOG) scale, was better with toremifene 60 mg than with tamoxifen.

A four-way randomized study was undertaken in 541 women in Russia comparing two doses of toremifene (60 mg and 240 mg) with tamoxifen (20 mg) and letrozole (2.5 mg)^[30]. Objective responses were most frequent in the toremifene 240 mg group (41.5%); objective responses were lower (though not statistically significantly so) in the toremifene 60 mg and letrozole groups (33.0% and 35.4% respectively). The proportion of responses in the tamoxifen group was statistically significantly lower than in the other three treatment groups. Similarly, the median duration of remission was longest in the toremifene 240 mg group (14.5 mo), shortest in the tamoxifen group (9.2 mo) and intermediate in the toremifene 60 mg and

Table 1 Randomized controlled clinical trials

Ref.	Type	Pts	Diagnosis	Receptor status	Follow-up	Treatment	Results
Nomura <i>et al</i> ^[39] , 1993	DB	114 women	Advanced or recurrent breast cancer	NS	NS	RR TOR 40 mg 26.3% TAM 20 mg 28.1%	Time to onset of CR 91 d Duration of efficacy 155 d 169 d ($P < 0.05$) 154.5 d
Hayes <i>et al</i> ^[29] , 1995	OL	648 post- or peri-menopausal women	Metastatic breast cancer	Positive or unknown	NS	Overall RR TOR 60 mg 50% TOR 200 mg 48% TAM 20 mg 44% (ns)	CR + PR 21% PFS 5.6 mo 23% 5.6 mo (ns) 19% (ns)
Gershanovich <i>et al</i> ^[31] , 1997	OL	463 post-menopausal women	Advanced breast cancer	Positive or unknown	Median 20.5 mo	RR TOR 60 mg 20.4% TOR 240 mg 28.7% TAM 40 mg 20.8%	PFS 49 61 50
Pyrhönen <i>et al</i> ^[32] , 1997	DB	415 post-menopausal women	Advanced breast cancer	Negative or unknown	Median 20.5 mo	CR+PR TOR 60 mg 31.3% TAM 40 mg 37.3%	TTF 6.3 mo TIP 7.3 mo 8.5 mo 10.2 mo
Holli <i>et al</i> ^[33] , 2000	OL	899 post-menopausal women	Early invasive breast cancer (adjuvant treatment)	Any	Median 3.4 yr	Time to recurrence TOR 40 mg 21.6 mo TAM 20 mg 23.5 mo	Overall recurrence rate 23.1% 26.1% 20.3% 24.3%
Milla-Santos <i>et al</i> ^[34] , 2001	DB	217 women	Advanced breast cancer	Positive	NS	CR (mo) TOR 60 mg 12.2 TAM 40 mg 8.1	PR (mo) 25.4 24.3 SD (mo) 26.4 19.6 Median TTP (mo) 11.9 12.3 (ns)
Pagani <i>et al</i> ^[35] , 2004	OL	1035 peri- or post-menopausal women	Lymph node positive breast cancer (adjuvant treatment)	ER positive	5.5 yr	5-yr DFS TOR 60 mg 72% TAM 40 mg 69%	5-yr OS 85% 81%
Zeinalov <i>et al</i> ^[30] , 2006	OL	541 post-menopausal women	Disseminated breast cancer	Positive	NS	CR + PR TAM 20 mg 25.6% ($P < 0.05$ compared with three other treatments) TOR 60 mg 33.0% TOR 240 mg 41.5% LTZ 2.5 mg 35.4%	Median duration of remission 9.2 mo 11.3 mo 14.5 mo 13.1 mo
Lewis <i>et al</i> ^[36] , 2010	OL	1813 peri- or post-menopausal women	Stage I or II Early primary invasive breast cancer (adjuvant treatment)	Positive	Median 59 mo	5-Yr DFS TOR 60 mg 91.2% TAM 20 mg 91.2%	Cumulative OS 97.5% 97.3% 90.6%
Kimura <i>et al</i> ^[37] , 2012	OL	253 post-menopausal women	Early phase breast cancer (adjuvant treatment)	Positive or unknown	Median 66.5 mo	5-yr survival TOR 40 mg 97% TAM 20 mg 96.7%	Cumulative DFS 88.4% 90.6%
Yamamoto <i>et al</i> ^[38] , 2013	OL	91 post-menopausal women	Advanced, Non-steroidal aromatase inhibitor resistant in metastatic breast cancer	Positive	Median 16.9 mo	CB TOR 120 mg 41.3% Exemestane 25 mg (ns)	ORR 10.80% 2.2% (ns) PFS 7.3 mo 3.7 mo ($P = 0.045$) OS 32.3 mo 12.9 mo (ns)

DB: Double blind; CR: Complete response; PR: Partial response; SD: Stable disease; LSD: Long stable disease; TOR: Toremifene; TAM: Tamoxifen; DFS: Disease-free survival; OS: Overall survival; (O)RR: (Objective) response rate; CB: Clinical benefit; DFS: Disease-free survival; PFS: Progression-free survival; R: Randomized; OL: Open label; UC: Uncontrolled; RA: Retrospective analysis; CC: Case control; Pr: Prospective; CS: Cohort study; AIU: Aromatase inhibitor; TTP: Time to progression.

Table 2 Non-randomized clinical trials (case reports or series with fewer than 10 patients excluded)

Ref.	Pts	Study type	Diagnosis	Treatment	Results						
Asaishi <i>et al</i> ^[68] , 1993	51 women	Not stated	Advanced breast cancer refractory to TAM	CR + PR TOR 120 mg	SD > 6 mo	Median duration of response 127 d	Median duration of SD > 6 mo 238.5 d				
Gams <i>et al</i> ^[69] , 2002	102 women peri- or post-menopausal women	Pr	Advanced breast cancer refractory to TAM	OR TOR 200 mg	11.8% 5% (TTF 10.9 mo)	15.7%	SD 23% (TTF 7.8 mo)				
Pyrhönen <i>et al</i> ^[70] , 1994	50 Women	Pr	Advanced breast cancer refractory to TAM	RR TOR 240 mg	Mixed response 4%	SD 6%	18% < 5 mo 26% > 5 mo				
Hietanne <i>et al</i> ^[71] , 1997	73 post-menopausal women	Pr	Advanced breast cancer	OR (CR + PR) TOR 240 mg	NC 59%	29%	PD 12%				
Yamamoto <i>et al</i> ^[72] , 2005	10 Women	RA	Metastatic breast cancer	OR TOR 120 mg	CB 30%	70%	Media TTP 9 mo	Median OS 21.5 mo			
Ohtake <i>et al</i> ^[73] , 2009	12 post-menopausal women who had failed AI therapy	RA	Advanced/recurrent breast cancer	CR TOR 120 mg	CB 16.70%	58.30%	Mean TTP 33.8 wk				
Okita <i>et al</i> ^[74] , 2009	15 women	Pr	Metastatic breast cancer	CR TOR 120 mg Paclitaxel 80 mg/m ² on 5 d	PR 0%	6.7%	No change 66.7%,	Stable > 6 mo 26.7%	PD 26.7%	Mean TTF 2.7 mo	
Koyama <i>et al</i> ^[75] , 2011	19 postmenopausal women	RA	Advanced or metastatic breast cancer	OR TOR 120 mg	CB 36.8% (1 CR, 6 PR 6)	47.4%					
Gu <i>et al</i> ^[40] , 2012	810 pre-menopausal women	RA	Early invasive breast cancer (adjuvant treatment)	5-yr OS TOR 60 mg TAM 20 mg	DFS 100% 98.4 (ns)%	97.2% 90.4% (P = 0.022)					
Sawaki <i>et al</i> ^[76] , 2012	13 post-menopausal women	Pr	Adjuvant aromatase inhibitor resistant metastatic breast cancer	CR TOR 120 mg	SD 7.7%	53.8%	PD 38.5%	CB 46.2%	PFS 5.9 mo		
Tokura <i>et al</i> ^[77] , 2012	18 women	Pr	Advanced/recurrent breast cancer	CB TOR 120 mg	PD 58% (5 PR, 5 long SD)	22%	Media PFS 5.5 mo				
Koike <i>et al</i> ^[78] , 2013	21	Pr	Recurrent or metastatic breast cancer	CR TOR 120 mg	PR/SD (12 wk) 0%	21.1%/47.4%					
Ogata <i>et al</i> ^[79] , 2013	23 women	Pr	Recurrent breast cancer who were receiving or had received adjuvant aromatase inhibitor therapy	PR TOR 120 mg	SD 13%	62%	CB 78.30%	Median TTP 8.1 mo			
Qin <i>et al</i> ^[41] , 2013	1847 pre-menopausal women	RA	Operable breast cancer (adjuvant treatment)	DFS TOR 60 mg TAM 20 mg	5-Yr DFS 10.3 yr 10.3 yr	87% 85%	5-Yr OS 94.3% 93.5%				

DB: Double blind; CR: Complete response; PR: Partial response; SD: Stable disease; LSD: Long stable disease; TOR: Toremifene; TAM: Tamoxifen; DFS: Disease-free survival; OS: Overall survival; (O)RR: (Objective) response rate; CB: Clinical benefit; DFS: Disease-free survival; PFS: Progression-free survival; R: Randomized; OL: Open label; UC: Uncontrolled; RA: Retrospective analysis; CC: Case control; Pr: Prospective; CS: Cohort study; AIU: Aromatase inhibitor; TTP: Time to progression.

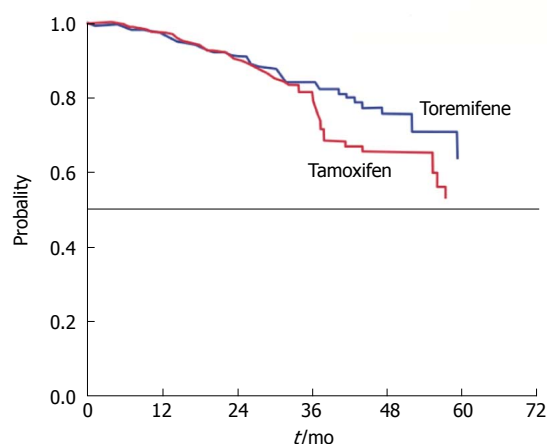


Figure 1 Time from randomization to recurrence in estrogen-receptor positive invasive breast cancer patients receiving adjuvant toremifene or tamoxifen^[33].

letrozole groups (11.3 and 13.1 mo, respectively), the difference between tamoxifen and the other treatments was statistically significant.

Another open study, carried out in the United States, compared toremifene 60 mg and 200 mg with tamoxifen 20 mg in 648 post- or peri-menopausal women with metastatic breast cancer^[30]. As in the previous studies, there were no significant differences between the treatments with regard to response rates (21%, 23% and 19% with toremifene 60 mg, toremifene 200 mg and tamoxifen, respectively), time to progression, response duration or overall survival. There was again a suggestion that the 60 mg dose of toremifene resulted in the greatest improvement in quality of life, although the differences were not statistically significant. Compared with tamoxifen, more patients given toremifene 60 mg reported an improvement in enjoyment of life, pain and mood.

A Spanish double-blind, randomized study in 217 postmenopausal women with advanced breast cancer reported a somewhat higher response rate with toremifene 60 mg than with tamoxifen 40 mg (64% *vs* 52%), although the difference did not achieve statistical significance^[34]. Time to progression and overall survival rates were similar in the two groups.

The most recent randomized study reported in 2013 and compared toremifene 120 mg with the aromatase inhibitor exemestane in 91 post-menopausal women with non-steroidal aromatase inhibitor-resistant breast cancer^[37]. After a median 16.9 mo follow-up there were advantages for toremifene over exemestane in clinical benefit [41.3% *vs* 26.7% respectively (ns)], objective response rate [10.8% *vs* 2.2% respectively (ns)], progression-free survival (7.3 mo *vs* 3.7 mo respectively; $P = 0.045$) and overall survival [32.3 *vs* 21.9 mo respectively (ns)].

Toremifene in early stage breast cancer

The International Breast Cancer Study group combined the results from two studies with almost identical protocols in which a total of 1035 peri- or post-menopausal

women with breast cancer received either toremifene 60 mg or tamoxifen 40 mg^[31]. In common with other similar studies the efficacy of toremifene and tamoxifen were approximately equal; 5-year disease-free survival was 72% and 69% respectively and 5-year overall survival was 85% and 81%. Likewise, in an even larger randomized study in [North American Fareston *vs* Tamoxifen Adjuvant (NAFTA) trial] 1813 peri- or post- menopausal women with invasive breast cancer toremifene 60 mg and tamoxifen 20 performed similarly (5-year disease-free survival 92.2% in both groups)

After a median follow-up of 3.4 years a Finnish study found a number of efficacy parameters showing advantages for toremifene compared with tamoxifen, although none achieved statistical significance. Overall, time to recurrence, recurrence rate, recurrence at distant sites and the number of patients dying during follow-up were similar in toremifene- and tamoxifen- treated patients. Although the Kaplan-Meier analysis of the time to recurrence or the time to progression or disease free survival in this study (Figure 1) appears to show a separation between toremifene and tamoxifen from 3 years onwards, the difference is not statistically significant (hazard ratio toremifene:tamoxifen 0.88 (95%CI: 0.70-1.09). The respective 5-year survival rates were 70.3% *vs* 65.6% (also not statistically significantly different)^[33].

Whilst most randomized controlled studies of toremifene have been performed in patients with advanced or metastatic disease, another small study has been undertaken in an adjuvant setting in women with early stage breast cancer; 91 post-menopausal women with early stage, lymph node negative breast cancer were randomized to adjuvant treatment with toremifene 120 mg or tamoxifen 20 mg^[36]. Five-year survival (97% and 96.7% respectively), cumulative disease-free survival (97.5% and 97.3% respectively) and cumulative disease-free survival (88.5% and 90.6% respectively) were very similar between the two groups after 66.5 mo follow-up.

Non-randomized trials

Whilst there are at least 14 published non-randomized studies of toremifene in advanced breast cancer many include rather few patients or have imperfect methodologies. However, there are two recent retrospective studies that merit further description

The study of Gu *et al*^[40] reviewed the records of 810 women with early invasive breast cancer and identified 240 eligible patients who had received tamoxifen and 212 who had received toremifene. Following median follow-up times of 50.8 mo, although 5-year overall survival rates were similar (100% for toremifene and 98.4% for tamoxifen), recurrence-free survival was significantly longer in the toremifene group than in the tamoxifen (97.2% and 90.4% respectively, $P = 0.022$).

Another retrospective study, this time including 1847 pre-menopausal women who had undergone surgery followed by chemotherapy toremifene or tamoxifen similarly found no significant differences between the

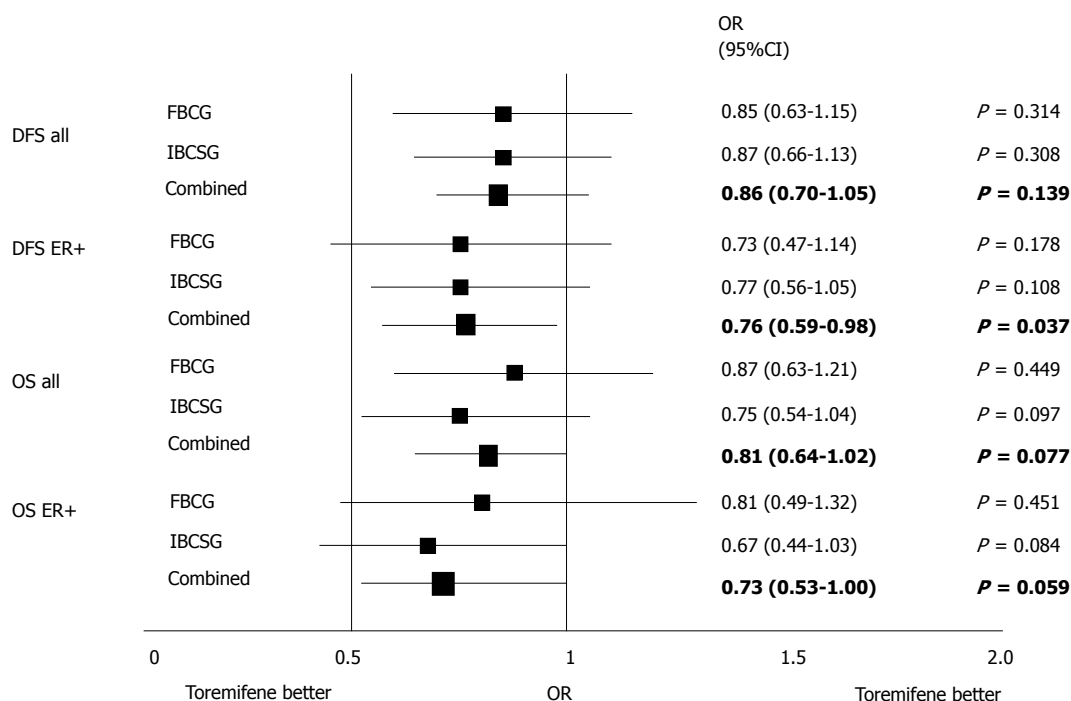


Figure 2 Disease-free survival and overall survival in patients receiving adjuvant toremifene and tamoxifen: meta-analysis of the Finnish Breast Cancer Group^[30] and International Breast Cancer Study Group^[34] (data on file). DFS: Disease-free survival; OS: Overall survival; IBCSG: International Breast Cancer Study Group; FBCG: Finnish Breast Cancer Group.

treatments^[41]. All survival figures were identical between toremifene and tamoxifen; disease-free survival (10.3 years in both groups). Five-year disease-free survival (87% *vs* 85%, respectively) and 5-year overall survival (94.3% *vs* 93.5%, respectively).

META-ANALYSES

Whilst individual clinical trials can provide invaluable data on the efficacy of a medication in restricted populations, meta-analysis can provide information about more generalized patient populations and increase statistical power. The randomized clinical trials described above have been the subject of several meta-analyses. The earliest combined two randomized studies comparing toremifene (200 or 240 mg) and tamoxifen (20 or 40 mg) and included a total of 733 patients with advanced breast cancer^[42]. Response rates were higher in toremifene patients than in tamoxifen patients (25.2% *vs* 19.8%), but not statistically significantly ($P = 0.87$). Disease progression and survival also showed no statistically significant differences between toremifene and tamoxifen.

A subsequent meta-analysis in 1999 included results from the five randomized studies completed to that date^[43]. This meta-analysis represented 1421 postmenopausal patients with previously untreated, locally advanced or metastatic breast cancer that were treated with either toremifene 40-60 mg ($n = 725$) or tamoxifen 20-40 mg ($n = 696$). As in the previous meta-analysis toremifene and tamoxifen proved to be broadly equivalent in terms of response rate (24.0% *vs* 25.3%, respectively),

time to treatment failure (4.9 *vs* 5.3 mo) and survival (31.0 *vs* 33.1 mo).

Another meta-analysis has been performed on the disease-free and overall survival findings from two pivotal randomized studies comparing toremifene and tamoxifen in an early breast cancer^[31,33] (data on file, Orion). As shown in Figure 2, there are indications that toremifene may be superior to tamoxifen, most notably in estrogen-receptor-positive patients. In this subgroup of patients, the combined data showed a significant ($P = 0.037$) benefit for toremifene with regard to disease-free survival, although the difference with regard to overall survival did not achieve statistical significance ($P = 0.059$).

A more recent meta-analysis using somewhat more restrictive criteria for inclusion analyzed three eligible randomized comparisons with tamoxifen^[44]. However, the overall results were similar in that no significant differences in efficacy between toremifene and tamoxifen were identified with risk ratios close to unity for overall survival and disease-free survival.

A recent meta-analysis identified 23 randomized clinical trials comparing toremifene with tamoxifen involving a total of 7242 patients with breast cancer^[45]. This large study found that although for most efficacy parameters there were no significant differences between toremifene and tamoxifen, toremifene was significantly more effective in terms of 5-year survival [odds ratio (OR) 1.25; 95%CI: 1.04-1.50] among patients with early stage breast cancer.

A Cochrane review on toremifene *vs* tamoxifen for advanced breast cancer compared randomized controlled comparisons providing data on objective response rate,

Table 3 Incidence of adverse events among 1847 women with invasive breast cancer treated with tamoxifen or toremifene^[41]

Adverse event	Adverse event incidence (%)		P value
	Tamoxifen (n = 1451)	Toremifene (n = 396)	
Flushing	480 (33.1)	39 (35.1)	0.450
Sweating	295 (20.3)	82 (20.7)	0.869
Nausea or vomiting	213 (14.7)	57 (14.4)	0.881
Fatigue	74 (5.1)	18 (4.5)	0.653
Insomnia	62 (4.3)	14 (3.5)	0.513
Dizziness	14 (1.0)	6 (1.5)	0.408
Dry eyes	60 (4.1)	17 (4.3)	1
Blurred vision	40 (2.8)	9 (2.3)	0.595
Cataracts	7 (0.5)	2 (0.5)	1
Weight gain	68 (4.7)	17 (4.3)	0.740
Vaginal discharge	241 (16.6)	69 (17.4)	0.701
Irregular menses	145 (10)	25 (6.3)	0.025
Endometrial cancer	1 (0.1)	0 (0)	0.601
Ovarian cyst	20 (1.4)	4 (1.0)	0.631
Thromboembolic events	22 (1.5)	5 (1.3)	0.709
Hypertriglyceridemia	76 (5.2)	19 (4.8)	0.725
Hyper-LDL cholesterolemia	65 (4.5)	16 (4.0)	0.783
Fatty liver	64 (4.4)	13 (3.3)	0.320
Elevated ast	59 (4.1)	15 (3.8)	0.802
Elevated alp	33 (2.3)	7 (1.8)	0.571
Hepatic cyst	29 (2.0)	6 (1.5)	0.550
Bilirubin	27 (1.9)	8 (2.0)	1

time to progression and overall survival^[46].

The review identified 2061 patients from seven studies (1226 patients received toremifene and 835 patients received tamoxifen). The pooled risk ratio for the objective risk ratio was 1.02 suggesting that there was no statistically significant difference between toremifene and tamoxifen [objective risk ratios (ORR) were 25.8% *vs* 26.9%, respectively]. Similarly the hazard ratio for time to progression was 1.08, again, implying no statistically significant difference between toremifene and tamoxifen (time to progression 6.1 mo and 5.8 mo respectively). Overall survival also showed equivalence between the two medications [hazard ratio (HR) 1.02; overall survival 27.8 mo and 27.6 mo, respectively]. The authors conclude that toremifene and tamoxifen are equally effective in the first-line treatment of patients with advanced breast cancer.

The results of the randomized clinical trials are remarkably consistent and supported by both meta-analyses and retrospective studies; toremifene is at least as effective as tamoxifen in the treatment of breast cancer. Rather few studies find significant differences between the two SERMS, those that do differentiate between the two treatments find significant differences in favor of toremifene^[31,37,38]. None find significant advantages for tamoxifen.

Safety and tolerability

Toremifene and tamoxifen have similar adverse event profiles and are well tolerated, both in women with advanced breast cancer and in those receiving adjuvant therapy. Hot flushes, sweating, nausea and vaginal dis-

Table 4 Frequency of subjective adverse events among 499 patients with invasive breast cancer randomised to adjuvant toremifene or tamoxifen therapy^[33]

	Toremifene		Tamoxifen	
	Number of patients	%	Number of patients	%
Sweating	247	53.8	225	51.1
Hot flashes	237	51.6	209	47.5
Vaginal discharge	193	42.0	156	35.5
Vaginal dryness	120	26.1	117	26.6
Itching	118	25.7	119	27.0
Depression	112	24.4	119	27.0
Rash	90	19.6	75	17.0
Nausea	78	17.0	85	19.3
Vaginal bleeding	40	8.7	37	8.4
Diarrhea	37	8.1	51	11.6
Weight increase	23	5.0	19	4.3

charge are among the most common adverse effects and serious adverse events are rare.

A large retrospective analysis of 1847 breast cancer patients treated with toremifene or tamoxifen showed the expected pattern of adverse events with sweating and nausea/vomiting as the most common undesirable effects (Table 3). Although the great majority of adverse effects occurred at similar rates in toremifene- and tamoxifen-treated patients, irregular menses were significantly more common in the tamoxifen group (10% *vs* 6.3%, $P = 0.025$)^[40].

In the largest randomized study comparing tamoxifen and toremifene there were few differences in thromboembolic, gynecological and ocular adverse events between the two treatment groups. Fever and chills were significantly more common among tamoxifen-treated patients^[35].

A safety analysis in the Finnish Breast Cancer Study Group data also illustrates the similar tolerability and safety profiles of toremifene; sweating and hot flashes being observed in more than half of the patients, followed by vaginal dryness and discharge with itching and depression observed in more than 20% of patients^[32]. (Table 4) In no case were significant differences between toremifene and tamoxifen observed. There was similarly little difference in the incidence or pattern of serious adverse events between toremifene and tamoxifen (Table 5).

The above described studies were conducted with the low dose toremifene (60 mg), however high dose toremifene 200 or 240 mg is not associated with a significantly increased incidence, or different profile, of adverse events compared with the 60 mg dose^[30,31]. For example, in a randomized study that involved 648 women with metastatic breast cancer, with the exception of a slightly greater incidence of nausea in the high dose group, the incidence of the most common side effects was similar with toremifene 60 mg and 200 mg^[30]. Similarly, there were no significant differences between the incidence of side effects with toremifene 60 mg and 240 mg in a randomized study of 463 women with advanced breast can-

Table 5 Frequency of serious adverse events among 499 patients with invasive breast cancer randomised to adjuvant toremifene or tamoxifen therapy^[33]

	Toremifene		Tamoxifen	
	Number of patients	%	Number of patients	%
Serious adverse events	72	15.7	74	16.8
Cardiac events	9	2.0	6	1.4
Myocardial infarctions	7	1.5	5	1.1
Angina pectoris	2	0.4	1	0.2
Thromboembolic events	16	3.5	26	5.9
Pulmonary embolisms	3	0.7	3	0.7
Deep vein thrombosis	8	1.7	11	2.5
Cerebrovascular events	5	1.1	12	2.7
Endometrial events	17	3.7	19	4.3
Polyps	8	1.7	7	1.6
Hemorrhage	2	0.4	3	0.7
Disorders	7	1.5	9	2.0
Subsequent cancers	12	2.6	8	1.8
Breast	3	0.7	1	0.2
Uterine	-	-	2	0.5
Gastrointestinal	3	0.7	1	0.2
Other	6	1.3	4	0.2
Cataracts	3	0.7	8	1.8
Increased liver enzyme levels	2	0.4	2	0.5
Bone fractures	13	2.8	5	1.1
Osteoporotic	2	0.4	3	0.7

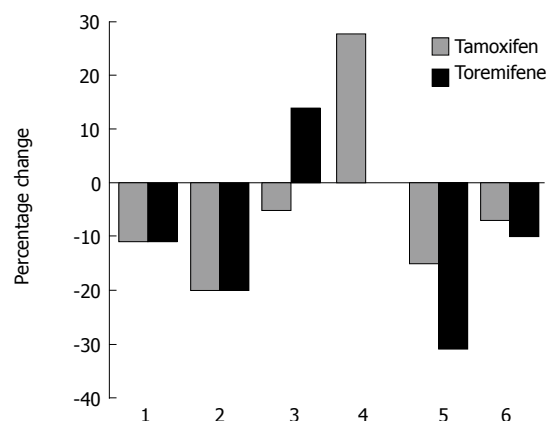
cer^[31]. In a pooled-analysis of two studies involving 733 women with advanced breast cancer^[41], high-dose toremifene 200 or 240 mg was tolerated as well as tamoxifen 20 or 40 mg.

In a meta-analysis of five studies significantly more tamoxifen- than toremifene-treated patients discontinued treatment prematurely (19.6% *vs* 13.7%; $P = 0.007$), predominantly due to greater non-compliance and protocol violations in the tamoxifen group^[42]. Not every study shows identical tolerability; in the Nordic study^[32], the percentage of patients discontinuing treatment prematurely was significantly lower with toremifene than with tamoxifen (14% *vs* 20% respectively; $P = 0.011$), mainly due to fewer adverse events/patients' refusals, loss to follow-up and deaths.

The recent meta-analysis of Chi *et al*^[45] however, showed that compared with tamoxifen, toremifene was associated with more vaginal discharge in patients with early stage breast cancer and more vaginal bleeding in patients with advanced disease, although the both drugs had a similar overall effect on quality of life.

Lipids

As the long-term prognosis for breast cancer patients improve, increasing attention has been focused on continuing quality of life and morbidity from other causes. This is particularly important when SERMs are used in an adjuvant setting in early-stage breast cancer where the probability of long-term survival is high. In this context, an attractive property of SERMs is their ability to improve cardiovascular risk factors. Toremifene reduces

**Figure 3** Percentage change in lipid parameters after one year with toremifene and tamoxifen^[47]. 1: Total cholesterol; 2: low-density lipoprotein (LDL) cholesterol; 3: high-density lipoprotein (HDL) cholesterol; 4: Triglycerides; 5: LDL:HDL; 6: Apo lipoprotein-B.

both total and low-density lipoprotein (LDL) cholesterol and increases high-density lipoprotein (HDL) cholesterol (Figure 3)^[47-50]. Particularly persuasive are the results from a crossover trial in which 197 women receiving adjuvant therapy with toremifene or tamoxifen were monitored for lipid levels^[51]. After one year of treatment the total cholesterol had decreased in both groups, but HDL-cholesterol increased only in the toremifene group ($P < 0.001$); indeed, HDL cholesterol significantly decreased in the tamoxifen-treated patients ($P < 0.001$). After one year of therapy patients who still had abnormal lipid levels were switched to the other medication. In patients switched from tamoxifen to toremifene total- and HDL-cholesterol increased and triglycerides decreased to pre-treatment levels whilst in the patients switched from toremifene to tamoxifen total cholesterol decreased and triglycerides increased. The authors conclude that the lipid profile changes associated with toremifene are better than those associated with tamoxifen^[50]. This finding was supported by the results of a recent meta-analysis of 23 clinical trials in which toremifene and tamoxifen were compared^[44]. In an early stage breast cancer patients' triglyceride levels were reduced more and HDL-cholesterol levels increased more by toremifene than by tamoxifen, although tamoxifen was more effective in reducing LDL-cholesterol. In patients with advanced disease toremifene also reduced triglyceride levels more than tamoxifen. Similar beneficial changes have also been reported from an extended randomized controlled investigation of the effects of toremifene *vs* the aromatase inhibitor anastrozole on lipid profile^[52].

The evidence seems rather clear that the effect of toremifene on patients' lipid profile is generally positive and better than that of the comparator treatments so far investigated.

Bone mineral density in breast cancer patients

Toremifene improves bone mineral density (BMD) and helps prevent osteoporosis in postmenopausal breast cancer patients. These effects are similar to those of

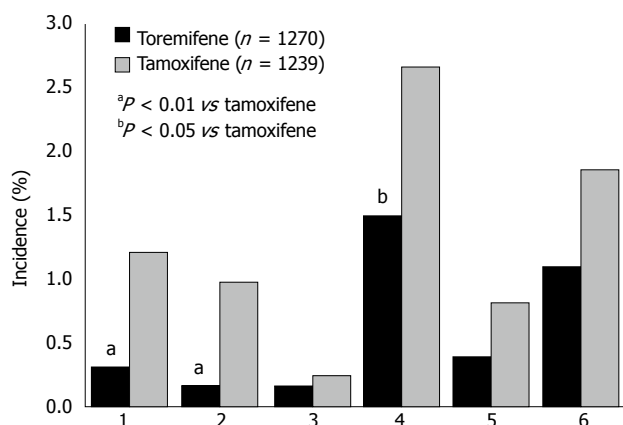


Figure 4 Incidence of serious vascular events in patients randomized to toremifene or tamoxifen adjuvant therapy in post-menopausal women^[17]. 1: Total cerebrovascular events; 2: Stroke; 3: Transient ischaemic attack; 4: Total thromboembolic events; 5: Pulmonary embolism; 6: Deep vein thrombosis.

tamoxifen. Comparative studies have shown that both toremifene and tamoxifen prevent reductions in BMD in the lumbar spine and proximal femur, and that these effects are reflected by changes in a wide range of bone biochemistry markers such as pyridinoline, deoxypyridinoline and urinary cross-linked aminoterminal telopeptide of type I collagen^[53-55]. Toremifene and tamoxifen have also been used successfully in combination with the bisphosphonate clodronate, with no significant differences between them^[56,57].

Some beneficial effects on BMD have been observed in premenopausal women at high risk for developing breast cancer taking toremifene 60 mg as chemoprevention, therefore making an attractive alternative to tamoxifen. A double-blind, placebo-controlled pilot study in 259 healthy premenopausal and postmenopausal women at high risk for breast cancer found a trend for a sustained increase in lumbar spine BMD after one year of toremifene therapy in premenopausal women^[58].

LONG TERM SAFETY

The long-term safety profile of toremifene was evaluated in detail in a review of all preclinical and clinical safety data from 1978 to 2004 and comparative clinical safety data between October 1995 and the end of 2004^[59]. At the time of this review, information was available from more than 350000 patient treatment years. The evidence indicated that toremifene has good long-term safety, with a lower incidence of endometrial cancer, stroke, pulmonary embolism, deep vein thrombosis and cataracts than tamoxifen.

A 3-year study specifically designed to compare the gynecological effects of toremifene 40 mg and tamoxifen 20 mg in 167 postmenopausal breast cancer patients showed that the incidence of proliferative endometrium was increased to a significantly ($P < 0.0001$) lesser extent by toremifene (from 20.0% to 32.2%) than by tamoxifen (from 20.4% to 46.8%)^[60].

Endometrial cancer

The finding that tamoxifen at high doses caused liver tumors in rats^[61] raised concern that it may be mutagenic in humans. The mechanism of this effect in laboratory animals was believed to be due to DNA adduct formation by metabolites of tamoxifen, although this has more recently been questioned^[62]. Nevertheless, endometrial cancer rates are increased in women taking tamoxifen^[13]. The chlorine substitution in the structure of toremifene alters its metabolism such that DNA adducts are much less likely to form^[13,63-66] and a case-control study based on records of 38000 Finnish breast cancer patients appears to suggest that toremifene is considerably less frequently associated with endometrial cancer than is tamoxifen [OR 2.9; 95%CI: 0.3-3.9 *vs* 0.9; 95%CI: 0.3-3.9]^[17]. However, a recent meta-analysis of studies involving a total of 7242 patients with early or advanced breast cancer found no difference in the number of endometrial cancers between patients treated with toremifene or tamoxifen, although the follow-up was relatively short in the majority of studies. There is clearly still much to be discovered concerning the oncogenicity of SERMs, but both laboratory and clinical evidence suggests an advantage for toremifene over tamoxifen in this regard.

Thromboembolic effects

A retrospective analysis of the serious vascular events reported in the manufacturer's Drug Safety Database^[67] revealed that cerebrovascular and thromboembolic events overall were significantly higher in tamoxifen than in toremifene-treated patients (Figure 4).

Other evidence suggests that toremifene may be associated with a lower risk of such thromboembolic events^[59]. A retrospective analysis of adjuvant treatment trials with toremifene 40 or 60 mg and tamoxifen 20 mg in more than 2500 postmenopausal women revealed a significantly lower incidence of ischemic stroke, total cerebrovascular events and total thromboembolic events with toremifene compared with tamoxifen.

DISCUSSION

For obvious ethical reasons the great majority of randomized clinical trials of toremifene have been undertaken with tamoxifen as the comparator, rather than placebo. The results of these studies, and the several meta-analyses that are based upon them, appear to characterize toremifene as being at least as effective as tamoxifen in the treatment of breast cancer both in the adjuvant setting and in patients with advanced and metastatic disease. Of the ten randomized controlled trials described in Table 1 all found toremifene to be at least as effective as tamoxifen. In some studies and for some parameters there was a statistically significant advantage for toremifene over tamoxifen; a shorter time to onset of complete response in Nomura *et al*^[39] 1993, a higher rate of objective response in Zejnalov *et al*^[30] 2006 and a longer progression-free survival in Yamamoto *et al*^[38] 2013. There

were no statistically significant efficacy advantages for tamoxifen in these studies. However, whilst it is tempting to claim at least a trend for better efficacy for toremifene, some statistically significant differences are likely to arise by chance when a large number of parameters are compared in several studies (68 individual parameters are represented in Table 1 far more were examined in the studies cited). So far as the efficacy of toremifene is concerned, the conclusion is that it is at least as effective as tamoxifen is reasonable seems conservative and reasonable. In addition, toremifene has been the subject of a number of meta-analyses using different criteria for inclusion of studies and all have come to the same conclusion that the efficacy of toremifene and tamoxifen are not statistically significantly different.

Modern hormonal treatment for breast cancer emphasizes continuing therapy with an anti-estrogen, or an anti-estrogen followed by a switch to an aromatase inhibitor after longer or shorter periods. Whilst toremifene appears to behave similarly to tamoxifen, there is a relative dearth of information on its use in these switch or extended adjuvant contexts.

So far as safety and tolerability are concerned, the simple substitution of a chlorine atom for a hydrogen atom appears to make a considerable difference. The altered pattern of metabolite formation with its strongly reduced DNA adduct formation is reflected in a lower incidence of endometrial cancer—a recent meta-analysis of studies involving a total of 7242 patients with early or advanced breast cancer found no difference in the number of endometrial cancers between patients treated with toremifene or tamoxifen, although the follow-up was relatively short in the majority of studies^[44]. On the other hand, the pattern of serum lipids is more favorably affected by toremifene with lower triglycerides, and an improved HDL/total cholesterol ratio. Thromboembolic events also show benefits in favor of toremifene. Overall, toremifene is well tolerated and the pattern of adverse events reported in clinical trials is rather similar between the two SERMs.

Taken together, the findings of clinical trials, meta-analyses and studies on specific aspects of the pharmacology of toremifene suggest that it is an effective and well tolerated agent for the treatment of early and advanced breast cancer. In comparison with tamoxifen, toremifene is at least as effective in all therapeutic contexts so far investigated and may have tolerability and safety advantages.

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WJCO 5th Anniversary Special Issues (2): Breast cancer

Exercise in patients coping with breast cancer: An overview

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tary treatment for achieving physiological and psychological improvements. Drawing clinicians' attention to this issue is important for improving patients' quality of life. We advise a multidisciplinary approach to encourage breast cancer patients into engaging in rehabilitation programs combining both strengthening and aerobic exercises for the most beneficial results.

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Abstract

Breast cancer is the most common type of cancer in women, but fortunately has high survival rates. Many studies have been performed to investigate the effects of exercise in patients diagnosed with breast cancer. There is evidence that exercise after the diagnosis of breast cancer improves mortality, morbidity, health related quality of life, fatigue, physical functioning, muscle strength, and emotional wellbeing. Based on scientific data, breast cancer patients should be recommended to participate in rehabilitation programs including aerobic and strength training. The aim of this article is to review the recently published data on the effect of exercise in patients with breast cancer in order to present the current perspective on the topic.

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Key words: Breast cancer; Exercise; Rehabilitation; Physical activity; Cancer

Core tip: Exercise is not merely safe and feasible for breast cancer patients, but is moreover a complemen-

INTRODUCTION

Breast cancer is the most frequent cancer in females, with an estimated more than 2.8 million breast cancer survivors in the United States alone^[1,2]. For many years, breast cancer has been incredibly difficult for both patients and health care providers due to its high mortality and morbidity rates^[3]. However, due to recent advances in the diagnosis and treatment of breast cancer, survival rates have increased^[3]. Overall, the 5-year survival rate for breast cancer in all stages has been reported as 89% by the American Cancer Society^[2]. Despite this successful increase in the rate of survival, there are still many problems arising from either the disease itself or relating to its treatment in patients living with the disease.

Due to the high prevalence of breast cancer, especially in developed countries, the increased rates of survival and high expectations in the quality of life for women with breast cancer ultimately led patients and health care providers to seek alternative or additional approaches in the management of the disease^[4]. All of these factors created a greater interest in the physical activity of cancer patients over time. Thus, there has been much research on the effect of exercise on breast cancer patients and survivors within the medical community in the last few

decades^[5].

The effect of exercise might be evaluated in some items according to the disease stage, which may all be considered different major topics of interest. Among them, the preventive effects of exercise for breast cancer and effectiveness of exercise in breast cancer patients are the most covered areas in the current literature. The preventive effect of exercise for breast cancer has been shown in epidemiological studies^[6,7]. Risk reduction with physical activity for breast cancer in females is estimated to be up to 25%-30%^[1,6]. To achieve such an effect, women are recommended to follow a 150-min per week exercise regime of moderate to vigorous intensity consisting of sports or other physical activity^[4].

Fortunately, in many cases, cancer patients are no longer considered isolated people in many aspects of life. The traditional approach of clinicians advising rest and the avoiding of physical activity to breast cancer patients and survivors has changed over time, and both patients and the medical community are seeking the ideal level, type, and intensity of physical activity^[8]. This brings us to the other aspect researched: the effect of exercise in breast cancer patients.

Although the additional effect of exercise in breast cancer has been investigated in numerous studies throughout the last few decades, due to the wide variety in the status of patients in such areas as disease stage, associating co-morbid conditions, and physical function, to date there has been no clear consensus or standard approach that has been agreed upon in terms of exercise in breast cancer patients^[5,8], despite recent systematic reviews and meta-analyses published on the effect of exercise interventions^[5,9,10]. Considering the quickly accumulating amount of literature on the subject, we felt the need to overview the effects of exercise specifically for breast cancer and discuss the need for research for future trials. Therefore, the aim of this article is to review the recently published data on the effect of exercise in patients with breast cancer in order to present the current perspective on the topic.

POSSIBLE MECHANISMS OF EXERCISE

A variety of robust studies have clearly showed the beneficial effects of exercise in a healthy population, with a varying spectrum of positive changes in physiological and psychological effects^[11]. Hence, it is important to determine how these positive effects of exercise impact breast cancer patients.

The effect of treatment of cancer on immune functions and the positive effects of good immune function on survival and morbidity in cancer patients have been linked^[12]. Scientific data has suggested cancer and its treatment are related with a disruption of immune functions^[12,13]. Pro-inflammatory cytokines are found in higher levels in advanced stage, metastatic, and recurrent disease compared with non-metastatic, non-recurrent, and early stage disease^[14]. Although researchers have investigated

the relation between inflammatory markers and exercise in cancer patients, there is no significant data showing the effects of exercise on immune system markers in cancer patients^[13,15]. Recently, a few studies have showed some positive results in cytokine and insulin levels with Tai Chi exercises in breast cancer patients^[16,17].

Angiogenesis and apoptosis are related to the progression and metastasis of tumors, with vascular endothelial growth factor being the most studied angiogenic molecule^[18]. Ergun *et al*^[19] have showed the positive effects on levels of angiogenic molecules with supervised and home exercises in breast cancer patients compared to an education-only-group. Unfortunately, many studies investigating the relationship between immune functions and exercise in breast cancer patients are limited (*e.g.*, due to a small sample size), and so should be investigated further in future randomized clinical trials (RCT) with large populations.

Being overweight/obese is clearly associated with an increased risk of developing many cancer types, including breast cancer in postmenopausal women^[4,20]. The mechanism underlying obesity and breast cancer is considered to be immune function, inflammation, and levels of estrogen and IGF-1^[21,22]. However, there are studies exploring the effects of reducing breast cancer risk with intentional weight loss^[23,24]. The American Cancer Association recommends a healthy diet and advises against gaining weight due to its negative effects on treatment success and recurrence^[25]. The established ways for losing weight are to reduce dietary intake and/or increase physical activity. Besides obtaining a healthy diet, regular exercise in breast cancer patients would be helpful in maintaining their ideal weight.

Hahn *et al*^[26] showed that disruption of the circadian rhythm, such as by performing night shift work, might have an association with the progression of breast cancer. Exercise also acts as a regulator on the circadian rhythm and sleep; therefore regular exercise might hypothetically have an indirect effect on preventing the progression of breast cancer. However, this theory needs be proven with clinical studies in breast cancer patients.

One descriptive study showed that the prevalence of fibromyalgia might be high in hospitalized breast cancer patients^[27]. Fibromyalgia symptoms additional to typical cancer symptoms, such as fatigue, might inversely influence the patients' quality of life. Thus, taking fibromyalgia into account when prescribing exercise to patients diagnosed with breast cancer should be considered.

EFFECTS OF EXERCISE IN BREAST CANCER

There is a growing interest among cancer patients in searching for alternative options in order to achieve a better life quality. If the effect of practicing exercise in breast cancer patients on mortality as well as morbidity were well understood, that would create an important clinical impact in the future.

Prospective observational studies have demonstrated that physical activity after cancer diagnosis is associated with a reduced risk of cancer recurrence and improved overall mortality among multiple cancer survivor groups, including breast, colorectal, prostate, and ovarian cancer^[25,28-30]. Several studies in breast cancer survivors have demonstrated that being physically active after the diagnosis of breast cancer led to a 24%-67% reduction in the risk of total deaths and a 50%-53% reduction in the risk of breast cancer deaths when compared to a sedentary lifestyle^[31-33]. Some studies have also shown that physical activity is inversely related with co-morbidities in patients diagnosed with breast cancer^[34].

Fatigue is an important symptom that occurs frequently in breast cancer patients and has a negative impact on the quality of life^[35]. Studies have reported a prevalence of fatigue in cancer patients of up to 96%^[36]. A Cochrane review published in 2006 concluded that aerobic and resistive exercise in breast cancer patients on adjuvant therapy had significant positive effects on cardiorespiratory fitness and non-significant effects on fatigue and weight gain^[37]. A meta-analysis published by McNeely *et al.*^[5] also showed the significant positive effect of exercise on symptoms of fatigue in breast cancer patients. An updated Cochrane review in 2012 showed the benefits of aerobic exercise in combating fatigue in breast cancer patients during or adjuvant to chemotherapy treatment^[38].

The literature suggests that exercise in breast cancer survivors or in patients receiving therapy improves cardiorespiratory fitness, physical function, and muscular strength^[5,37-39]. However, there is a need for long-term studies for a better interpretation of these results.

Studies show that exercise and physical activity improve depression and anxiety in breast cancer patients receiving adjuvant therapy^[38-40]. Carayol *et al.*^[40] suggested that, according to their meta-analysis, relatively low doses consisting of 90-120 min of weekly moderate physical exercise is efficacious for such improvements in patients receiving adjuvant therapy.

A meta-analysis performed for trials on the effect of exercise in breast cancer patients showed a significant pooled effect of exercise on patients' quality of life^[5]. Confirming this data, recent Cochrane reviews evaluating the effects of exercise on health-related quality of life in cancer patients and survivors in randomized and controlled trials have suggested that cancer patients may benefit from exercise in some domains, including physical function, role function, social function, and fatigue^[38,39].

INDICATIONS AND CONTRAINDICATIONS

Indications for exercise treatment in this patient population include regaining or improving physical functions, aerobic capacity, strength, flexibility, body image, body composition, quality of life, the ability to physically and psychologically withstand to any current and/or future

cancer treatments, and to withstand anxiety due to living with current or recurrent disease^[41]. Indications also include the reduction of long-term and late effects of cancer treatment, and the potential delay in any recurrence or progress of the disease^[41].

Contraindications for exercise prescription in breast cancer patients include, but are not limited to: acute post-operative period (up to 8 wk); acute arm and shoulder problems for upper body exercises; patients with extreme fatigue, anemia, or ataxia; and general cardiovascular and respiratory contraindications for an exercise regimen^[41].

Traditionally, upper extremity exercises were avoided in breast cancer patients with lymph node dissection and radiotherapy. However, some recent studies have shown that upper body exercises do not have a negative impact on lymphedema^[42].

EXERCISE TYPE AND CONTENT

There is no standard approach to exercise regimens for breast cancer patients. This is mainly due to the wide spectrum of such patients in terms of age, stage of the disease, co-morbid situation, physical function, etc. Considering this variety, it seems impossible to ever have a standard approach for each individual patient. In addition, the prior functional status and exercise habits of each patient should always be taken into account when an exercise program is being prescribed.

There are various studies concerning the effects of different types of exercise on breast cancer patients. Types of exercises studied in breast cancer patients range from regular aerobic exercise to such activities as Tai Chi^[16,17,42]. The most frequently studied in these patient populations are aerobic exercises (*i.e.*, group, home, walking, and cycling), resistive exercises, and special types of exercise like Pilates, Tai Chi, and Yoga^[17,42-44]. Exercise regimens can be prescribed as either group exercises instructed by a trainer or self-practiced home exercises.

Resistance exercises in breast cancer patients are gaining more attention due to their ability to decrease muscle waste and fatigue^[45]. A combined Aerobic and Resistance Exercises (CARE) trial has investigated standard dose aerobic (25-30 min per session of 3 d per week), high dose aerobic (50-60 min per session of 3 d per week), and combined aerobic and resistive exercise (standard aerobic in addition to standard resistance training; 3 d per week, 2 set of 10-12 repetitions) schemes in breast cancer patients. The results showed that higher intensity and resistance exercises are safe in this population, and that high and combined resistive-aerobic exercise regimens are superior to standard aerobic ones in terms of certain domains such as muscle strength, endocrine symptoms, and quality of life aspects like bodily pain^[45].

Pilates exercises improve physical strength, flexibility, and postural control, and it is commonly-accepted by women as a fitness activity in developed countries^[43]. In a randomized clinical trial, it was shown that Pilates exercises have significant effects in females with breast cancer

in terms of functional capacity, fatigue, flexibility, and quality of life compared to the control group.

Tai Chi exercises are respected as exercises of mindfulness, and are known to improve physical and psychological well-being. Some studies demonstrated that Tai Chi exercises for 10-12 wk improved fatigue, body composition, and quality of life, as well as muscular, memory, and cognitive functions, in breast cancer survivors^[16,17].

Yoga is another type of meditative physical activity including breathing, posture, flexibility, and core strength exercises that can be used in breast cancer patients. In 2013, Sudarshan *et al*^[46] published a study on breast cancer patients which reported that weekly Yoga therapy improved physical function.

According to the data pooled from cancer studies, the American Cancer Society recommends that cancer survivors engage in regular physical activity, avoid inactivity, and exercise for at least 150 min per week, including strength-training for at least 2 d per week, to obtain a healthy weight^[25].

Kirkham *et al*^[47] compared different intensity arrangement methods for the precise prescription of aerobic exercise regimens. The American College of Sports Medicine's metabolic equation for treadmill walking and heart rate reserve are found to be the most accurate methods for exercise intensity prescription in breast cancer patients and survivors^[47].

Enjoyable exercises like Pilates, Tai Chi, Yoga, Nordic walking, and dance may be chosen according to the expectations and motivations of patients by carefully adjusting intensity on an individual-by-individual basis.

ADVERSE EVENTS

In most clinical trials that evaluate different types of exercises, adverse events are not even reported^[38,39]. In the clinical studies that have reported safety associated with exercise, most of them either reported no adverse events at all or only those that are very rare and usually non-serious. The reported exercise-related adverse events are mainly dizziness, dyspnea, musculoskeletal injuries, or lymphedema, which were similar to the control group in terms of numbers^[38-39]. In general, no differences whatsoever were observed regarding recurrence, disease progression, or increased mortality in exercise *vs* control groups. Although limited, these studies show that appropriate exercise regimens in breast cancer patients are quite safe to implement. However, the adverse events of exercise regimens in this patient group should be further studied and reported more thoroughly in future trials.

LIMITATIONS AND RECOMMENDATIONS

Although many studies have investigated the effects of exercise in breast cancer patients, there is still a need for randomized controlled trials for the clarification of exercise type, duration, and intensity for program standardization.

Most reported trials are conducted in small groups

and for short-term periods. Therefore, the results of these trials are difficult to implement for a long-term period and large populations. While advantageous that most cancer trials on the impact of exercise are researched in breast cancer survivors, it is unfortunate that very few of these are powered randomized controlled trials with large enough sample sizes^[9,10].

Considering the heterogeneity of breast cancer patients, individualized programs according to disease stage, treatment status, and co-morbid situation should be tailored and addressed in future research questions. Along with RCTs, long-term real life data from registries and databases on the outcome of exercises in female breast cancer patients might help in directing these questions.

Additionally, compounding factors that would have an effect on exercise and its outcome, such as the patients' previous exercise habits, co-morbid situation (*i.e.*, lymphedema), and functional status need to be evaluated.

Lack of knowledge on the effects of exercise and physical activity among the medical community and fear in creating an exercise regimen with regards to the seriousness of the disease is a social burden that results in unnecessary avoidance from physical activity in patients diagnosed with breast cancer. Therefore, in order for this substantial issue to be settled, the mindset of physicians should be changed, which should happen naturally over time as the evidence-based data accumulates.

Raising awareness and improving information in this area for patients, caregivers, and healthcare providers would have a great impact in approaching these patients' exercise needs. This emphasizes the importance of a multidisciplinary approach in overcoming the barriers in this area and helps patients get the best outcome from treatment. Little to no data is available on the cost-effectiveness of exercise treatment for breast cancer patients, which in the long-term needs to be evaluated.

Despite the crucial need for physical activity and exercise in this patient population, patients' demand for physical treatment greatly varies from 2%-81%^[3,48]. Patient awareness about physical activity after diagnosis can be a vital factor in determining this variation.

CONCLUSION

Exercise is not merely safe and feasible for breast cancer patients, but is moreover a complementary treatment for one to achieve physiological and psychological improvements. There is increasing evidence that regular exercise after the diagnosis of breast cancer might have a substantial positive impact in mortality, morbidity, prognosis, and quality of life. We advise a multidisciplinary approach in order to encourage breast cancer survivors into engaging in rehabilitation programs combining both strengthening and aerobic exercises for the most beneficial results.

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WJCO 5th Anniversary Special Issues (2): Breast cancer

Biological subtypes of breast cancer: Prognostic and therapeutic implications

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Core tip: Breast cancer is a heterogeneous disease with many subtypes that have different treatment responses and clinical outcomes. The present review summarizes current knowledge in breast cancer molecular biology, focusing on novel classification, prognostic and predictive factors.

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Abstract

Breast cancer is a heterogeneous complex of diseases, a spectrum of many subtypes with distinct biological features that lead to differences in response patterns to various treatment modalities and clinical outcomes. Traditional classification systems regarding biological characteristics may have limitations for patient-tailored treatment strategies. Tumors with similar clinical and pathological presentations may have different behaviors. Analyses of breast cancer with new molecular techniques now hold promise for the development of more accurate tests for the prediction of recurrence. Gene signatures have been developed as predictors of response to therapy and protein gene products that have direct roles in driving the biology and clinical behavior of cancer cells are potential targets for the development of novel therapeutics. The present review summarizes current knowledge in breast cancer molecular biology, focusing on novel prognostic and predictive factors.

INTRODUCTION

Breast cancer is a heterogeneous complex of diseases, a spectrum of many subtypes with distinct biological features that lead to differences in response patterns to various treatment modalities and clinical outcomes. Traditional classification systems regarding biological characteristics, such as tumor size, lymph node involvement, histological grade, patient's age, estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor 2 (HER2 or c-erbB2) status, may have limitations for patient-tailored treatment strategies. Furthermore, the histological appearance of the tumors may not be sufficient to establish the underlying complex genetic alterations and the biological events involved in cancer development and progression. Tumors with similar clinical and pathological presentations may have different behaviors. Therefore, recent studies have

focused on defining more detailed biological characteristics to improve patient risk stratification and to ensure the highest chance of benefit and the least toxicity from a specific treatment modality. Global gene expression profiling (GEP) studies have provided evidence for classifying breast cancer into distinct biological classes associated with patient survival, based on gene expression patterns^[1,2].

Population based screening programs have resulted in a significant shift to early stage disease and increased the interest in studying biological prognostic and predictive factors^[3]. Novel molecular studies have opened a broad field in cancer research that allows basic and translational researchers to look for new potential targets. Analyses of breast cancer with new molecular techniques now hold promise for the development of more accurate tests for the prediction of recurrence. Gene signatures have been developed as predictors of response to therapy and protein gene products that have direct roles in driving the biology and clinical behavior of cancer cells are potential targets for the development of novel therapeutics^[4]. The present review summarizes current knowledge in breast cancer molecular biology, focusing on novel classification, prognostic and predictive factors.

IDENTIFICATION OF BREAST CANCER SUBTYPES BY GEP STUDIES

Gene expression microarray studies have identified distinct molecular tumor classes based on simultaneous expression analyses of thousands of genes in a single experiment. Perou *et al*^[5] first analyzed gene expression patterns in grossly dissected normal or malignant human breast tissues in 65 tumor samples from 42 individuals with locally advanced breast cancer treated with neoadjuvant doxorubicin, using complementary microarrays representing 8102 human genes. The authors selected 496 genes based on the criteria of significantly greater variation in expression between different tumors and minimum variation between paired samples from the same patient and these genes were termed the intrinsic gene subset. Samples and genes were aggregated according to the similarity to each other (unsupervised clustering). Subset cluster analysis revealed a dendrogram with two main branches that were clinically described as *ER-positive* and *ER-negative*. The tumors in the *ER-positive* group were characterized by the relatively high expression of many genes expressed by breast luminal cells (ER-responsive genes, luminal cytokeratins and other luminal associated markers), so they were termed the *luminal* group. The *ER-negative* group was further divided into *basal-like*, *ErbB2-positive* and *normal-like* subclasses. *Basal-like* tumors expressed many of the characteristics of breast basal epithelial cells that did not express ER and showed staining with basal keratins. Another cluster of tumors was characterized by the expression of high levels of HER2 oncogene, which also showed low levels of ER expression and other genes associated with ER expression. Eventu-

ally, the authors identified four groups of samples using the intrinsic gene set that might be related to different molecular features of mammary epithelial biology and they named them *ER-positive luminal-like*, *basal-like*, *ErbB2-positive* and *normal-like*. These results were confirmed in follow up experiments using larger numbers of cases^[6].

Subsequent studies revealed that similar molecular subtypes of breast cancer could be identified in multiple cohorts of breast cancers and that *luminal* cancers could be subclassified into 2 or 3 groups and different molecular subtypes were shown to have distinct clinical outcomes. Sørlie *et al*^[7] investigated the clinical relevance of gene expression profiles in 78 breast carcinoma patients. Of these patients, 51 were part of a prospective study with locally advanced (T3-T4 and/or N2) tumors and had received doxorubicin based chemotherapy before the surgery. The authors showed a highly significant difference in overall survival between the subtypes. Both the *basal-like* and *ErbB2-positive* subtypes were associated with the shortest survival times. The authors subclassified the *luminal-like* breast cancer into three subclasses comprising *luminal-A*, *luminal-B* and *luminal-C* and identified *luminal-A* subgroup of *ER-positive* tumors as being associated with the best outcome. Van't Veer *et al*^[8] also investigated node-negative breast cancer patients and found 231 genes significantly associated with disease outcome, as defined by the presence of distant metastasis at the 5th year. These data revealed that each breast tumor has its own unique molecular portrait, providing the basis for an improved molecular taxonomy of the disease.

SUBCLASSIFICATION OF LUMINAL LIKE BREAST CANCER

Approximately 75% of breast cancers are positive for ER and/or PR. The *ER-positive* tumors express ER, PR, ER responsive genes and other genes that encode typical proteins of luminal epithelial cells so they are termed the *luminal* group. Characterization of *luminal-like* breast cancer varied between various studies, probably due to the identification and use of distinct intrinsic gene sets for cluster analysis. Hu *et al*^[9] evaluated an intrinsic gene set derived from three independent studies (Sørlie *et al*^[7], 2001; Van't Veer *et al*^[8], 2002; Sotiriou *et al*^[10], 2003), joined them together into a combined data set and identified two main *luminal-like* subclasses corresponding to *luminal-A* and *luminal-B*. Most subsequent studies have supported the concept of two *luminal-like* subclasses^[10-12].

Luminal-A

The *luminal-A* is the most common subtype and represents 50%-60% of all breast cancers. These tumors frequently have low histological grade, low degree of nuclear pleomorphism, low mitotic activity and include special histological types (*i.e.*, tubular, invasive cribriform, mucinous and lobular) with good prognosis. *Luminal-A* is characterized by higher levels of ER and lower levels of proliferation related genes. It is characterized by the

expression of luminal epithelial cytokeratins (CK) 8 and 18, other luminal associated markers including ER1, genes associated with ER function such as *LIV1* (zinc transporter ZIP6 or *SLC39A6*; solute carrier family 39 zinc transporter, member 6), *hepatocyte nuclear factor 3 alpha* (*FOXA1*), *X-box binding protein 1* (*XBPI*), *GATA binding protein 3* (*GATA3*), *B cell lymphoma 2* (*BCL2*), *erbB3* and *erbB4*^[13]. *Luminal-A* subtype is defined as ER-positive and/or PR-positive tumors with negative HER2 and low Ki67 (proliferating cell nuclear antigen) index by immunohistochemistry^[14].

Patients with *luminal-A* breast cancer have a good prognosis; the relapse rate is significantly lower than the other subtypes. Recurrence is common in bone, whereas liver, lung and central nervous system metastases occur in less than 10% of patients and treatment is mainly based on hormonal therapy^[15,16].

Luminal-B

Luminal-B tumors comprise 15%-20% of breast cancers and have a more aggressive phenotype, higher histological grade, proliferative index and a worse prognosis^[17]. This subtype has a higher recurrence rate and lower survival rates after relapse compared to *luminal-A* subtype^[18].

The main difference between both *luminal* subgroups is increased expression of proliferation-related genes such as *avian myeloblastosis viral oncogene homolog (v-MYB)*, *gamma glutamyl hydrolase (GGH)*, *lysosome-associated transmembrane protein 4-beta (LAPTMB4)*, *nuclease sensitive element binding protein 1 (NSEP1)* and *cyclin E1 (CCNE1)* in *luminal-B* breast cancers. *Luminal-B* tumors also demonstrate increased expression of growth receptor signaling genes^[19]. Approximately 30% of *HER2-positive* tumors defined by immunohistochemistry are assigned to the *luminal-B* subtype^[20].

It should be noted that expression levels of proliferation related genes in ER-positive disease form a continuum; therefore, the cutoffs to define *luminal-A* and *luminal-B* cancers are set in an arbitrary manner rather than emerging from a bimodal distribution of these genes' expression levels^[21]. Various studies conducted to differentiate *luminal-A* and *luminal-B* subtypes defined more pragmatic criteria that can be broadly applied to clinical practice. The Ki67 index is suggested as a potential proliferation marker that could successfully differentiate *luminal-B* tumors from *luminal-A* in clinical practice. Cheang *et al*^[22] studied 357 breast cancer subtypes by using microarray based gene expression profiling and the Ki67 hormone receptor and HER2 status by immunohistochemistry. The authors determined the Ki67 cut off point (14%) that distinguishes *luminal-A* from *luminal-B* tumors, then applied it to an independent microarray series of 4046 breast cancers and concluded that the two subtypes could be distinguished by the Ki67 index. However, Ki67 immunohistochemistry has known limitations, such as low intra- and inter-laboratory reproducibility, arbitrary selection of optimal antibodies for testing and different methods of cell counting (manual

vs automated) in addition to potential problems resulting from tumor heterogeneity^[23]. There is also an urgent need to standardize the Ki67 expression analysis and validate its clinical utility.

From the immunohistochemical point of view, *luminal-B* subtype is defined as ER-positive, HER2-negative and Ki 67 high or ER and HER-2 positive tumors, but this definition does not include all *luminal-B* tumors as up to 6% of them are negative for both ER and HER2. Moreover, the Ki67 cut off point to distinguish *luminal-A* and *-B* has not been standardized^[24].

Overall survival in untreated *luminal-B* breast cancers is similar to the *basal-like* and *HER2-positive* subtypes which are widely recognized as high-risk tumors^[9]. *Luminal-B* tumors have poorer outcomes with hormone therapy. Several studies have suggested that *luminal-B* breast cancer was relatively insensitive to endocrine therapy compared to *luminal-A* breast cancer and to paclitaxel- and doxorubicin-containing preoperative chemotherapy compared with *HER2-positive* and *basal-like* breast cancers. However, *luminal-B* breast cancer responds better to neo-adjuvant chemotherapy than *luminal-A* subtype, achieving higher pathological complete response rates^[25-29]. Increased relapse rates observed in *luminal-B* tumors are limited to the first 5 years after diagnosis^[30].

Recent evidence suggests that certain alternative growth factor pathways, such as *fibroblast growth factor receptor 1 (FGFR1)*, *HER1*, *phosphoinositide 3 kinase (PI3K) catalytic alpha polypeptide*, and *sarcoma proto-oncogene (Src)*, may contribute to the higher proliferation and poorer prognosis of *luminal-B* breast cancer and related therapeutic agents are in active clinical development^[31].

In breast cancer, changes to fibroblast growth factor signaling are considered important for oncogenesis, mainly through amplification of *FGFR1* and *FGFR2*. *FGFR1* is amplified in 10% of all breast cancers. Recent data suggest that the *luminal-B* subtype is enriched for *FGFR1* gene amplification^[32]. Studies suggested that *FGFR1* gene amplification might be a contributor to the poor prognosis observed in *luminal-B* breast cancer through increased proliferation and resistance to endocrine therapy. Several antibodies and small molecule inhibitors of *FGFR* are currently under clinical study processes.

In breast cancer, the PI3K pathway is frequently activated. Amplification of upstream receptors such as HER2, loss of negative regulators such as PTEN, amplification of downstream targets such as protein kinase B (PKB or Akt) and activating mutations or genetic amplification of the alpha catalytic subunit of PI3K have all been described in breast cancer. Targeting the PI3K pathway appears promising, although more extensive studies are required^[33].

HER2-positive

Human epidermal growth factor receptor-2 is a member of the family of four membrane tyrosine kinases. The HER2 receptor is encoded by the HER2 gene, which is a proto-oncogene mapped in chromosome 17q21. Upon

ligand binding to their extracellular domains, HER proteins undergo dimerization and transphosphorylation of their extracellular domains. HER2 does not have a ligand and relies on heterodimerization with another family member or homodimerization with itself to be activated when expressed at very high levels. These phosphorylated tyrosine residues interact with numerous intracellular signaling molecules, leading to activation of downstream second messenger pathways and crosstalk with other membrane signaling pathways. Transcription factors activated by the pathway regulate many genes involved in cell proliferation, survival, differentiation, angiogenesis, invasion and metastasis^[34-37].

HER2-positive cancer accounts for 15-20% of breast cancer subtypes. HER2 positivity confers more aggressive biological and clinical behavior. These tumors are characterized by high expression of the HER2 gene and other genes associated with the HER2 pathway and/or HER2 amplicon located in the 17q12 chromosome. Morphologically, these tumors are highly proliferative, 75% have a high histological and nuclear grade and more than 40% have p53 mutations^[38]. Nearly half of *HER2-positive* breast cancers are positive for ER but they generally express lower ER levels.

The immunohistochemical profile of *ER-negative* and *HER2-positive* does not correspond perfectly with the intrinsic subtype since only 70% of HER2 tumors by microarray have the protein overexpressed on immunohistochemistry. Conversely, all tumors with HER2 amplification or overexpression are not included in the HER2 cluster by microarray analysis^[39,40].

Staaf *et al.*^[41] identified three separate subtypes of *HER2-positive* tumors, one with a clearly poor prognosis with a 12% 10 year survival compared to the 50-55% survival in the other two groups using HER2 derived prognostic predictor (HDPP) gene analysis. The HDPP was not directly related to the expression of proliferation gene and HER2 pathway but was mostly associated with genes related to immune response to tumor invasion and metastasis.

In the absence of treatment, *HER2-positive* tumors have a poor prognosis. They have increased sensitivity to certain cytotoxic agents such as doxorubicin, relative resistance to hormonal agents and a propensity to metastasize to the brain and visceral organs. Doxorubicin sensitivity is possibly due to coamplification of the topoisomerase-2 gene which is near the HER2 locus on chromosome 17 and is the target of this drug^[42,43]. Advances in translational science have led to the development of a large spectrum of HER directed therapies.

Basal-like

The *basal-like* subtype represents from 8% to 37% of all breast cancers, depending on the proportion of poorly differentiated G3 cases included in the population studied^[44]. *Basal-like* cancers are associated with high histological and nuclear grade, poor tubule formation and the presence of central necrotic or fibrotic zones,

pushing borders, conspicuous lymphocytic infiltrate and medullary features with exceptionally high mitotic and proliferative indices. Most of these tumors are infiltrating ductal tumors with solid growth pattern, aggressive clinical behavior and high rate of metastasis to the brain and lung^[45].

Tumors belonging to the *basal-like* subgroup express high levels of basal myoepithelial markers, such as CK5, CK 14, CK 17 and laminin, and do not express ER, PR and HER2, hence referred to as *triple-negative*. They also overexpress P-cadherin, fascin, caveolins 1 and 2, alpha-beta crystallin and epidermal growth factor receptor (EGFR). *Basal-like* cancers present with frequent mutations in the *tumor protein 53* (TP53) gene, evidence of genomic instability and inactivation of the *retinoblastoma* (Rb) pathway. Deregulated integrin expression has also been detected and may contribute to aggressive cell behaviors and progression in this subtype^[45].

It is important to clarify that the terms *triple-negative* and *basal-like* are not completely synonymous and there is approximately 20%-30% discordance across studies. The term *triple-negative* refers to the immunohistochemical classification of breast tumors lacking ER, PR and HER2 protein expression, whereas the *basal-like* subtype is defined *via* gene expression microarray analysis. The *basal-like* classification is available only in the research setting to date and thus the *triple-negative* phenotype currently is a reliable surrogate in the clinical setting^[46].

There are several reported biomarkers associated with the *basal-like* group as well as putative candidates suitable for immunohistochemical screening. However, currently there is no specific international consensus on complementary biomarkers that can define *basal-like* cancers^[47].

Several genes related to the *basal-like* subtype have been implicated in promoting cellular proliferation, cell survival, cell migration and invasion. Despite the wide diversity of the involved pathways, signaling molecules, such as the mitogen activated protein kinase (MAPK), PI3K, Akt and nuclear factor kappa B (NF- κ B), are commonly deregulated as seen in other breast cancer subtypes. Other alterations such as cytoplasmic and nuclear accumulation of beta catenin were also observed in *basal-like* cancers, being the marker suggested as a potential therapeutic target for this cancer^[48].

Microarray and immunohistochemical analyses demonstrated that basal-like subtype constitute approximately three quarters of *breast cancer 1* (BRCA1) gene related breast cancers. This gene, often termed the *caretaker of the genome*, is located on chromosome 17 and is related with both inherent DNA damage sensing processes and DNA repair mechanisms. Breast cancers related to BRCA1 often express *triple-negative* phenotype and are frequently positive for Ki67 basal cytokeratins, TP53, EGFR and P cadherin and X chromosome abnormalities. Outcomes for women with *basal-like* tumors and BRCA1 related breast cancers are similar, in particular for early relapse and pattern of metastatic disease^[49]. *Basal-like* cancers with deficient BRCA1 pathway may respond to specific

Table 1 2013 St. Gallen - intrinsic subtypes of breast cancer

Intrinsic subtype	Clinicopathological surrogate definition	
Luminal-A	"Luminal-A-like" all of: ER and PgR positive HER2 negative Ki-67 "low" ^a Recurrence risk "low" based on multi-gene-expression assay (if available) b	a A level of < 14% best correlated with the gene-expression definition of Luminal A based on the results in a single reference laboratory ^b PgR cut-point of ≥ 20% to best correspond to Luminal A subtype
Luminal-B	Luminal-B-like (HER 2 positive) ER positive HER2 negative and at least one of: Ki-67 "high" PgR "negative or low" Recurrence risk "high" based on multi- gene-expression assay (if available) Luminal-B (HER 2 negative) ER positive HER2 over-expressed or amplified Any Ki-67 Any PgR	
Erb-B2 overexpression	HER 2 positive (non-luminal) HER2 over-expressed or amplified ER and PgR absent	
Basal-like	Triple negative (ductal) ER and PgR absent HER2 negative	There is an 80% overlap between "triple-negative" and intrinsic "basal-like" subtype

therapeutic regimens such as poly-ADP ribose polymerase (PARP) enzyme inhibitors. Also, BRCA1 deficient cells have defects in DNA double strand break repair mechanisms that could render them particularly sensitive to therapeutic agents that generate DNA double strand breaks such as PARP enzyme inhibitors^[50]. As often overexpressed in *basal-like* cancer, EGFR may also be another potential therapeutic target. Dong *et al.*^[51] identified notch pathway as one of the mechanisms of resistance to EGFR inhibition in *basal-like* breast cancer as valuable information to overcome this resistance. Dual pathway inhibition may be a viable clinical strategy in *basal-like* cancers.

As one of the *triple-negative* subtypes, *claudin-low* breast cancer was described by Herschkowitz *et al.*^[52]. This subtype is characterized by low expression of genes involved in tight junctions and cell-cell adhesions including claudins 3, 4 and 7, occludin and E cadherin showing high expression of epithelial to mesenchymal transition genes and stem cell features. Currently, it has been reported that patients with *claudin-low* tumors also have poor clinical outcomes like other *triple-negative* tumors.

Normal breast-like

These tumors account for about 5%-10% of all breast carcinomas. They are poorly characterized and have been grouped into the classification of intrinsic subtypes with fibroadenomas and normal breast samples. They express gene characteristics of adipose tissue presenting an intermediate prognosis between *luminal* and *basal-like* cancers and usually do not respond to neoadjuvant chemotherapy. As they lack the expression of ER, PR and

HER2, these tumors can also be classified as *triple-negative* but they are not considered to be *basal-like* cancers as they are negative for CK5 and EGFR. There are few studies on this subtype and their clinical significance remains undetermined. There are doubts about their existence as a breast cancer subtype and some researchers believe they could be a technical artifact from high contamination with normal tissue during the microarrays^[53]. In fact, in a large series of samples where the neoplastic cells were isolated by microdissection, no cases of *normal breast-like* subtype were found, supporting this hypothesis.

The implications of the molecular classification in the therapeutic era have been accepted by international panels. In the 2011 and the latest 2013 St. Gallen International Breast Cancer Conferences, the expert panel members agreed that therapeutic decisions should be made based on the recognition of the intrinsic subtypes of breast cancer. Panel members agreed that the different breast cancer subtypes can be defined only by genetic array testing but approximation to this classification can be made by immunohistochemistry^[54,55] (Table 1).

Although molecular taxonomy of breast cancer has attracted great attention, to date, actual practical adaptation seems limited. Certain critical issues have been raised, such as validation, reproducibility and clinical utility. The four main molecular classes frequently reported can be considered an oversimplification of a novel molecular classification system and add little to our understanding of the biology and behavior of breast cancer. Sub classification of the largest *luminal* class remains unresolved. Most *luminal* tumors are hormone receptor positive and can be identified in routine practice using immunohisto-

chemistry. Hormone receptor expression in *luminal* phenotype is recognized as a validated predictor to hormonal treatments. The difference between *basal-like* and *triple-negative* is disputed, with triple negativity in clinical practice providing a more practical and routinely applicable classification. Similarly, strongly *HER2-positive* breast cancer patients by immunohistochemistry are likely to be offered anti-HER2 therapy, especially if their tumors show evidence of HER2 gene amplification, regardless of their molecular classification. Furthermore, the *normal breast-like* class is not well defined and the proportion of some classes defined by GEP varies substantially. Finally, the contribution of this molecular taxonomy to current clinical practice is just the modification of treatment protocols related to ER, PR, HER2 and Ki67 status of breast cancer. Molecular classification based on combination of the classical well-defined immunohistochemical markers can be considered a simpler and more practical approach and it is expected to remain as such unless novel target molecules driving individual classes are identified.

BIG 1-98 is a randomized, phase III study that compared five years of tamoxifen or letrozole and their sequences in post-menopausal women with ER positive early breast cancer. Metzger *et al.*^[56] updated benefit of endocrine treatment among *Luminal* subgroups in this trial. ER positive subtypes were defined as *Luminal-A* (ER+ and/or PR+ HER2- and Ki67 < 14%) or *Luminal-B* (ER+ and/or PR+, HER2- and Ki67 ≥ 14%). In the invasive ductal carcinoma subset, 1436 (44%) and 1163 (36%) were classified as *Luminal-A* and *Luminal-B*, while in the invasive lobular carcinoma subset, 237 (59%) and 87 (22%) were classified as *Luminal-A* and *Luminal-B*, respectively. In lobular carcinoma patients, disease free survival hazard ratios for letrozole *vs* tamoxifen were 0.51 (95%CI: 0.33 to 0.79) for *Luminal-A* and 0.35 (95%CI: 0.21 to 0.56) for *Luminal-B* subtypes. The disease free survival hazard ratios for letrozole *vs* tamoxifen were 0.93 (95%CI: 0.74 to 1.77) for invasive ductal carcinoma *Luminal-A* and 0.64 (95%CI: 0.52 to 0.78) for invasive ductal carcinoma *Luminal-B*. A greater reduction in risk of a disease free survival event was shown in women with Luminal B for both invasive ductal carcinoma and invasive lobular carcinoma^[56].

Currently, the available molecular tests have offered the opportunity to challenge the molecular complexity of breast cancer but do not provide sufficiently robust information to modify established treatment schemes. These tests require validation in large series and comparison with traditional classification systems in the context of comprehensive clinical trials.

CLINICAL GENE EXPRESSION BASED ASSAYS

Although up to 70% of patients with early breast cancer currently receive adjuvant chemotherapy, only a specific subgroup of these patients derive benefit from this treatment. Therefore, in parallel with the advances in the

molecular sub classification of breast cancer, several multigene predictors of outcome have been developed (Table 2). It was conceived that microarray based gene signatures were able to identify a subgroup of patients sufficiently with a good prognosis that would not be treated with adjuvant chemotherapy. Currently, many classifiers have been generated by using various technologies such as cDNA and oligonucleotide arrays and multiplex polymerase chain reaction (PCR) analysis. These genomic tests assess expression of different but sometimes overlapping sets of genes. Despite differences in candidate genes in each of the assays, most of them can quite reliably predict the biology of tested tumors. In fact, when some of these tests were compared with each other, they were found to be quite similar in their abilities to predict metastases-free and overall survivals. Five different prognostic signatures were shown to have a high correlation, even among tests utilizing expression of very few genes in common. One important finding from analyses of various genomic tests is the fact that they assign almost all patients with hormone receptor negative disease as high risk. Therefore, most of these tests are more applicable to patients with ER-positive cancers who constitute a more heterogeneous group for prognosis and probability of response to chemotherapy. Given this distinction, the utility of these tests in practice will still depend on clinical and histological assessments to identify specific patients who would then be appropriate for additional testing with gene expression signatures.

PAM 50

PAM 50 is a 50 gene expression assay based on microarray and quantitative real time (qRT)-PCR that was developed by analyzing 189 breast tumor samples to separate them into four molecular breast cancer subtypes (*luminal-A*, *luminal-B*, *HER2-positive* and *basal-like*)^[57].

PAM 50 assay can provide a risk of relapse score that predicts relapse-free survival for node-negative breast cancer patients who had not received adjuvant systemic therapy. The validation study revealed that patients with *luminal-A* subtype had better prognosis in contrast to the other types and were less responsive to chemotherapy^[58].

The most well described, albeit investigational, classifier for the intrinsic subtypes that can be performed on the fixed tissue available in most pathology laboratories is the PAM 50 assay; however, this assay requires further validation for routine clinical practice^[59].

MammaPrint

MammaPrint is a microarray based gene expression profiling assay that was developed after analyzing data from 78 patients with ER-positive, node-negative breast cancer patients who had not received adjuvant systemic therapy. Of the 78 patients, 34 developed distant metastasis and 44 were disease free at the 5th year. The tumors' mRNA was extracted for reverse transcription into cDNA, which was tested on microarray containing 25000 human genes. Seventy genes that had the strongest association with

Table 2 First generation gene expression signatures

Gene signature	MammaPrint	OncotypeDX	MapQuantDX	Breast cancer index	PAM 50 assay
Starting material	FF or stabilized RNA, FFPE	FFPE	FFPE, FF	FFPE	FFPE
Analytical platform	Microarray, RT-PCR	qRT-PCR	Microarray, qRT-PCR	qRT-PCR	nCounter
Number of genes	70	21	97/9	7	50
Indications	Stage I / II, 5 cm, ER (+), Node (-)/[1-3 Node (+)]	ER(+), Node (-)	ER (+), G2	ER (+)	All, Node (-) untreated
Application	Clinical outcome	Clinical outcome, benefit from chemotherapy	Molecular grading prediction of response to TMX	Clinical outcome, prediction of response to TMX	Subtype definition, risk of relapse without treatment
FDA approved	Yes	No	No	No	No
ASCO and NCCN recommendation	No	Yes	No	No	No

FF: Fresh frozen; FFPE: Formalin fixed paraffin embedded; G: Grade; TMX: Tamoxifen.

outcome *i.e.*, predicted good and poor risk disease were accurately selected^[60]. The genes that comprise the MammaPrint assay are proliferation genes and genes associated with invasion and angiogenesis. This test is based on microarray results and hence requires high quality RNA from freshly collected tissues^[61]. The expression of the selected genes defines a prognostic classification of patients as having a good or poor prognosis. This test was approved by Food and Drug Administration (FDA) for lymph node-negative breast cancer patients younger than 61 years of age with tumors smaller than 5 cm in size.

The microarray-prognostics-in-breast-cancer (RASTER) study is the first study designed to prospectively evaluate the performance of the 70-gene signature. 427 patients with cT1-3N0M0 breast cancer were treated based on the Dutch CBO 2004 guidelines, the 70-gene signature and doctors' and patients' preferences. Five year distant recurrence-free interval probabilities were compared between subgroups based on the 70-gene signature and Adjuvant! Online. Fifteen percent (33/219) of the 70-gene signature low risk patients and 81% (169/208) of the 70-gene signature high risk patients received adjuvant chemotherapy. The 5 year distant recurrence-free interval probabilities for 70-gene signature low risk ($n = 219$) and high risk ($n = 208$) patients were 97.0% and 91.7%. The 5 year distant recurrence-free interval probabilities for adjuvant online low risk ($n = 132$) and high risk ($n = 295$) patients were 96.7% and 93.4% respectively. For 70-gene signature low risk adjuvant online high risk patients ($n = 124$), of whom 76% ($n = 94$) had not received adjuvant chemotherapy, 5 year DRFI was 98.4%. In this prospective community-based observational study, the 5 year distant recurrence-free interval probabilities confirmed the additional prognostic value of the 70-gene signature to clinicopathological risk estimations^[62].

MammaPrint has not yet been sufficiently evaluated as a predictive tool. MINDACT (microarray in node negative and 1 to 3 lymph node-positive disease may avoid chemotherapy) is a large prospective randomized trial designed to document when chemotherapy can be omitted if genomic information and conventional clinical risk

assignment system are discordant^[63].

Oncotype DX

Oncotype Dx is the most widely used prognostic and predictive clinical 21 gene qRT-PCR based assay for women with hormone receptor positive, node-negative breast cancer^[64]. The test is based on qRT-PCR technology that utilizes short and homogeneous amplicons. This method accurately measures gene expression even in the presence of mRNA fragmentation that occurs in archived formalin fixed paraffin embedded tissues. The test is based on 21 selected genes essentially related to proliferation, ER and HER2 signaling and was developed and validated through a retrospective analysis of formalin fixed paraffin embedded materials from three independent clinical trials^[65,66]. The gene expression pattern was translated into a quantitative recurrence score used as a continuous variable to estimate the probability of recurrence. Recurrence score divided patients into 3 groups as low, intermediate and high risk categories. The 21 gene signature has been subsequently evaluated in other cohorts of breast cancer patients and was shown to be an independent prognostic parameter in patients with ER-positive tumors with up to 3 positive nodes receiving adjuvant chemotherapy and in postmenopausal patients with ER-positive tumors treated with anastrozole^[67].

Multiple retrospective validation studies in various clinical settings established the prognostic and predictive accuracy of Oncotype Dx assay. Examination of the genes of the 21 gene profile by intrinsic subtype suggests that virtually all *luminal-B* tumors would have high recurrence scores, whereas 29% of *luminal-A* tumors would have high recurrence scores due to relative endocrine resistance^[68]. A high recurrence score is able to predict poorer outcome among hormone receptor positive tumors despite endocrine therapy and also predicts sensitivity to a variety of adjuvant cytotoxic regimens^[69]. For this reason, the recurrence score is thought to predict general chemosensitivity in hormone receptor positive breast cancer and is a reasonable assay for decision making on chemotherapy, particularly in the node-negative popula-

tion.

Oncotype DX is suggested by the American Society of Clinical Oncology and the National Comprehensive Cancer Network for the decision of adjuvant chemotherapy in ER-positive, node-negative breast cancer patients^[70,71]. Tailor X is a large prospective randomized trial set to validate OncotypeDX in clinical practice by better defining the intermediate risk stratum^[72].

GENOMIC GRADE INDEX

MapquantDx is a predictor test that defines the tumoral histological grade by gene expression features, used to assign a grade index to ER-positive breast cancers in attempt to refine their molecular classification. It was derived by identifying 97 genes from grade 1 and 3 breast tumors. The test was able to classify grade 2 tumors into low and high genomic grades with a statistically significant difference in relapse free survival^[73,74]. Most of the genes in this signature are involved in cell cycle regulation and proliferation. Genomic grade index (GGI) was strongly associated with recurrence risk among patients with grade 2 tumors. This assay is microarray based and requires freshly prepared tissues.

BREAST CANCER INDEX

The breast cancer index (BCI) prognostic assay provides an assessment of the likelihood of distant recurrences in patients diagnosed with ER-positive, node-negative breast cancer. This assay has been developed from the combination of two indices: the ratio of HOXB13/IL17BR genes, which predicts distant recurrence in ER-positive patients treated with tamoxifen, and a proliferation related five gene molecular grade index, which discriminates grade 1 from grade 3 disease. The test is based on qRT-PCR using RNA from paraffin embedded tissues^[75,76].

The biological roles of the genes included in most of these tests are not completely understood and it is often unclear which clinical or tumor characteristics are being measured. Although proliferation related genes are essential components of most classifiers, there is little overlap and instabilities exist among different gene series.

These prognostic profiles have been far better examined in the node-negative population as estimating the risk according to signature may be more difficult in node-positives. The expression patterns of hormone receptors and Ki67 may especially show differences in the tumor cells at the lymph nodes and the primary lesion, probably due to tumor cell heterogeneity in parallel to increased tumor burden^[77].

Although research results indicate that these multi-gene molecular assays can reclassify some breast cancer patients who are ranked as high risk using the traditional classification systems into low risk (*i.e.*, reducing the number of patients who might unnecessarily undergo chemotherapy) and vice versa, available data are insufficient to challenge classical classification systems and to

justify withholding chemotherapy for high risk patients if classified as low risk using multigene assays. However, it should be realized that these assays can potentially provide important prognostic information in clinically indeterminate subgroups and, in such situations, combining these tests with conventional predictors may yield valuable information. For instance, high grade but small (10 mm) sized, node-negative breast cancer may be offered systemic therapy if it is classified as high risk using multigene assays as staging information in such cases may be insufficient to reflect the behavior of these early detected tumors.

NEXT GENERATION SEQUENCING

Gene expression profiling and microarray analysis led to new molecular classification systems in breast cancer. In recent years, research has moved from gene expression profiling to a more detailed overview through the biological mechanism of carcinogenesis and tumor progression by mutational profiling. Technological advances such as array comparative genomic hybridization (array-CGH), single nucleotide polymorphism (SNP) and high throughput screening (HTS) are applied to further *in vitro* and *in vivo* research in order to improve knowledge on breast cancer biology and understand the complex process of metastasis^[78].

Next generation sequencing is based on deep sequencing which produces billions of short sequences at a time. It is quantitative and can analyze the entire genome at base pair resolution without the limitations of microarrays^[79].

Sanger sequencing was the first approach for sequencing the genome but it was both expensive and time consuming. Next generation sequencing (NGS), known as massive parallel sequencing, can be applied to study the whole genome (exons, introns and intergenic regions for about 22000 genes) more specifically to whole exome or to the 200-400 potentially targetable exons. High sensitivity of this technique allows the evaluation of single nucleotide variants, small insertions, deletions, copy number alternations (gain and losses) and structural variations (translocations, inversions). NGS can also be applied to the RNA for expression level analysis and to alternative splicing, RNA editing and fusion transcripts. NGS can be applied to the tumor to identify somatic mutations compared to normal tissues or to the peripheral blood samples to investigate germ line alterations. The study of germ line aberrations may give more information about germ line actionable mutations, toxicity susceptibility, drug metabolism and familial disease susceptibility^[80].

Application of NGS has led to the extension of knowledge to produce a comprehensive catalogue of likely genomic drivers of the most common breast cancer subtypes. The Cancer Genome Atlas Network analyzed more than 800 primary breast cancers using all the cutting edge technologies. They demonstrated four main breast

cancer classes, each of which shows significant molecular heterogeneity. They showed that somatic mutations in only three genes (TP 53, PIK3A and GATA 3) occurred at 10% incidence across all breast cancers. There were numerous subtype associated novel gene mutations, including the enrichment of specific mutations in GATA3, PIK3CA and MAP3K1 with the *luminal-A* subtype^[81].

Although NGS creates a massive amount of information, each mutation/alteration is not a good candidate to become a target for specific therapeutics. Molecular Taxonomy of Breast Cancer International Consortium (METABRICK) study revealed ten different subtypes, each characterized by common genetic alterations such as PPR2A, MAP2K4 and MTAP deletions that are potentially targetable and linked to survival^[82]. Alterations in the gene expression landscape can also be useful to guide treatments with conventional and experimental therapeutics.

Recently, the prospective multicenter molecular screening trial SAFIR 01 (High Throughput Technologies to Drive Breast Cancer Patients to Specific Phase I / II Trials of Targeted Agents) analyzed 423 patients with metastatic breast cancer. Metastatic sites were biopsied and profiled using the copy number changes array and Sanger sequencing PIK3CA (exon 10/21) and AKT1 (exon 3). A targetable genomic alteration was identified in 204 patients. The most frequent genomic alterations were PIK3CA mutations, CCND1, FGF4 and FGFR1 amplifications. In this study, 46 out of 277 (17%) patients with genomic analyses received a targeted therapy matched to the genomic alteration, covering twelve different targets^[83].

The clinical applications of NGS have many difficulties. Searching for every single gene alteration or pathway abnormality is uncertain. There are biological issues due to tumor heterogeneity, clonal evolution and the difficulty of discriminating between driver and passenger mutations. There are also some technical problems in terms of tumor tissue availability, stromal interference and laboratory reproducibility of the results.

CONCLUSION

One of the main contributions of the breakthrough in cancer research is the integration of molecular studies into clinical trials. Advances in molecular biology of breast cancer over the past decade have led to the classification of the disease from a molecular point of view. Incorporation of multigene molecular classifiers to conventional breast cancer classification systems seems more realistic and practical to support more effective tailoring of therapy. These multigene classifiers can complement traditional methods through provision of additional biological prognostic and predictive information by identifying important, clinically relevant, biological processes better than that determined using morphological factors or individual molecular markers.

New molecular techniques hold promise for improv-

ing diagnosis and sub typing, better assessment of recurrence risk, careful selection of therapy and identification of targets involved in carcinogenesis and function of tumor cells, leading to the discovery of selective drugs. Understanding the pathways regulating the processes involved in neoplastic development helps in the design of clinical trials aimed at patients with specific characteristics that are candidates to benefit from specific treatments. Protein gene products that have direct roles in driving the biology and clinical behavior of cancer cells are potential targets for the development of novel therapeutics. Research efforts have focused on the investigation and identification of new molecular factors, which can improve the predictability of risk of metastasis and the likelihood of response to therapies.

Probably in the near future, the tumoral key mechanisms of regulation will be identified individually and treatments will be more specific and effective, with minimal toxicity. Numerous agents targeting various biological pathways are currently under clinical development to achieve an ideal, personalized medical therapeutic approach in breast cancer.

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WJCO 5th Anniversary Special Issues (2): Breast cancer

Current role of modern radiotherapy techniques in the management of breast cancer

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Key words: Breast cancer; Radiotherapy; Intensity modulated radiotherapy; Partial breast irradiation; Hypofractionation

Core tip: Several randomized trials provided evidence for the feasibility of modern radiotherapy techniques in the management of breast cancer. Current review will provide an up-to-date evidence based data on the role of modern radiotherapy techniques in the management of breast cancer.

Abstract

Breast cancer is the most common type of malignancy in females. Advances in systemic therapies and radiotherapy (RT) provided long survival rates in breast cancer patients. RT has a major role in the management of breast cancer. During the past 15 years several developments took place in the field of imaging and irradiation techniques, intensity modulated RT, hypofractionation and partial-breast irradiation. Currently, improvements in the RT technology allow us a subsequent decrease in the treatment-related complications such as fibrosis and long-term cardiac toxicity while improving the loco-regional control rates and cosmetic results. Thus, it is crucial that modern radiotherapy techniques should be carried out with maximum care and efficiency. Several randomized trials provided evidence for the feasibility of modern radiotherapy techniques in the management of breast cancer. However, the role of modern radiotherapy techniques in the management of breast cancer will continue to be defined by the mature results of randomized trials. Current review will provide an up-to-date evidence based data on the role of modern radiotherapy techniques in the management of breast cancer.

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INTRODUCTION

Radiotherapy (RT) has a major role in the management of breast cancer for many years. It significantly reduces the risk of loco-regional recurrences after surgery by at least 70%^[1]. RT has been shown to improve overall survival both for early stage breast cancer after breast-conserving surgery (BCS) and locally advanced disease after mastectomy^[1]. However, its use is usually limited by late toxicity. In patients with a long life expectancy, only modern RT techniques could obtain survival benefit that is mostly dependent on the radiation dose to the cardiac structures^[1-4].

During the past 15 years, several developments took place such as imaging and irradiation techniques, hypofractionation and partial-breast irradiation (PBI). Improvements in the RT technology now frequently allow us a subsequent decrease in treatment-related complica-

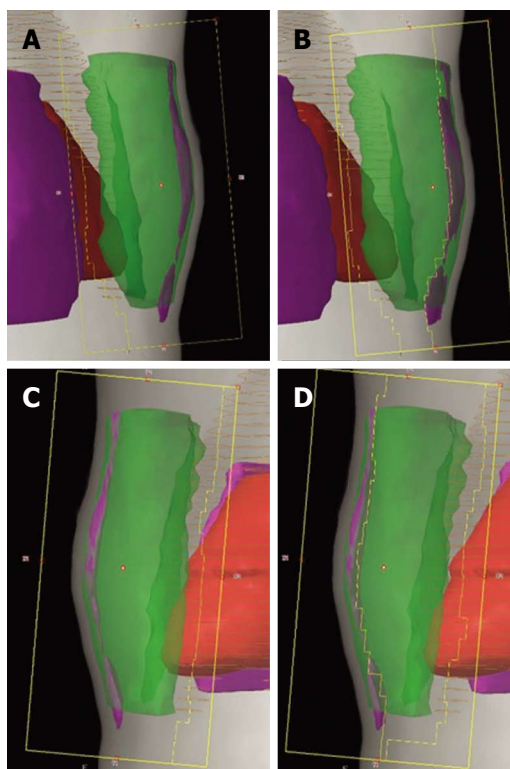


Figure 1 BEV shaped forward-planned intensity modulated radiotherapy (IMRT) (field-in field technique). Open tangential fields (A and C). DRR showing the multileaf collimator segments closing the volumes receiving $\geq 110\%$ of the prescribed dose (B and D).

tions such as fibrosis and long-term cardiac toxicity while improving the loco-regional control rates and cosmetic results^[5-7]. Computed tomography (CT) simulators, modern-day linear accelerators, three-dimensional (3D) planning techniques and treatment verification modalities provides improved targeting and smaller irradiated volumes of normal tissue.

Depending on the type of surgery and pathology reports, traditionally, conventional two-dimensional (2D) beams were used for whole-breast or chest wall irradiation^[8]. The first important challenging step in the RT technique came with the introduction of the CT-based treatment planning and 3D conformal RT (3DCRT) that provides us precise target volume definition, dose distribution calculation, and virtual simulation. Optimal shielding of organs at risk (OARs), including the heart, lungs, brachial plexus, esophagus, trachea, thyroid, and spinal cord decreased normal tissue exposure. Additionally, more homogeneous dose distribution in the clinical target volume could be obtained.

INTENSITY MODULATED RADIOTHERAPY

Intensity modulated RT (IMRT) is an advanced form of 3DCRT that became increasingly available for breast cancer. Several important studies has been carried out on the use of IMRT for breast cancer patients requiring

complex breast treatments^[9]. Patients with larger breasts are more likely to have dose inhomogeneities and most likely to benefit from IMRT. It can also be the best alternative for left-sided breast cancers to decrease cardiac dose, re-irradiation, contralateral breast irradiation, PBI, and deeply seated tumor bed irradiation.

The modulation of beam intensities could be determined by allowing sculpting the dose to fit a patient's anatomy. The major goal for IMRT technique is providing more homogenous dose distribution throughout the breast and concave structures such as the chest wall^[10,11]. This technology also allows better conformity of dose to the target and better sparing of OARs compared to non-IMRT plans^[7,10,12-17]. However it has some drawbacks, including decrease in surface build up dose which could adversely affect local control and increase risk for secondary malignancies^[18-20].

For each individual case, adequate coverage of the primary tumor site and most of the breast can be achieved by changing the gantry angle, the collimator angle, or shaping [with small cardiac blocks or multileaf collimator (MLC) leaves] the borders of the medial and/or lateral tangential fields while the heart and lung can be excluded from the high dose region at the same time. The normal tissue anatomy, the location of the primary tumor bed, and the contour of the breast should be taken into consideration when applying treatment field modifications for each individual patient. Forward planning by using the "field-in-field" technique provides excellent dose homogeneity in the irradiated areas. Thus, it is the most widely used technique in breast IMRT^[10,21] (Figure 1). There are also other methods, including forward-planned step-and-shoot breast IMRT, and an inversely planned breast IMRT technique, which can all improve dose homogeneity (Figure 2).

IMRT planning should be performed based on 3D visualization of contours delineated on planning CT images. Proper patient positioning, immobilization, target localization, and management of breathing-related motion are essential for IMRT due to sharp dose gradient changes^[22]. Also, sophisticated technical resources and longer period of time are required both for planning and quality assurance tests. In addition, radiation delivery turns out to be more complex. It requires specialized software to automate the process to reduce treatment time and the risk of delivery error. As a result of longer beam-on time, whole body and contralateral breast doses may increase.

Image-guided RT (IGRT) is required to precise localization of both target and normal tissues during planning and treatment procedure. The main advantage of IGRT is that it allows more accurate targeting in breast cancer by providing correct target volume delineation, obtaining simulation images, and set-up correction using images with the patient in the treatment position immediately prior to or during the treatment. A variety of imaging methods are used: (1) gantry-mounted systems [MV-electronic portal imaging device (EPID), kV/MV cone

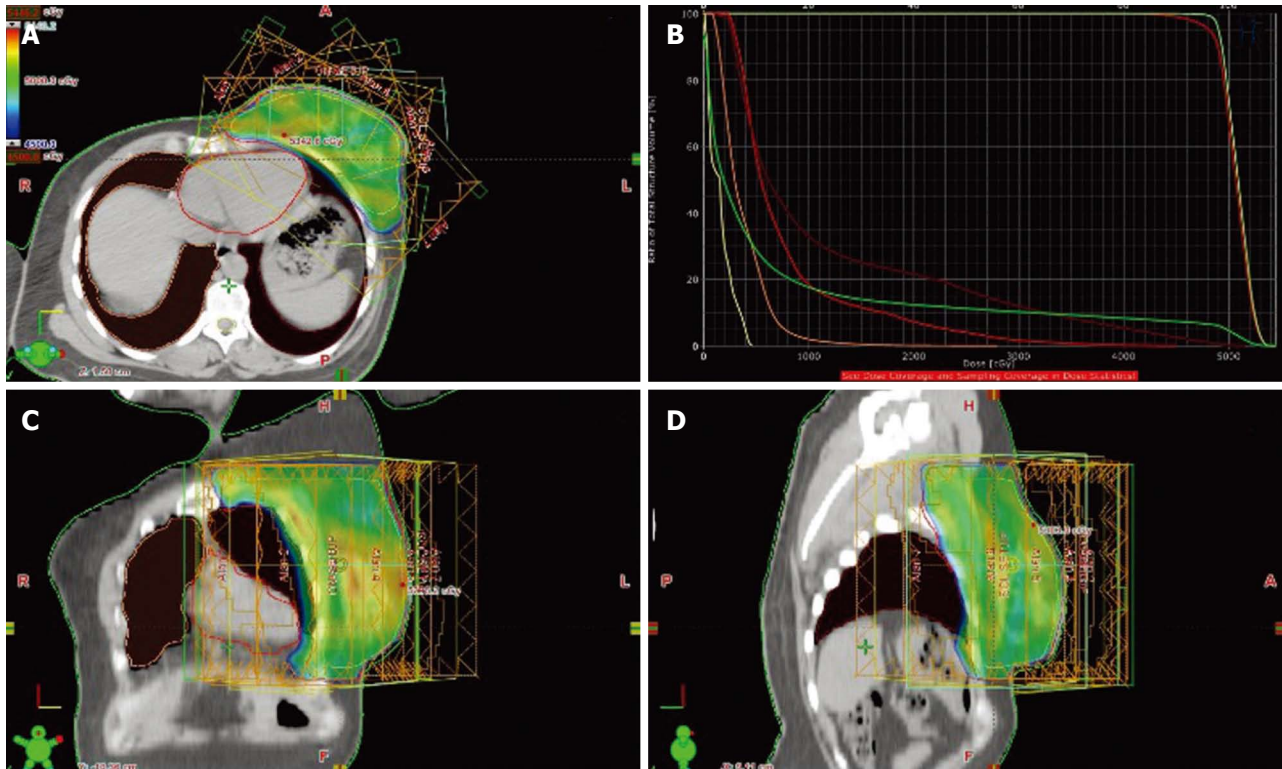


Figure 2 Treatment fields and dose distribution of inverse-planned intensity modulated radiotherapy: Axial section (A), dose-volume histogram (B), coronal section (C) and sagittal section (D).

beam CT, MV systems-tomotherapy]; (2) room-mounted systems; and (3) non-ionizing systems (ultrasound, video-based systems)^[23]. Besides, breath-holding techniques (using “active breathing control” devices or unassisted) and respiratory gating can effectively limit motion and decrease the dose to the heart and lungs, especially in cases in which the tumor bed is very close to the heart^[24-26].

There are several IMRT techniques; dynamic or static MLC based IMRT, arc therapy, tomotherapy, and topotherapy^[27,28]. Radiation is delivered to the patient as gantry rotates continuously around the patient in intensity modulated arc therapy. In tomotherapy, there is a helical radiation delivery that continues during treatment couch movement and binary MLC motion creates fluency. Topotherapy is performed with static gantry while as the patient translates through the treatment field instead of rotational delivery^[28].

TREATMENT OUTCOMES AND TOXICITY AFTER IMRT

IMRT has changed our RT practice and it is used for palliative and curative indications throughout several tumor types. The highest level of evidence by using IMRT exists particularly for breast and nasopharyngeal carcinomas^[29]. Although the clinical outcome of IMRT in breast cancers came mainly from retrospective studies, it has been assessed in three prospective randomized studies^[30-33] (Table 1). The primary aim of these studies was to investigate treatment-induced toxicity and patients’ quality of life

(QOL). They showed that IMRT increased the dose homogeneity and decreased the frequency and severity of toxicity in early-stage breast cancer after BCS. However, larger sample size and longer follow-up is required to see long-term clinical outcomes and evaluate for late deleterious effects.

There is only one randomized study of treatment efficacy comparing IMRT and non-IMRT^[33]. Mukesh *et al*^[33] reported 5-year results of 815 patients randomized to either standard wedged-based tangential fields or forward-planned IMRT. In this study, there was no statistically significant difference in 5-year loco-regional recurrence (2.56% and 1.35%) or overall survival (92.5% and 91.7%) rates. Additionally, there has been two reported trial designed to investigate breast cancer-related outcomes, the retrospective cohort study ($n = 240$) by McDonald *et al*^[34] and the prospective cohort study ($n = 332$) by Morganti *et al*^[35]. Findings did not show a statistically significant differences between IMRT and non-IMRT techniques for breast cancer related outcomes like survival, disease-specific survival and freedom from contralateral breast cancer recurrence.

Conventional RT causes acute skin toxicity, specifically moist desquamation in 30%-50% of patients^[36,37]. An inhomogeneous dose distribution and consequential hot spots of whole breast irradiation increases the rate of acute and late skin toxicity including erythema, edema, desquamation, pain, telangiectasia and fibrosis, and effects negatively cosmetic results and patient’s QOL^[32,38,39]. Large breast size and dose inhomogeneities > 10% as-

Table 1 Randomized phase III trials of intensity modulated radiation therapy for breast cancer

Ref.	N		FU (mo)	End-points	IMRT	Non IMRT	P value	Outcomes reported
	IMRT	2D RT						
Donovan <i>et al</i> ^[32] , 2007	150	156	N/A	Distribution of any change in breast appearance between the presence or absence of doses > 105%	OR, 2.6; 95%CI: 1.1-6		0.03	DC, LAE, QOL
				Photographic assessment of any change in breast appearance at 1, 2 and 5 yr	OR, 1.7; 95%CI: 1.2-2.5		0.008	
				Physician assessment of breast induration at 5-yr, %				
				Centre of the breast	21	32	0.02	
				Pectoral fold	22	29	0.006	
				Inframammary fold	17	24	0.009	
				Boost site	37	61	< 0.001	
Pignol <i>et al</i> ^[30] , 2008	170	161	N/A	Acute skin toxicity (Gr 3-4), %	27.1	36.7	0.06	DC, AAE, QOL
				Moist desquamation (all breast), %	31.2	47.8	0.002	
				Moist desquamation (inframammary crease), %	26.5	43.5	0.001	
				Pain (Gr 2-4), %				
Barnett <i>et al</i> ^[31] , 2012	411	404	24 ¹	Photographic assessment of breast shrinkage at 2 yr	OR, 1.51; 95%CI: 0.83-1.58		0.41	AAE, LAE, QOL
				Acute toxicity (Gr ≥ 2)	OR, 1.00; 95%CI: 0.76-1.34		0.97	
				Telangiectasia	OR, 1.68; 95%CI: 1.13-2.50		0.009	
				Moderate or poor overall cosmesis (good baseline surgical cosmesis)	OR, 0.63; 95%CI: 0.39-1.03		0.061	
				Patient reported				
				Breast pain, %	46.7	37.3	0.98	
				Oversensitivity, %	47.1	35	0.43	
Mukesh <i>et al</i> ^[33] , 2013	228	237	60 ¹	Photographic assessment of breast shrinkage at 5 yr	OR, 0.79; 95%CI: 0.55-1.14		0.21	LAE, TRO
				Teleangiectasia	OR, 0.58; 95%CI: 0.36-0.92		0.021	
				Overall cosmesis	OR, 0.68; 95%CI: 0.48-0.96		0.027	
				Breast edema	OR, 0.74; 95%CI: 0.48-1.15		0.18	
				Tumor bed induration	OR, 0.76; 95%CI: 0.54-1.06		0.11	
				Pigmentation	OR, 0.80; 95%CI: 0.46-1.38		0.42	
				5-yr overall survival, %	91.7	92.5	0.88	
				5-yr locoregional recurrence, %	1.35	2.56	0.36	

¹Minimum average follow-up. IMRT: Intensity modulated radiotherapy; 2D RT: Two dimensional radiotherapy; N: Number of patients; FU: Follow-up; DC: Dosimetry characteristics; AAE: Acute adverse effects; LAE: Late adverse effects; TRO: Treatment related outcomes; QOL: Quality of life; Gr: Grade; N/A: Not available; NS: Not significant.

sociated with a poorer clinical outcomes^[40-42]. IMRT significantly improved dose homogeneity with a median of 0.1% of the treatment volume receiving ≥ 110% of the prescribed dose *vs* 10% with conventional wedge-based breast RT^[10,11]. Therefore, IMRT has been established as an effective treatment for adjuvant RT after BCS with a decrease in moderate or severe skin reaction of up to 44% and better cosmetic results as compared with the standard wedged tangential field techniques^[30-32,34,42,43].

There are eight studies reported on acute radiation toxicity of IMRT^[30,34,35,42-44]. Vicini *et al*^[10] reported a prospective trial of breast IMRT and demonstrated a reduction in acute skin reactions. Similarly, Freedman *et al*^[43] reported the results of a matched-pair analysis of 131 patients treated using either breast IMRT (*n* = 73) or standard wedge-based RT (*N* = 58). This study found a significant reduction in the rate of acute desquamation using IMRT compared with the wedge-based treatment. Harsolia *et al*^[42] reported toxicity results of 172 patients at a median follow-up of 4.7 years, and demonstrated that the use of IMRT resulted in significantly less acute ≥

Grade 2 toxicity for dermatitis (41% *vs* 85%, *P* < 0.001), breast edema (1% *vs* 28%, *P* < 0.001), and hyperpigmentation (5% *vs* 50%, *P* < 0.001), compared with patients treated with conventional wedge-based plans (2D RT)^[42]. In patients with larger breasts (≥ 1600 cm³, *n* = 64), use of IMRT was associated with a statistically significant decrease in ≥ Grade 2 acute breast edema (0% *vs* 36%, *P* < 0.001), and hyperpigmentation (3% *vs* 41%, *P* = 0.001), and chronic long-term edema (3% *vs* 30%, *P* = 0.007) compared with conventional wedge-based plans. McDonald *et al*^[34] reported cohort analysis on long-term outcomes of IMRT (*n* = 121) with conventional RT (3D RT) (*n* = 124)^[34]. Median dose to the whole breast was 50 Gy, and median total dose to the tumor cavity was 60 Gy for both IMRT and conventional RT patient groups. IMRT resulted in reduced Grade 2 or 3 dermatitis compared with conventional RT (39% *vs* 52%, *P* = 0.047) at median 6.3 years of follow-up. Pignol *et al*^[30] reported the first multicenter randomized trial demonstrating a successful reduction an acute radiation skin toxicity using IMRT^[30]. Three hundred and fifty-eight patients

were randomized to forward-planned IMRT or standard conventional wedge technique after complete excision of an early-stage breast cancer. IMRT significantly reduced the occurrence of moist desquamation anywhere in the breast and in the inframammary fold, with an absolute reduction of 16.6% ($P = 0.002$) and 17% ($P = 0.001$), respectively. Smaller breast size ($P < 0.001$) and use of IMRT ($P = 0.003$) strongly associated with a decreased risk of moist desquamation. Despite no statistically significant difference in the QOL or pain between the two treatment arms, there was a highly significant correlation between the development of moist desquamation and grade 2 to 3 pain score ($P < 0.0001$), a decrease in the global health status scale ($P = 0.0019$), and an increase in the breast symptoms scale ($P = 0.0028$). The retrospective cohort study reported by Morganti *et al.*^[35] found that all skin-related acute toxicity reduced when standard 3D wedges were compared to simplified step and shoot IMRT technique ($P < 0.05$), despite with a lower total dose in the IMRT group^[35]. Only the retrospective cohort study by Freedman *et al.*^[44] reported on the proportion of treatment time with acute dermatitis, finding a significant benefit for IMRT compared with conventional wedged-based plans (18% *vs* 71%, $P < 0.0001$)^[44]. In that trial, 405 patients treated with conventional RT and 399 patients treated with IMRT. A subgroup analysis demonstrated that the time spent with radiation induced Grade 2-3 dermatitis was decreased in IMRT for all patients regardless of breast size (all $P < 0.05$). Barnett *et al.*^[31] reported the randomized trial demonstrating no significant differences were found in the incidence of any acute toxicity and development of any photographically assessed breast shrinkage between the IMRT or standard RT groups [odds ratio (OR), 1.51; 95% confidence interval (CI): 0.83-1.58; $P = 0.41$]^[31].

The decrease in acute toxicity achieved with IMRT translates into a decrease in late toxicity. There are two randomized trials showing a beneficial effect of forward-planned IMRT on late toxicity^[31-33]. The first prospective randomized clinical trial testing the role of forward-planned IMRT in terms of 5 year outcome for adverse effects was reported by Donovan *et al.*^[32]. They randomized 306 patients after BCS to standard 2D wedge-based RT or to IMRT (either IMRT with “step-and-shoot” fields or a physical 3D compensator). All were treated with a dose of 50 Gy in 25 fractions followed by a 10-Gy boost with electrons. Forward-planned IMRT significantly decreased dose inhomogeneity ($\geq 105\%$ of the prescribed dose) comparing standard 2D wedge-based plans (19% *vs* 92%). Results of treatment were evaluated using photographic assessment performed before RT and at 1, 2, and 5 years follow-up. The standard arm patients were 1.7 times more likely to have a change in breast appearance than the IMRT arm patients after adjustment for the year of photographic assessment (95%CI: 1.2-2.5, $P = 0.008$). However, there were no significant differences in outcome between randomized groups in any of the self-assessed parameters, including breast pain, discomfort,

hardness, body image, or QOL as measured by EORTC QLQ C30 and BR23 modules. The highest levels of dose inhomogeneity in the 2D RT were seen in the upper and lower third of the breast. This suggested that dose inhomogeneity in the breast increases late adverse events. The second one designed to investigate the effect of forward-planned IMRT on the incidence of late radiation toxicity reported by Barnett *et al.*^[31] from Cambridge. In their study, 815 patients with early stage breast cancer were randomized to either standard wedged-based tangential fields or forward-planned IMRT. Patients were randomized if $\geq 2 \text{ cm}^3$ receiving $> 107\%$ of prescribed dose. In this study, breast dosimetry was significantly improved with the forward-planned IMRT. All patients were treated to a dose of 40 Gy in 15 fractions. The patients in the standard RT group were more likely to develop telangiectasia than those in the IMRT group at early follow-up of only 2 years after RT completion (OR, 1.68; 95%CI: 1.13-2.40; $P = 0.009$). In patients who had good baseline surgical cosmesis, those randomized to IMRT were less likely to deteriorate to a moderate or poor overall cosmesis than those in the standard RT group (OR, 0.63; 95% CI: 0.39-1.03, $P = 0.061$). Recently, Mukesh *et al.*^[33] reported 5-year results of this study^[33]. On univariate analysis, patients receiving IMRT had superior overall cosmesis (OR, 0.68; 95%CI: 0.48 to 0.96; $P = 0.027$) and reduced skin telangiectasia (OR, 0.58; 95%CI: 0.36 to 0.92; $P = 0.021$) as compared with patients receiving standard RT arm. However, no significant difference was observed in the development of photographically assessed breast shrinkage or clinically assessed breast edema, tumor bed induration, or pigmentation. On multivariate analysis, use of IMRT was significantly associated with improved overall cosmesis (OR, 0.65; 95%CI: 0.44 to 0.98; $P = 0.038$) and decreased risk of skin telangiectasia (OR, 0.57; 95%CI: 0.34 to 0.95; $P = 0.031$). Large breast volume, poorer baseline surgical cosmesis, and tumor bed boost were also associated with suboptimal overall cosmesis on multivariate analysis. Patients with moderate to poor baseline surgical cosmesis more frequently developed suboptimal final cosmesis, tumor bed induration, and photographically assessed breast shrinkage at 5 years in the study.

Late toxicity results have been reported in only two retrospective cohort studies and one prospective study^[34,42,45]. The study by Harsolia *et al.*^[42] ($n = 172$) showed a significant difference between IMRT and conventional wedged-based plans in favor of IMRT for \geq Grade 2 breast edema (1% *vs* 25%, $P < 0.001$), with no differences in hyperpigmentation, fat necrosis, induration/fibrosis or overall cosmetic score^[42]. The study by McDonald *et al.*^[34] ($n = 240$) found a trend towards a reduction in lymphedema rates (0% *vs* 4%, $P = 0.06$), with no differences in the reported occurrence of radiation pneumonitis, fat necrosis or second malignancies^[34]. Freedman *et al.*^[45] reported the 5-year results of a phase II study of IMRT. Seventy-five patients were treated with simultaneous integrated boost (SIB)-IMRT; the whole

breast received 2.25 Gy per fraction for a total of 45 Gy and the tumor bed received 2.8 Gy per fraction for a total of 56 Gy in 20 treatments over four weeks. After a median follow-up of 69 mo, the 5-year rate of local recurrence was 2.7%. There were no significant differences over time in patient-reported cosmesis, pain and arm function and physician-reported cosmesis through the 5-year period of the study.

ACCELERATED PARTIAL BREAST IRRADIATION

Multiple prospective randomized trials have demonstrated that BCS followed by whole breast RT (WBRT) as a standard treatment approach in early stage breast cancer^[46-51]. A large meta-analysis showed that a reduction in ipsilateral breast tumor recurrence (IBTR) translated into a survival benefit of 5.4% after 15 years in early stage breast cancer patients treated with BCS^[1]. This treatment allows preservation of the breast with equivalent survival to mastectomy. In WBRT, entire breast is treated with a standard fractionation, which consists of 45-50 Gy, daily Monday to Friday over a 5- to 6-wk period. Despite being well tolerated and good cosmetic results, some patients does not receive WBRT due to long treatment duration, limited geographical access, and cost of the treatment^[52]. To address some of these issues accelerated partial breast irradiation (APBI) has gained popularity in selected patients. However, more data are needed about the use of different methods of APBI defining the optimal patient selection criteria, technique, dose and fractionation, side effects, and long-term outcomes.

APBI is a reasonable alternative to WBRT in patients with BCS who have more favorable tumor characteristics. Published studies indicated that 70%-90% of IBTRs after breast conservation therapy occurred at or in close proximity to the lumpectomy cavity^[49,50,53]. Multiple phase II and several phase III trials confirmed that APBI may offer equivalent local control to WBRT with shortening conventional treatment duration from 5 to 6 wk to a single fraction or few days (1-3 wk). In APBI, only the lumpectomy cavity treated with a limited margin for potential microscopic spread. Potential advantages of APBI include shorter treatment interval, improved cosmesis due to the decreased volume of breast tissue treated, decreased heart and lung volume, and reduced cost compared with standard fractionation^[54]. Additionally, decreased volume allows acceleration and hypofractionation which might be has some theoretical radiobiological advantages.

A consensus group by American Society for Radiation Oncology (ASTRO) and the European GEC-ESTRO Cancer Working Group developed guidelines for patient selection to APBI on the basis of a variety of clinical and pathologic factors (Table 2)^[55,56]. These guidelines categorized patients into three groups: Low-risk or suitable, intermediate-risk or cautionary group, and high risk or un-

suitable group. ASTRO defined suitable group which was include patients age ≥ 60 years, without BRCA mutation, T1 (≤ 2), > 2 mm surgical margins, no lymphovascular space invasion, ER positive, unicentric, invasive ductal or other favorable histology, no extensive intraductal component, and lymph node negative optimally as part of a clinical trial. Application of APBI to intermediate or cautionary group is considered acceptable only in the context of prospective clinical trials^[57]. It is compatible with GEC-ESTRO recommendations except that tumor size (T1-2; ≤ 3 cm) and age (≥ 50 years).

Modalities for APBI include brachytherapy; interstitial brachytherapy (multi-catheter interstitial implant), intracavitary brachytherapy [one balloon catheter (MammoSite®), multiple balloon catheter (Contura®), hybrid BRT (SAVI®)], intraoperative RT (IORT); intraoperative electrons (Liac®, Mobetron®, or Novac-7®; 3-10 MeV) or low energy X-rays (Intrabeam®; 50 kV), external beam RT; 3DCRT and IMRT.

Multicatheter interstitial brachytherapy

The most mature follow-up and experience of all APBI technique is multicatheter interstitial brachytherapy (MIB) which is commonly used as a boost treatment^[55-58]. It is an invasive approach that multiple interstitial catheters (up to 20) are placed surrounding the tumor bed at the time of surgery or postoperatively under ultrasound guidance. The number of the catheters may vary according to the size and the shape of the target volume. After catheter placement, tumor bed plus a 1-2 cm margin is treated with LDR, HDR or PDR devices. Radioactive sources are inserted temporarily into the catheter during treatment and then removed. Most commonly used regimen is 34 Gy in 10 fractions (twice daily) over 5 d. This modality only performed at a few institutions because of the specialized training required, costly equipment, and more complex technical support needed for the procedure. It is well tolerated but dose heterogeneity within the target volume can potentially lead to fat necrosis and subcutaneous toxicity^[59]. Acute complications include pain and infection. Often oral antibiotic treatment is required and rarely requires removal of the catheters.

Intracavitary brachytherapy: Intracavitary brachytherapy is the most common form of brachytherapy for APBI because of the less invasive, simple, and requires less experience. It can be applied with a single-lumen (MammoSite®) or multilumen (Contura®) balloon catheter, and elliptically shaped cluster of catheters such as SAVI®^[60]. It can be inserted into the lumpectomy cavity either at the time of surgery or postoperatively using ultrasound guidance^[61]. The balloon is then filled with saline, and an HDR radioactive source (commonly ¹⁹²Ir) is inserted. After the balloon is inflated, it should be symmetric and conform to the cavity. Dose is usually prescribed to 1 cm from the balloon surface. The minimum distance between the balloon and the skin/chest wall should be ≥ 5 mm, with a shorter distance leading to a poorer cosmesis.

Table 2 American Society for Radiation Oncology and GEC-ESTRO recommendations on patient selection criteria for Accelerated Partial Breast Irradiation

Factor	ASTRO Suitable	GEC-ESTRO Low-risk	ASTRO Cautionary	GEC-ESTRO Intermediate-risk	ASTRO Unsuitable	GEC-ESTRO High-risk
Patient factors						
Age (yr)	≥ 60	> 50	50-59	40-50	< 50	< 40
BRCA1/2 mutation	Not present	Not defined	Not present	Not defined	Present	Not defined
Pathologic factors						
Tumor size (cm)	≤ 2	≤ 3	2.1-3.0	≤ 3	> 3	> 3
T stage	T1	T1-2	T0 or T2	T1-2	T3-4	T2 (> 3 cm), T3-4
Histology	IDC or other favorable subtypes	IDC, mucinous, tubular, medullary and colloid carcinoma	ILC allowed	ILC allowed	Any	Any
Grade	Any	Any	Any	Any	Any	Any
Pure DCIS	Not allowed	Not allowed	≤ 3 cm	Allowed	> 3 cm	Any
EIC	Not allowed	Not allowed	≤ 3 cm	Not allowed	> 3 cm	Allowed
Associated LCIS	Allowed	Allowed	Allowed	Allowed	Allowed	Allowed
Multicentricity	Unicentric	Unicentric	Unicentric	Unicentric	Multicentric	Multicentric
Multifocality	Clinically unifocal ≤ 2 cm	Unifocal	Clinically unifocal 2.1-3 cm	Multifocal (limited within 2 cm of the index lesion)	Clinically multifocal, > 3 cm	Multifocal (> 2 cm from the index lesion)
LVSI	No	Not allowed	Limited/focal	Not allowed	Extensive	Allowed
ER status	Positive	Any	Negative	Any	Any	Any
Surgical margins	≥ 2 mm	≥ 2 mm	< 2 mm	< 2 mm	Positive	Positive
Nodal factors						
N stage	pN0 (i-, i+)	pN0	pN0 (i-, i+)	pN1mi, pN1a	≥ pN1	pNx, ≥ pN2a
Nodal surgery	SN biopsy or ALND				None performed	
Neoadjuvant therapy	Not allowed	Not allowed	Not allowed	Not allowed	If used	If used

DCIS: Ductal carcinoma in situ; EIC: Extensive intraductal component; LCIS: Lobular carcinoma *in situ*; ASTRO: American Society for Radiation Oncology.

Therefore, this technique may not be suitable for small breast size. The most commonly used regimen is 3.4 Gy per fraction, given twice daily, total of 34 Gy over 5 consecutive days^[58]. Potential advantage of this technique is that final pathology is known. Multi-lumen balloon catheters are more suitable for irregularly shaped lumpectomy cavities^[62]. The morbidity rates were significantly higher in patients treated with intracavitary brachytherapy with reported infection rates of 9.5% and seroma formation of 26.8% than IORT which were 1.3% and 12.9%, respectively^[63,64]. Fat necrosis was observed less compared to interstitial brachytherapy^[65,66].

External beam radiotherapy: The newest of the three major techniques with the most amounts of ongoing randomized studies is APBI with 3DCRT or IMRT. Potential advantages include noninvasiveness, knowledge of final pathology, a more homogenous dose distribution, widespread availability, less user experience, less seroma formation and infection. Additionally irregular cavities can be treated without concern for distance from the skin^[55,67]. The most common regimen is 38.5 Gy in 10 fractions given twice daily over 5 d. Shortcomings are the delivering more radiation to uninvolved quadrants of the breast and critical organs compared to other forms of APBI, short follow-up, uncertainty regarding the optimal dose and fractionation, and patient set-up requirement before each fraction.

Intra-operative radiotherapy: The least prevalent

technique is IORT using electrons (Liac[®], Mobetron[®], or Novac-7[®]; 3-10 MeV) or low energy X-rays (Intrabeam[®]; 50 kV). This technique is most commonly used in Europe and firstly used as a boost treatment. Most commonly applied after quadrantectomy and sentinel lymph node biopsy. A single fraction treatment can be delivered with either electrons (21 Gy in one fraction) or low energy photons (20 Gy in one fraction) immediately after surgery in the operating room^[68]. Direct visualization of the operative bed before treatment delivery reduces the likelihood of missing the target. This modality allows shielding of the skin. The potential disadvantages include increased operating times, the lack of final pathological result before delivering the RT, technical expertise and limited availability of this technique. Long-term radiobiological and cosmetic effects of such a single high fraction dose to the breast are largely unknown; however, an acceptable toxicity is achieved based on a randomized trial and a large, nonrandomized cohort studies^[63,69]. The risk of toxicity was low: 1.3% infections, 12.9% seroma formation, and 4.2% fat necrosis^[63].

Accelerated partial breast irradiation trials: Multiple modern phase II studies regarding APBI have reported promising local control and excellent cosmetic results^[57,70-77]. These studies showed 3%-6% of patients with 5-year local recurrence rates and 56%-99% of patients with good or excellent cosmesis. However, many Phase III studies have not been completed and primary

Table 3 Prospective randomized phase III trials of Accelerated Partial Breast Irradiation

Institution/trial	Number of patients	Inclusion criteria	Control arm	Experimental arm
National Institute of Oncology, Budapest, Hungary ^[80]	258	Wide local excision, > 40 yr, Tm ≤ 20 mm, Invasive ductal carcinoma (non-lobular), Node negative, Margin negative	WBRT (50 Gy in 25 fx)	(1) MIB (36.4 Gy in 7 fx) (2) Electrons (50 Gy in 25 fx)
European Institute of Oncology ELIOT ^[63,82]	1305	Quadrantectomy, ≥ 48 yr, Tm ≤ 2.5 cm, Invasive carcinoma, Node negative	WBRT (50 Gy in 25 fx) ± 10 Gy boost	IORT (21 Gy in 1 fx, electrons up to 9 MeV)
TARGIT-A ^[69,81]	3451	Lumpectomy, ≥ 45 yr, Invasive ductal carcinoma (non-lobular), Node negative	WBRT 40–56 Gy ± 10–16 Gy boost	IORT (20 Gy in 1 fx, low-energy X-rays)

Tm: Tumor; WBRT: Whole breast radiotherapy; fx: Fraction; MIB: Multicatheter interstitial brachytherapy; IORT: Intraoperative radiotherapy; DCIS: Ductal carcinoma *in situ*; 3DCRT: Three dimensional conformal radiotherapy; Gy: Gray.

outcome data of the largest randomized trial of WBRT *vs* APBI with the longest follow-up (NSABP B-39/RTOG 0413) is not yet reported. Regarding the efficacy of APBI in a higher risk population of patients, this trial including patients with > 18 years of age, DCIS, 1–3 positive lymph nodes, and ER negative tumors will provide valuable data on literature. This trial also allows comparison of the efficacies and toxicities of three most common techniques of APBI: MammoSite®, MIB and 3DCRT.

So far, only 5 randomized controlled trials have presented the final results of a completed trial^[69,78–81] (Table 3). The first two published studies from the United Kingdom have important limitations including patient selection criteria and variety of techniques^[78,79]. At 8-year follow-up, local recurrence was increased using APBI. However, surgical margins and axillary nodal status were not evaluated and larger tumors (< 4 cm) were included in the Christie Hospital study^[78]. Similarly, in the Yorkshire Hospital trial, surgical margin status was not assessed^[79]. After the publication of these trials, there has been growing interest and published studies on APBI using more strict patient selection criteria and modern radiation technique. The first of these studies was Hungarian trial compared WBRT with APBI using either HDR MIB or electrons in 258 patients with early stage breast cancer^[80]. At a median follow-up of 66 mo, the 5-year local recurrence rate was 4.7% for APBI and 3.4% for WBRT arm ($P = 0.50$). Excellent to good cosmesis was noted in 77.6% and 62.9%, respectively ($P = 0.009$). There were no significant differences in disease-free or overall survival. Since another study opened with the same patients group covering GEC-ESTRO trial, this study was stopped early. The second completed randomized trial, the TARGIT-A trial ($n = 2232$), compared WBRT with single dose IORT with or without boost after BCS^[69]. With a median follow-up of 2 years, the estimated 4-year local recurrence rate was 1.2% for IORT group and 0.95% for WBRT group ($P = 0.41$). The incidence of major toxicities were 3.9% and 3.3%, respectively ($P = 0.44$). Fourteen percent of patients received WBRT in addition to IORT according to the final pathology report. Five-year results of this trial recently published^[81]. Supplemental WBRT after IORT was applied in 15.2% of patients who received IORT in

the prepathology stratum. The 5-year risk for local recurrence in the conserved breast was 3.3% for IORT *vs* 1.3% for WBRT ($P = 0.042$). Overall, breast cancer mortality was similar between two groups (2.6% *vs* 1.9%, $P = 0.56$) but there were significantly fewer non-breast cancer deaths with IORT (1.4% *vs* 3.5%, $P = 0.0086$). Grade 3–4 skin complications were significantly reduced with IORT ($P = 0.029$). The last randomized trial was reported by Veronesi *et al*^[82] in 2013. They randomized 1305 patients after BCS to WBRT or IORT with electrons (21 Gy). After a median follow-up of 5.8 years, 35 patients in the IORT group and four patients in the WBRT group had had an IBTR ($P < 0.0001$). Five-year overall survival did not differ between the groups (96.8% in the IORT *vs* 96.9% in the WBRT, $P = 0.59$). Skin complications were significantly reduced with IORT ($P = 0.0002$). However, longer follow-up is required before routinely adopting these modern radiation techniques into clinical practice.

With regard to cosmesis, the analysis of all major studies shows conflicting data about the outcomes with APBI. Vicini *et al*^[83] reported the long-term experience of APBI with Mammosite A total of 1440 patients were treated. With a median follow-up of 4.5 years, 5-year local recurrence rate was 3.8% and 91% of patients had a good or excellent cosmetic result. The prospective study by Jaggi *et al*^[84] reported an early closure of an APBI study with IMRT. They showed unacceptable cosmesis in 7 of 34 patients with a median follow-up of 2.5 years. Toxicity of 3DCRT APBI was reported by Hepel *et al*^[85] in accordance with the technique and dose-volume constraints of the NSABP/RTOG 0413 protocol. At 15 mo, grade 2–4 late toxicity was observed in 10% of patients. The preliminary results of the RAPID study was presented at American Society for Radiation Oncology (ASTRO) 2012 and showed that the toxicity with 3DCRT APBI (32%) was more severe than WBRT (19%) at 3 years^[86]. In another prospective study ($n = 50$), 54% of incidence of moderate to severe fibrosis and 35% of fat necrosis was seen with long-term follow-up after the use of interstitial brachytherapy^[65]. The total dose was significantly correlated with the poor cosmetic results. Livi *et al*^[87] detected significant improvements in acute grade 1–2 skin toxicity, favoring APBI with IMRT over convention-

Table 4 Prospective randomized phase III trials of whole breast radiotherapy vs conventional fractionation radiotherapy

Institution/trial	N	Median F/U	Eligibility criteria	Treatment arms	Primary endpoint	Secondary endpoint
Royal Marsden Hospital/ Sutton and Gloucestershire Oncology Centre ^[90,91]	1410	5 yr ¹	Invasive breast cancer, T1-3N0-1M0, < 75 yr, BCS (complete macroscopic resection), Level II / III AD	50 Gy in 25 fx 39 Gy in 13 fx 42.9 Gy in 13 fx	Late changes in breast appearance	Palpable breast induration Ipsilateral tumor recurrence
UK START A ^[92,94]	2236	9.3 yr	Invasive breast cancer, T1-3aN0-1M0, > 18 yr, Clear tm margins (≥ 1 mm), No immediate surgical reconstruction, Available for follow-up	50 Gy in 25 fx 41.6 Gy in 13 fx 39 Gy in 13 fx	Loco-regional tumor recurrence	Late normal tissue effects QOL
UK START B ^[93,94]	2215	9.9 yr	Invasive breast cancer, T1-3aN0-1M0, > 18 yr, Clear tm margins (≥ 1 mm), No immediate surgical reconstruction, Available for follow-up	50 Gy in 25 fx 40 Gy in 15 fx	Loco-regional tumor recurrence	Late normal tissue effects QOL
Ontario Clinical Oncology Group ^[95]	1234	12 yr	Invasive breast cancer, BCS + Level I / II AD, Tm ≤ 5 cm, Negative axillary nodes, Maximum width of breast tissue ≤ 25 cm, No multicentric disease	50 Gy in 25 fx 42.5 Gy in 16 fx	Local recurrence	Regional and distant recurrence Second cancers Breast cosmesis Late toxic effects of radiation

¹Minimum follow-up. N: Number of patients; F/U: Follow-up; WBRT: Whole breast radiotherapy; RT: Radiotherapy; BCS: Breast conserving surgery; AD: Axillary dissection; fx: Fraction; Gy: Gray; UK START: United Kingdom Standardization of Breast Radiotherapy; QOL: Quality of life.

ally fractionated WBRT. The preliminary results of the NSABP B-39/RTOG 0413 trial also have been reported equivalence of cosmesis to WBRT^[88].

Veronesi *et al.*^[63] reported on 1822 patients treated with IORT (electron, 21 Gy) after quadrantectomy. With a mean follow-up of 36.1 mo, the local recurrence rate and the recurrence rate outside the treatment area were 2.3% and 1.4%, respectively. Recently, ELIOT study was evaluated regarding GEC-ESTRO recommendations and local recurrence rates were reported^[89]. They found that 5-year local recurrence was 1.9% for good candidates, 7.4% for possible candidates, and 7.7% for contraindication groups. It clearly shows for this technique that accurate patient selection is so important. In order to reach a conclusion that APBI is an acceptable alternative to WBRT, further studies with longer follow-up are needed. Until this date; when treating patients with APBI, consensus guidelines should be considered.

HYPOFRACTIONATED WHOLE BREAST RADIOTHERAPY

Hypofractionated RT involves fewer treatments, delivers a higher dose per treatment, and shorter overall treatment time (approximately 5 wk) compared to conventional RT. The role of hypofractionated WBRT after BCS has been clearly defined by four prospective randomized trials (Table 4)^[90-95]. The Royal Marsden Hospital and Sutton and Gloucestershire Oncology Centre trial randomizing patients in the same 5-wk length of treatment between conventional RT (50 Gy in 25 fractions) and hypofractionated WBRT (39 Gy or 42.9 Gy in 13 fractions)^[90,91]. There was a significant reduction in the rate of local recurrence using hypofractionated WBRT (12.1% for 50 Gy, 14.8% for 39 Gy, and 9.6% for 42.9 Gy; $P = 0.027$). However,

there was a statistically significant change in breast appearance with the largest daily fraction size to 42.9 Gy compared with 39 Gy and 50 Gy. START trials (A and B) reported the experience of WBRT and hypofractionation^[92,93]. Trial A compared 50 Gy in 25 fractions, 41.6 Gy in 13 fractions, or 39 Gy in 13 fractions within same 5 wk length of treatment^[92]. Trial B compared 50 Gy in 25 fractions over 5 wk *vs* 40 Gy in 15 fractions over 3 wk^[93]. The 5-year local control, disease free survival, and overall survival with the hypofractionation arms similar to conventional RT arm. Ten-year results of these studies have been published recently and similar breast cancer related outcomes have been reported^[94]. In trial A, moderate or marked breast induration, telangiectasia, and breast edema were significantly less common in the 39 Gy group than in the 50 Gy group. Normal tissue effects did not differ significantly between 41.6 Gy and 50 Gy groups. In trial B, breast shrinkage, telangiectasia, and breast edema were significantly less common in the 40 Gy group than in the 50 Gy group. Whelan *et al.*^[95] randomized 1234 patients to either 42.5 Gy in 16 fractions over 22 d *vs* 50 Gy in 25 fractions over 35 d. At 10 years, a non-significant trend was seen for a lower local recurrence in the hypofractionated arm than in the conventional RT arm (6.2% and 6.7%, respectively). There were no differences in the survival, breast cancer mortality, and cosmetic outcomes. Additionally, ongoing UK FAST trial comparing 50 Gy in 25 fractions *vs* 28.5 or 30 Gy in 5 once-weekly fractions of 5.7 or 6 Gy, respectively^[96]. Preliminary results of this study showed inferior outcome for the ultra short fractionation regimen.

In a meta-analysis of the START A and B trials and pilot study from the Ontario Clinical Oncology Group found no significant difference between the hypofractionated WBRT and conventional RT for grade 3 tumors^[97].

Cochrane review comparing the major trials of hypofractionated WBRT with conventional RT have shown that there is no difference in local recurrence rate (RR, 0.97; $P = 0.78$), breast appearance (RR, 1.17; $P = 0.09$), or 5-year survival (RR, 0.89; $P = 0.16$)^[98]. However, acute skin toxicity was significantly lower with conventional RT (RR, 0.21; $P = 0.007$).

Despite the successful outcomes of hypofractionated WBRT, there are many unanswered questions regarding this issue. Firstly, all of these randomized studies did not have routine boost irradiation which was standard after WBRT for invasive breast cancer. Therefore, the optimal boost method after hypofractionated WBRT is still unknown. However, three phase I - II studies have reported favorable early local control and cosmesis of hypofractionated WBRT with a concurrent boost for early-stage breast cancer^[99-101]. Grade 3 or higher skin toxicity was not reported in these trials. Secondly, patients who underwent hypofractionated WBRT are often low risk patients, and have small breast size and small chest wall separation. Especially, application of this technique to high risk patients whom required chemotherapy remains investigational until mature data from IMPORT and RTOG 1005 can provide efficacy and safety of hypofractionated WBRT in this group. Finally, Coles *et al.*^[102] suggested that hypofractionated WBRT should be applied to only right-sided breast cancer to reduce the RT doses per fraction received by the heart and coronary arteries.

Nowadays, the ASTRO has published guidelines for the implementation of hypofractionated WBRT in early-stage breast cancer^[103]. Hypofractionated WBRT can be an acceptable treatment option outside of a clinical trial including patients with pT1-2 tumors, N0 nodal disease, age > 50 years old, patients who do not receive chemotherapy, and patients who do not require tumor bed boost.

CONCLUSION

Currently, phase III randomized trials demonstrated superiority of IMRT over conventional techniques in terms of both acute and late complications after breast conserving surgeries. Dosimetric trials showed that IMRT also improves breast and regional lymphatic coverage while decreasing radiation doses to heart, lungs, and contralateral breast tissues compared to old-fashioned radiotherapy techniques.

Hypofractionated regimens such as APBI may improve therapeutic index after breast conserving surgery. Furthermore, the duration of therapy will be shorter, and the workload in radiotherapy department will be minimized by those hypofractionated regimens. However, current standard of care after breast conserving surgery is still whole breast irradiation, not APBI. The role of hypofractionated regimens will be defined by mature results of both completed and ongoing randomized trials in the next decade.

Finally, it is noteworthy that quality assurance is cru-

cial for the application of those challenging radiotherapy techniques. Even minor errors may result in catastrophic outcomes. Therefore, planning and implementing of modern radiotherapy techniques in breast cancer should be carried out with maximal care.

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WJCO 5th Anniversary Special Issues (2): Breast cancer

Therapeutic options for HER-2 positive breast cancer: Perspectives and future directions

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Abstract

During the last 15 years we have witnessed an unprecedented expansion in the drugs developed to target human epidermal growth factor receptor-2 (HER-2) positive breast cancer. Trastuzumab, pertuzumab, ado-trastuzumab emtansine and lapatinib are currently food and drug administration (FDA)-approved for the treatment of breast cancer patients with HER-2 over-expressed. However, given the amount of information gathered from years of uninterrupted clinical research, it is essential to have periodic updates that succinctly recapitulate what we have learnt over these last years and help us to apply that information in our daily practice. This review will pursue that objective. We will summarize the most relevant and updated information

related to the state of the art management of HER-2 positive breast cancer in all the clinical scenarios including the adjuvant, neoadjuvant and metastatic settings. But we will also critically appraise that literature in order to highlight some key clinical concepts that should not be overlooked. Lastly, this review will also point out some of the most promising strategies that are currently being tested and may soon become available.

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Key words: Breast cancer; Human epidermal growth factor receptor-2; Trastuzumab; Pertuzumab; Ado-trastuzumab; Lapatinib

Core tip: This is a review manuscript with the most updated information regarding the state of the art management of human epidermal growth factor receptor-2 positive breast cancer. It summarizes the most relevant and updated information derived from more than 40 phase II and III clinical trials that constitute the theoretical framework to support our daily practice. It also highlights some key clinical concepts that should not be overlooked by critically appraising the current literature. Finally, it gives the reader with a compilation of potential new agents that are currently being tested and may soon become the next step in the battle against this disease.

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INTRODUCTION

Breast cancer is the most prevalent cancer in women, representing approximately 25% of all new cancers worldwide and accounting for one sixth of the total cancer-related deaths^[1]. Human epidermal growth factor receptor-2 (HER-2) positive breast cancer represents a special subtype that has clear epidemiological, clinical, molecular and prognostic differences that make it a separate entity with recognized worse prognosis and poor response to conventional chemotherapy agents alone.

The epidermal growth factor receptor (EGFR) family is composed by four different receptors: EGFR (ErbB1/HER-1), ErbB2 (HER-2/Neu), ErbB3 (HER-3) and ErbB4 (HER-4)^[2]. These membrane receptors have an intracellular domain with tyrosine kinase activity. Following the union of its ligands, ErbB receptors are activated by dimerization which can happen between identical or different type of receptors, a process named homo- and hetero-dimerization, respectively^[3]. Following dimerization, multiple tyrosine residues located in the membrane receptors become phosphorylated, ultimately leading to activation of downstream signaling pathways. EGFR activation has been linked to the regulation of key processes involved in tumor growth, proliferation, differentiation, adhesion, motility and migration. Multiple intracellular substrates participate in essential pathways such as Ras, Raf, mitogen-activated protein kinase (MAPK) and PI3K/AKT^[4-6]. To understand the role of HER-2 in breast cancer, it is important to highlight that this is a ligand-free receptor. HER-2 is always present in an active configuration and prepared to dimerize with any other family member. In breast cancer cells, HER-2 and EGFR are frequently over-expressed, conferring an aggressive tumor behavior and consequently, increased mortality in this population^[7]. HER-2 can be amplified in 20%-25% of breast cancers and is associated with adverse prognostic outcomes in early and advanced disease. The study of HER-2 and its intracellular and extracellular domains has led us into a deeper understating of the tumor biology and helped to develop pharmacological agents able to block this pathway.

Trastuzumab (Herceptin[®], Genentech, United States/Hoffman-Roche, Switzerland) was the first monoclonal antibody approved for breast cancer treatment directed against HER-2. It binds to HER-2 in its extracellular domain. Pertuzumab (Perjeta[®], Genentech, United States/Hoffman-Roche, Switzerland) is a humanized recombinant monoclonal antibody that binds HER-2 at a different extracellular domain than trastuzumab. Trastuzumab blocks homo-dimerization but cannot inhibit hetero-dimerization. Pertuzumab prevents also hetero-dimerization, resulting in more potent growth inhibition^[8]. Ado-trastuzumab emtansine (Kadcyla[®], Genentech, United States/Hoffman-Roche, Switzerland) is a conjugation of trastuzumab with a potent microtubule inhibitor agent, derivative of maytansine (DM-1)^[9]. This molecule has 3 properties, anti-HER-2 inhibition by trastuzumab, cytotoxic effect by DM-1 and certain level of tissue specific-

ity by directing the cytotoxic agent only to those cells that express HER-2. It has recently being approved for refractory metastatic disease. Lapatinib (Tykerb TM, GlaxoSmithKline, Research Triangle, NC, United States) is the only intracellular blocker approved. It is a dual reversible tyrosine kinase inhibitor of HER-2 and EGFR. It acts on both receptors simultaneously, achieving greater inhibitory effects^[10].

Given the enormous amount of information accumulated from almost 20 years of continuous basic and clinical research, it is important to have periodic updates in this topic that can succinctly recapitulate what we have learnt over the last years and help us to apply that information in our daily practice. This review will pursue that objective and also will allow the reader to envision some of the promising agents that may soon become part of our armament against this disease.

STATE OF THE ART ANTI-HER-2 THERAPY

In this section we will summarize all those relevant clinical trials that constitute the theoretical framework to support our daily practice. For practical reasons we will subdivide this section according to the clinical setting: adjuvant, neoadjuvant and metastatic disease. Also, given the breadth of this topic we will mainly focus only on phase III and some phase II clinical trials. Tables 1-3 recap the most important published clinical trials.

Adjuvant treatment

In the adjuvant scenario, treatment with trastuzumab is the standard of care for patients with HER-2 over-expressing breast cancer. Trastuzumab can be administered in combination with paclitaxel or docetaxel following an anthracycline-based chemotherapy (*i.e.*, doxorubicin and cyclophosphamide) or be given concurrently with carboplatin and docetaxel. Several phase III trials have consistently validated trastuzumab as the cornerstone of adjuvant chemotherapy. The question of the optimal therapy duration has been addressed and even though the answer is still pending, the actual evidence suggests that treatment for one year is probably the most appropriate. Cardiac toxicity is a major concern since anti-HER-2 therapy can result in decreased left ventricular ejection fraction (LVEF) and symptomatic heart failure. However, this is usually reversible after treatment discontinuation.

The National Surgical Adjuvant Breast and Bowel Project (NSABP) B-31 and the North Central Cancer Treatment Group (NCCTG) N9831 were two pivotal studies^[11]. The NSABP B-31 compared four cycles of doxorubicin and cyclophosphamide (AC) followed by four cycles of paclitaxel every three weeks to the same regimen plus trastuzumab given for 52 wk starting concurrently with paclitaxel. The NCCTG N9831 randomized patients to receive four cycles of AC followed by weekly paclitaxel for 12 cycles with or without trastuzumab, administered concurrently or sequentially

Table 1 Selected clinical trials in the adjuvant setting for human epidermal growth factor receptor-2 positive breast cancer

Drug or study name	Population included	No. of patients	Comparison	Median follow-up	DFS	OS	CHF/ Drop LVEF	Ref.
Trastuzumab (H)								
NCCIG N9831	LN (+) or high risk LN (-)	1087 949	AC → T <i>vs</i> AC → T → H (52 wk) <i>vs</i> AC	72 mo	71.8% (5-yr) 80.1% (5-yr)	88.4% (5-yr) 89.7% (5-yr)	0%/0% 2.2%/7%	Perez <i>et al</i> ^[14]
HERA	LN (+) or high risk LN (-)	954 1552 1553 1697	→ TH (H then 40 wk) Std QT → H (52 wk) <i>vs</i> Std QT → H (104 wk) <i>vs</i> Std QT → Observation	96 mo	84.4% (5-yr) 75.9% (5-yr)	91.9% (5-yr) 86.9% (5-yr)	1.5%/3.6% 1%/7.2%	Goldhirsch <i>et al</i> ^[16]
FINHER	LN (+) or high risk LN (-)	58 58 54 61	Docetaxel → FEC <i>vs</i> Vinorelbine → FEC <i>vs</i> Docetaxel + H → FEC <i>vs</i> Vinorelbine + H → FEC	62 mo	74.1% (5-yr) 72.0% (5-yr) 92.5% (5-yr) 75.2% (5-yr)	82.0% (5-yr) 82.8% (5-yr) 94.4% (5-yr) 88.4% (5-yr)	1.7%/10.5% (QT only) 0.9%/6.8% (QT + H)	Joensuu <i>et al</i> ^[18]
BCIRG 006	LN (+) or high risk LN (-)	1073 1074 1075	AC → Docetaxel <i>vs</i> AC → Docetaxel + H <i>vs</i> TCH	65 mo	75% (5-yr) 84% (5-yr) 81% (5-yr)	87% (5-yr) 92% (5-yr) 91% (5-yr)	0.7%/11.2% 2.0%/18.6% 0.4%/9.4%	Slamon <i>et al</i> ^[19]
PACS 04	LN (+)	260	FE100C or ED75 → Obser <i>vs</i>	62 mo	77.9% (3-yr)	96% (3-yr)	0.3%/14.2%	Spielmann <i>et al</i> ^[20]
PHARE	HER-2 (+) early breast cancer	268 1690 1690	FE100C or ED75 → H Std QT → H (26 wk) <i>vs</i> Std QT → H (52 wk)	42.5 mo	80.9% (3-yr) 91.1% (2-yr) 93.8% (2-yr)	95% (3-yr) 96.1% (2-yr) 94.5% (2-yr)	1.5%/35.4% 5.7% (both) 1.9% (both)	Pivot <i>et al</i> ^[21]
Lapatinib (L)								
TEACH	Stage I - III c - H naïve	1230 (HER-2 +) 1260 (HER-2 +)	Std QT → L (52 wk) <i>vs</i> Std QT → Observation	47.4 mo 48.3 mo	87% (4-yr) 83% (4-yr)	94% (4-yr) 94% (4-yr)	3.0% (both) 3.0% (both)	Goss <i>et al</i> ^[22]

LN: Lymph nodes; AC → T: Adriamycin cyclophosphamide paclitaxel; FEC: 5-FU epirubicin cyclophosphamide; ED: Epirubicin docetaxel; Std QT: Standard chemotherapy; OS: Overall survival; DFS: Disease free survival; CHF: Cardiac heart failure; LVEF: Left ventricular ejection fraction.

Table 2 Selected clinical trials in the neo-adjuvant setting for human epidermal growth factor receptor-2 positive breast cancer

Study	Neo-adjuvant chemotherapy	No. of patients	Pathological complete response (%)	Comments	Ref.
Trastuzumab (H)					
MD Anderson Group	T → FEC <i>vs</i> T → FEC + H	19 <i>vs</i> 23	26% (95%CI: 9%-51%) <i>vs</i> 65% (95%CI: 43%-84%)	Probably the first study to emphasize better pCR with H	Buzdar <i>et al</i> ^[24]
The NOAH Trial	A + T → T → CMF <i>vs</i> A + T → T → CMF + H	117 HER-2 (+) <i>vs</i> 118 HER-2 (+)	22% (95%CI: not reported) <i>vs</i> 43% (95%CI: not reported)	Not originally designed to test the effects of neoadjuvance	Gianni <i>et al</i> ^[26]
The TECHNO Trial	EC → TH	217	38.7% (95%CI: 32%-45%)	Suggest pCR correlate with DFS	Untch <i>et al</i> ^[27]
The Z1041 Trial	FEC → TH <i>vs</i> T + H → FEC + H	138 <i>vs</i> 142	56.5% (95%CI: 48%-65%) <i>vs</i> 54.2% (95%CI: 46%-62%)	Concurrent use of H with anthracyclines is not better	Buzdar <i>et al</i> ^[28]
The HannaH Trial	Doc + H (SQ) → FEC + H <i>vs</i> Doc + H (IV) → FEC + H	260 <i>vs</i> 263	45.4% (95%CI: 39%-52%) <i>vs</i> 40.7% (95%CI: 35%-47%)	H can be administered subcutaneously	Ismael <i>et al</i> ^[30]
Lapatinib(L) +/- (H)					
The GeparQuinto Trial	ECH → TH <i>vs</i> ECL → TL	309 <i>vs</i> 311	30.3% (95%CI: 25%-36%) <i>vs</i> 22.7% (95%CI: 18%-28%)	Lapatinib is less effective than H	Untch <i>et al</i> ^[31]
The NeoALLTO Trial	TH <i>vs</i> TL <i>vs</i> THL	149 <i>vs</i> 154 <i>vs</i> 152	29.5% (22%-37%) <i>vs</i> 24.7% (22%-37%) <i>vs</i> 51.3% (43%-59%)	Suggested that combination H + L could be quite effective	Baselga <i>et al</i> ^[32]
The NSABP B-41 Trial	AC → TH or TL or THL	181 <i>vs</i> 174 <i>vs</i> 174	52.5% (50%-59.5%) <i>vs</i> 53.2% (45%-60%) <i>vs</i> 62.0% (54%-69%)	H + L no better. All patients received anthracyclines	Robidoux <i>et al</i> ^[33]
Pertuzumab (P)					
The NeoSphere Trial	Do + H <i>vs</i> Do + P + H <i>vs</i> Do + P <i>vs</i> P + H	107 <i>vs</i> 107 <i>vs</i> 107 <i>vs</i> 96	29.0% (21%-38.5%) <i>vs</i> 45.8% (36%-56%) <i>vs</i> 24.0% (16%-34%) <i>vs</i> 16.8% (10%-25%).	Combination P + H result in better pCR.	Gianni <i>et al</i> ^[34]
The Tryphaena Trial (Abstract Only)	FEC + HP → Do + HP <i>vs</i> FEC → Do + HP <i>vs</i> TCHP	223 patients in total	62% <i>vs</i> 57% <i>vs</i> 66%	TCH + P is an active combination	N/A

T: Paclitaxel; F: 5-FU; E: Epirubicin; C: Cyclophosphamide; A: Adriamycin; M: Methotrexate; Do: Docetaxel; TC: Docetaxel carboplatin.

Table 3 Selected clinical trials in metastatic human epidermal growth factor receptor-2 positive breast cancer

Drug or study name	Population included	No. of patients	Comparison	Median OS (mo)	Median TTP (mo)	ORR	1-yr Survival	Ref.
Single agents								
Trastuzumab (H)	Phase II, first line, MBC	114	None-2 doses of H used	24.4	3.8	26% (18%-34%)	N/A (approximately 65%)	Vogel <i>et al</i> ^[45]
Ado-trastuzumab	Phase II, refractory MBC	110	None	N/A	6.9 (4.2-8.4)	34% (26%-44%)	N/A	Krop <i>et al</i> ^[48]
Anti-HER-2 + QT H + Paclitaxel (T)	Phase III, first line, MBC	469	QT (AC or T) + H vs QT	25.1 vs 20.3	7.4 vs 4.6	50% vs 32%	78% vs 67%	Slamon <i>et al</i> ^[44]
Cont. Anti-HER-2 after failing 1st line								
Lapatinib (L)	Phase III, failed to H	324	Cape + L vs Cape alone	N/A	8.4 vs 4.4	22% vs 14%	N/A (approximately 60%)	Geyer <i>et al</i> ^[54]
EMILIA Trial (Ado-trastuzumab)	Phase III, MBC who failed TH	991	Ado-T vs Cape + L	30.9 vs 25.1	9.6 vs 6.4	43.6% vs 30.8%	85% vs 78%	Verma <i>et al</i> ^[55]
Dual Anti-HER-2 CLEOPATRA	Phase III, first line, MBC	808	Do + H + P vs Do + H	Not reached	18.5 vs 12.4	80% vs 69%	N/A (approximately 90%-95%)	Baselga <i>et al</i> ^[57]
Lap + Trastuzumab	Phase III, failed to H	296	L + H vs L alone	11.8 vs 8.9	2.75 vs 1.85	10% vs 7%	70% vs 36%	Blackwell <i>et al</i> ^[59]
H + Pertuzumab (P)	Phase II, failed to H	66	None-H + P single arm	N/A	5.5	24.20%	N/A	Baselga <i>et al</i> ^[61]
Anti-HER-2 + AI Anastrozole + H	Phase III, HR and HER-2 positive, 1 st line in MBC	207	Anastrozole + H vs Anastrozole alone	28.5 vs 23.9	4.8 vs 2.4	20% vs 6.8%	N/A (approximately 78%)	Kaufman <i>et al</i> ^[63]
Letrozole + L	Same	219	Letrozole + L vs Letrozole alone	33.3 vs 32.3	8.2 vs 3.0	28% vs 15%	N/A	Johnston <i>et al</i> ^[64]

QT: Chemotherapy; AI: Aromatase Inhibitor; MBC: Metastatic breast cancer; HR: Hormones receptor; Do: Docetaxel; AC: Adriamycin cyclophosphamide; T: Paclitaxel; OS: Overall survival; TTP: Time to progression; N/A: Not available or not reported.

to paclitaxel, for 52 wk. A joint analysis of these clinical trials was first presented in 2005. Initially both trials were designed to include patients with node positive breast cancer. But later on, the NCCTG trial included patients with high-risk node negative disease defined as tumors ≥ 2 cm and positive for hormone receptors or tumors larger than 1 cm with negative hormone receptors. The primary endpoint of these trials was disease-free survival (DFS) and overall survival (OS) was part of the secondary endpoints. On the initial report with a median follow-up of two years the hazard ratio (HR) for DFS was 0.48 (95%CI: 0.39 to 0.59; $P < 0.001$). At four years, 85.3% of patients treated with trastuzumab were alive and free of disease compared to only 67.1% in the control group. Mortality was reduced by 33%. Updated results were consistent with previous observations^[12]. The final analysis of these studies was presented at the 2012 San Antonio Breast Cancer Symposium (SABCS) and reported a 10-year DFS of 73.7% vs 62.2% ($P < 0.001$) and a OS of 84% vs 75.2% ($P < 0.001$) all favoring trastuzumab^[13]. Overall, treatment with trastuzumab resulted in a 40% risk reduction benefit in terms of 10-year DFS and 37% in OS. The NCCTG trial compared, as well, the efficacy of concurrent vs sequential administration of trastuzumab, showing a trend toward improvement in DFS in the concurrent arm^[14]. However, sequential was still better

than placebo ($P < 0.001$).

Published simultaneously, the Herceptin Adjuvant Trial (HERA), a randomized phase III trial designed to compare adjuvant treatment with trastuzumab for one or two years vs observation reported similar results. At the first interim analysis DFS was superior in the trastuzumab treated population^[15]. An absolute 8% difference between arms in DFS events was achieved ($P < 0.001$). Unlike other studies, patients included in this trial had already undergone adjuvant (90%) or neoadjuvant treatment and could have nodal involvement. Following the interim analysis, 52% of patients in the observation arm crossed over to trastuzumab before an event had occurred^[16]. At a median follow-up of 8 years, the HRs for DFS and OS in the trastuzumab arm compared to observation were 0.76 for both outcomes. Importantly, given the persistent benefits in terms of DFS and OS found even after a mean time from randomization to crossover of 22.7 mo, in clinical practice trastuzumab must be used even in delayed scenarios. This study also proved that 2-year adds no benefit to 1-year of treatment, but adds a slight increment in cardiologic adverse events.

The Finland Herceptin (FinHer) trial aimed to determine the role of vinorelbine compared to docetaxel in the adjuvant setting in patients with node positive and high risk node negative breast cancer^[17]. A total of 1010

patients were randomized to treatment with vinorelbine or docetaxel for 3 cycles followed by three cycles of 5-FU, epirubicin and cyclophosphamide. A group of 232 patients with HER-2 amplified tumors were again randomized to receive nine weekly cycles of trastuzumab concurrently with docetaxel or vinorelbine. The primary end point was distant DFS and with a median follow-up of 5 years, it favored treatment with docetaxel over vinorelbine ($P = 0.010$)^[18]. OS also tended to be better in patients treated with docetaxel compared to vinorelbine (39 *vs* 55 death, respectively; $P = 0.086$). In HER-2 positive patients, trastuzumab arms had favorable recurrence free survival irrespectively of the chemotherapy used (80% *vs* 73%; $P = 0.12$). This benefit was maintained when adjusted for nodal involvement and in patients treated with docetaxel over vinorelbine. The main limitation of this trial is the small number of patients with HER-2 positive tumors included, undermining the study power to detect a statistically significant benefit with trastuzumab. Also, even though the results suggested a benefit in patients treated with trastuzumab in combination with chemotherapy, the short course of treatment might have underestimated the real efficacy of the drug in this population.

Cardiotoxicity is the most important adverse effect derived from treatment with trastuzumab, which is worse when combined with anthracyclines. Therefore, there has been a special interest in studying anthracycline-free regimens in order to avoid synergistic deleterious cardiac toxicities. The BCIRG 006 study was designed to provide information on this matter^[19]. This phase III clinical trial randomized 3222 patients with HER-2 positive and node positive or high-risk node negative tumors to treatment with doxorubicin, cyclophosphamide and docetaxel (AC \rightarrow T), the same regimen plus trastuzumab (AC \rightarrow TH) or docetaxel, carboplatin and trastuzumab (TCH). The primary end-point was DFS and secondary end-points were OS, safety and cardiac toxicity. With a median follow-up of 65 mo, 5-year DFS was 84% ($P < 0.001$) in the ACTH arm, 81% ($P = 0.04$) in the TCH arm and 75% in the ACT arm (control). OS was also improved with trastuzumab (92% with ACTH, 91% with TCH and 87% in the ACT; $P < 0.001$ and 0.04, respectively). ACTH was slightly better than TCH, though this study was not designed to compare efficacy between these two regimens. The incidence of cardiac toxicity was five times more with ACTH (2%) compared with TCH (0.4%). Reductions in LVEF, over 10% from basal measurements, were more frequently associated with ACTH than with TCH (18.6 *vs* 9.4%; $P < 0.001$). As well, the rate of symptomatic congestive heart failure favored treatment with TCH ($P < 0.001$). According to these results, anthracycline-free chemotherapy is efficient and is a valid treatment option in patients at high risk of cardiac toxicity. Although the combination of doxorubicin and trastuzumab resulted in better DFS and OS, the difference seemed to be insignificant. To confirm that one regimen is better than the other, further evidence is required.

The French PACS 04 was the only negative study re-

ported^[20]. A total of 3010 patients with early stage breast cancer were randomly assigned to adjuvant treatment with anthracycline-based chemotherapy with or without docetaxel. Patients with HER-2 over-amplified tumors ($n = 528$) were subsequently randomized to receive trastuzumab sequentially every 3 wk. The primary endpoint was DFS. Treatment with trastuzumab resulted in a non-significant 14% reduction in the risk of relapse ($P = 0.41$) and there was no difference in OS. However, 10% of the patients assigned to trastuzumab were never treated and 25% of patients discontinued before the 16th cycle. Also, sequential use seems to be inferior to concurrent.

Duration of adjuvant treatment in HER-2 positive breast cancer is a matter of current discussion. Based on the previously analyzed HERA trial, two years of treatment with trastuzumab is not superior to one year. There is a special interest in investigating whether treatment duration could be shortened. The PHARE trial is a non-inferiority study designed to answer this question. This study evaluated adjuvant treatment length with trastuzumab for 6 mo compared to one year^[21]. A total of 1691 patients were treated with trastuzumab for 12 mo and 1693 for 6 mo after receiving at least 4 cycles of adjuvant chemotherapy. Patients were stratified according to sequential or concurrent treatment and ER status. The primary endpoint was DFS and with a median follow-up of 42.5 mo, 2-year DFS was 93.8% for the 12-mo group and 91.1% for the 6-mo group (HR = 1.28; 95%CI, 1.05-1.56), concluding that 6 mo of treatment did not reach the non-inferiority criteria. However, cardiac events were more common in the 12 mo treatment arm (5.7% *vs* 1.9%; $P < 0.001$). Two other ongoing phase III trials, SHORT-HER (NCT00629278) and PERSEPHONE (NCT00712140), will contribute to clarify this issue. Nonetheless, on the basis of the current available evidence, 12 mo of adjuvant treatment with trastuzumab remains the standard of care.

Lapatinib is currently approved for metastatic disease but its use in the adjuvant setting could be interesting due to its oral bioavailability. The TEACH trial studied the efficacy of lapatinib in trastuzumab-naïve patients as adjuvant treatment^[22]. A total of 3147 patients were randomized to 12 mo of treatment, or until progression, with lapatinib or placebo. DFS was non-significantly prolonged in patients treated with lapatinib (87% *vs* 83%; $P = 0.09$). In patients with centrally confirmed HER-2 status the HR was 0.92 ($P = 0.94$). In conclusion, single agent lapatinib seems to be quite ineffective in the adjuvant treatment. The ongoing ALTO trial is evaluating the efficacy of lapatinib in combination with trastuzumab and lapatinib sequential to trastuzumab *vs* trastuzumab alone. A treatment arm with single agent lapatinib was prematurely closed by an independent data and safety committee (NCT00490139).

Neo-adjuvant treatment

The original rationale behind the use of neo-adjuvant chemotherapy was to attempt tumor volume reduction

whenever the tumor size precluded an optimal surgical resection. However, clinical researchers rapidly understood that pre-surgical chemotherapy could represent an excellent way to assess the real *in vivo* response of the tumor, a fact obviously impossible to achieve in the adjuvant setting. Moreover, it is at least theoretically feasible that the response obtained in the primary tumor could be a good surrogate to what happens with the invisible micrometastases. In that sense, if some cells remain viable in the primary site, then the chances of having resistant micrometastases are higher. On the contrary, complete pathological response (pCR) should correlate with fewer future relapses. This concept has been more strongly established in triple negative breast tumors and though quite appealing intellectually, there are some caveats in this assumption that requires further discussion (see later)^[23].

Probably the first evidence addressing the role of anti-HER-2 therapies in the neo-adjuvant scenario came from a small randomized trial published by the MD Anderson group^[24]. With only 42 patients randomized, the addition of trastuzumab to sequential paclitaxel followed by FEC (P x 4 → FEC x 4) resulted in an impressive 2.5 times higher rate of pCR than chemotherapy alone (66.7 *vs* 25%; *P* = 0.02). Given the small sample, the confidence intervals were wide and also no clinical endpoints were reported. An updated version of the study confirmed the findings^[25]. The results of the NOAH trial, a randomized phase III study, helped to give further enthusiasm to this approach^[26]. The study was originally designed to compare neoadjuvant chemotherapy plus trastuzumab followed by 1-year trastuzumab to neoadjuvant chemotherapy alone in patients with locally advanced or inflammatory HER-2 positive tumors. But with the approval of trastuzumab all patients were allowed to receive this drug as adjuvant therapy. From 238 patients originally randomized to neoadjuvant treatment with or without trastuzumab, the addition of anti-HER-2 therapy improved the pCR from 22% to 43% (*P* < 0.001). Trastuzumab also resulted in a 40% risk reduction of recurrence, progression or death when compared to chemotherapy alone, but this was already known due to the adjuvant studies. Moreover, in 2011 German investigators published the results of an open label, phase II study - TECHNO trial - where 217 patients with HER-2 positive and ≥ 2 cm tumors received four cycles of epirubicin and cyclophosphamide, followed by four cycles of paclitaxel and trastuzumab before surgery^[27]. Complete pathological response was achieved in 38.7% of the cases. However, the most relevant observation from this study was that pCR correlated with better 3-year DFS (88% *vs* 71%; *P* = 0.003) and 3-year OS (96% *vs* 85%; *P* = 0.007). Obviously, the small number of patients and the fact that this was a phase II study limited the generalization of these findings but sparked the idea the pCR could correlate with harder clinical endpoints. Also, the American Z1041 trial randomized 282 women with similar inclusion criteria as the TECHNO study to receive trastuzumab and paclitaxel concurrently with FEC-75 or after this regimen^[28]. Both arms showed a high propor-

tion of pCR (54% and 56%) but the concurrent use of anthracyclines and trastuzumab resulted in a greater drop in the cardiac ejection fraction (2.9 % *vs* 0.8% at 12 wk, respectively). Lastly, similar rates of pCR were described in patients treated with chemotherapy and trastuzumab in the GeparQuattro study (32%) as well as in the HannaH trial (41% and 45% for intravenous *vs* subcutaneous trastuzumab, respectively)^[29,30].

In an attempt to improve the efficacy of anti-HER-2 therapies, some researchers started exploring the use of lapatinib or its addition to trastuzumab. Recently, at least three randomized phase III clinical trials were published testing that hypothesis. In the German GeparQuinto study, 620 patients received four cycles of epirubicin and cyclophosphamide (EC) followed by docetaxel and were randomized to have trastuzumab or lapatinib^[31]. All patients received standard of care trastuzumab for 1-year after surgical resection. Primary outcome was pCR and trastuzumab showed approximately 7% more complete responses than lapatinib (30.3% *vs* 22.7%; *P* = 0.04). Given these results and the significant number of adverse events described in this study, it is unlikely that lapatinib could replace trastuzumab in the neoadjuvant setting; however, dual HER-2 inhibition seems to be a better option. The NeoALLTO international, randomized, phase III study compared the use of single agent lapatinib, trastuzumab or the combination of both in addition to paclitaxel for neo-adjuvant treatment^[32]. Interestingly, the combination arm showed a remarkable improvement in pCR almost duplicating the two other single agent anti-HER-2 arms (51% *vs* 29.5% trastuzumab *vs* 24.7% lapatinib; *P* < 0.001). As expected the addition of lapatinib resulted in worse side effects, mainly related to diarrhea and rash. However, in contraposition to the NeoALLTO, the freshly published NSABP B-41 study showed no statistical difference with the combination of trastuzumab and lapatinib when compared to either drugs used as single agent^[33]. Two issues though deserves further discussion. First, even though the population included in both trials was similar, the chemotherapy regimens were not. In the NSABP study all patient received four cycles of AC and then they were randomized to paclitaxel plus trastuzumab, lapatinib or both. Second, the rates of pCR in all three arms were unusually high (62% for the combination, 53% for trastuzumab and 52.5% for lapatinib).

Up to this date, lapatinib has not received FDA approval to use in the neoadjuvant setting. Nonetheless, the FDA has recently granted accelerated approval to pertuzumab for its use before surgery when combined with trastuzumab and chemotherapy. This controversial decision was based on the results of two phase II clinical trials. The already published NeoSphere trial was a multicenter, open-label, randomized phase II study where 417 patients were randomized to one of four possible arms: pertuzumab (P) + trastuzumab (T) + docetaxel (Do); T + Do; P + Do or P + T alone^[34]. All eligible patients then underwent surgical resection followed by adjuvant FEC and 1-year of trastuzumab. The primary

endpoint was pCR and no information regarding harder clinical outcomes (*i.e.*, DFS, OS, *etc.*) was reported. The three drug arm (P + T + Do) showed the maximal rate of pCR (46%) and was statistically different from T + Do (29%; $P = 0.014$). Pertuzumab + docetaxel resulted in a 24% pCR and the chemotherapy-free arm had a 17% pCR. Importantly, the addition of pertuzumab did not produce any significant drop in the cardiac function (4-5% EF drop across all groups). The second study - TRYPHAENA trial - is only available as an abstract but its importance relies on the fact that it incorporates the trastuzumab + pertuzumab combination to an anthracycline-free regimen^[35,36]. One of its three arms use the very popular TCH regimen (carboplatin, docetaxel and trastuzumab) with pertuzumab for six cycles before surgery followed by one year of trastuzumab. Even though more information will be available when the study become fully published, preliminary data suggest that all three arms achieved > 55% pCR.

The preceding paragraph served to describe the current available evidence supporting the use of anti-HER-2 drugs as a neoadjuvant treatment. But, how should we apply all this information? In our own opinion, there are a couple of important points to emphasize. First, dual blockage of the HER-2 receptor, even without chemotherapy, results in an at least 15% pCR (NeoSphere Trial), meaning that 1 in 6 patients may not need chemotherapy. This certainly represents an attractive option for patients who cannot tolerate more than targeted agents. Second, the addition of chemotherapy leads to a more robust effect with values between 40%-50% when trastuzumab alone is used or even more than 50% when dual blockage is applied. Moreover, anthracyclines seem to play a significant role in HER-2 positive tumors; however, results from NeoALLTO and TRYPHAENA trials suggest that when dual blockage is used, anthracyclines toxicity might be spared. In this regard clinicians have now two options; to follow the NeoSphere protocol that requires the use of FEC post-surgically or to use TCH + pertuzumab (TRYPHAENA). Until further information is available, the anthracycline-free option might better serve the lower risk tumor (*i.e.*, small tumors with negative lymph nodes) or in patients with serious cardiovascular comorbidities and leave the anthracyclines for more advanced disease and younger women. Third, in all of the clinical trials available pCR is markedly diminished in tumors expressing hormone receptors in addition to HER-2.

Nonetheless, probably the most important question to ask ourselves is how reliable is pCR as a valid surrogate for real clinical outcomes. It is important to consider that these drugs are utilized in potentially curable disease and there are still some doubts about whether pCR always correlates with DFS and OS. It has been established by many authors, including the FDA, that pCR is a surrogate of survival in patients with localized breast cancer previously treated with chemotherapy^[37]. This may well be the case for anti-HER-2 therapy when used in this fashion. A Cochrane meta-analysis evaluating preoperative with

postoperative chemotherapy evidenced that the risk of dying among patients who had a pCR was approximately 50% less than that in patients with residual disease^[38]. The FDA has presented in SABCS 2012 and is in process to publish a meta-analysis with 12900 patients enrolled in randomized neoadjuvant trials, with the objective to identify those patients in whom a pCR was most likely to predict survival^[39]. There was a marked correlation between pCR and relapse free survival in all the subgroups analyzed. However, a recent German study showed that pCR was correlated with OS in triple negative breast cancer (TNBC) as well as HER-2 positive and non-luminal tumors but was not the case in the cohort of HER-2 positive and luminal patients^[40]. In this last group pCR may not be a good surrogate for survival; but, from a hypothetical view, it is also true that ER positive disease tends to progress more slowly. It is possible that more events would be needed to have mature results. Perhaps, with longer follow-up curves might separate, becoming more consistent with the meta-analysis presented in SABCS 2012 by Cortazar and colleagues. Up for today, neoadjuvant with anti-HER-2 agents is a valid and approved option especially in those patients with locally advanced, unresectable tumors. Its use in small resectable cancer is probably appropriate but has to be balanced with the practical consideration as well as the patient's own preferences.

Lastly, some studies have also reported on different biomarkers which could predict pCR after neoadjuvant chemotherapy. They include estrogen receptor status, the PI4K pathway and p95HER-2 among others. However, no one is currently validated and available to use. An interesting niche where the use of biomarkers could be tremendously useful would be on patients with residual cancer cells at surgery after optimal neoadjuvant therapy. Since these patients have a poor prognosis, the analysis of the residual tumor cells for predictive biomarkers could unfold potential targets^[41].

Metastatic disease

It has now been close to 15 years since the first publications reporting the use of trastuzumab for metastatic breast cancer^[42-44]. Since those seminal studies, we have made major progress in our understanding of the anti-HER-2 therapies and some concepts should be highlighted.

Anti-HER-2 therapies are active even as single agents: Single agent anti-HER-2 molecules seem to have a modest but consistent activity even when used alone. This has been well proven for the case of trastuzumab (ORR = 15%-25%)^[45], but also for pertuzumab (ORR = 5%)^[46], lapatinib (ORR = 5%-7%)^[47] and ado-trastuzumab emtansine (ORR = 35%)^[48]. For the newer drugs, this holds true even after failing first line trastuzumab containing regimens. The vast majorities are only partial response, but some patients experience sustained stable disease which also adds to the clinical benefit.

The addition of conventional chemotherapeutics maximizes the efficacy of anti-HER-2 drugs: The addition of chemotherapy to anti-HER-2 agents increases the ORR up to 60%-70%. Paclitaxel is probably the most frequently used drug, but the benefit is seen regardless of the type of chemotherapy administered and there is solid evidence to support the combination of trastuzumab with vinorelbine or docetaxel^[49] and even carboplatin^[50]. Lapatinib is also frequently combined with capecitabine^[51].

The HER-2 target should remain blocked even after progression: Patients seem to achieve considerable clinical benefit if the HER-2 axis remained blocked even after experiencing progression from regimes containing anti-HER-2 therapies. This was categorically proven by the German Breast Group in the BIG 305 trial where 146 patients were randomized to capecitabine or capecitabine plus trastuzumab after having progressed to a trastuzumab containing regimen^[52]. The median progression-free survival (PFS) was 3 mo longer (8.2 *vs* 5.6; $P = 0.033$) and there was better ORR (48% *vs* 27%; $P = 0.015$) in the trastuzumab continuation arm. However, updated results showed no significant difference in OS^[53]. These results are somehow comparable with the earlier tested combination of capecitabine and lapatinib. In the study reported by Geyer and colleagues in 2006, patients with locally advanced or metastatic disease who had failed trastuzumab regimens (> 95% of the population enrolled) were randomly assigned to either capecitabine alone or combined with lapatinib^[54]. Time to progression (TTP) was similar in both trials, BIG 305 and the lapatinib study, in terms of the control arm (single agent capecitabine 4.5 and 5.5 mo, respectively) as well as the experimental arm (8.2 mo in trastuzumab + capecitabine and 8.5 mo in lapatinib + capecitabine). Irrespectively of the obvious difference in side effects of each drug and some intrinsic disparities in these two studies, the proof of concept brought by these investigations was the fact that the HER-2 target must continue to be attacked probably indefinitely. In accordance to this principle, a new molecule has recently gained FDA approval for trastuzumab refractory cases. Ado-trastuzumab emtansine, an interesting conjugation between the trastuzumab antibody and the chemotherapy agent DM-1, proved to be better than lapatinib and capecitabine combination. The EMILIA trial randomized 991 patients with metastatic (84%) or locally advanced (16%) disease, who have been previously treated with trastuzumab and a taxane, to T-DM1 or lapatinib plus capecitabine^[55]. PFS, the primary endpoint of the study, was significantly improved with T-DM1 (9.6 *vs* 6.4 mo; $P < 0.001$) as it was OS (31 *vs* 25 mo; $P < 0.001$) and ORR (44 *vs* 31%; $P < 0.001$). Currently, ado-trastuzumab emtansine is considered the best option for patient progressing after first line trastuzumab. Nonetheless, early data from phase II studies showed promising results with ado-trastuzumab as a front line option^[56]. What happened

after progression with T-DM1? Options will include either switching to trastuzumab + lapatinib (see below) or to consider lapatinib and capecitabine. But irrespectively of the option selected, the HER-2 targeted therapy should continue.

Two different anti-HER-2 therapies could be combined to exploit the benefit: Apparently, the conjunction of two different anti-HER-2 agents could result in better outcomes. Three combinations are particularly important: (1) Pertuzumab + trastuzumab + docetaxel; (2) Trastuzumab + lapatinib; and (3) Pertuzumab + trastuzumab alone. The CLEOPATRA study randomly assigned 808 patients with metastatic and chemotherapy naïve HER-2 positive breast cancer to either what was up to that moment the standard of care (trastuzumab + docetaxel) or the same combination plus pertuzumab^[57]. The study was strongly positive with almost 50% prolongation in PFS favoring the experimental arm (18.5 *vs* 12.4 mo; $P < 0.001$). Updated results also confirmed a significant improvement in OS^[58]. Currently, pertuzumab, trastuzumab and docetaxel are considered the standard of care for first line treatment in metastatic disease. Notably, this regimen takes advantage of two of the previously mentioned concepts: dual HER-2 blockage and addition of a chemotherapy agent. However, even without adding a chemotherapeutic, dual blockage is still an active alternative especially on those heavily pretreated patients who may not tolerate chemotherapy. The EGF104900 was an open-label, phase III study where women vastly treated with trastuzumab-containing schemas (median 3 prior regimens) were randomly assigned to single agent lapatinib or lapatinib and trastuzumab^[59]. The dual blockage arm showed longer PFS (12 *vs* 8 wk; $P = 0.008$) as well as OS (14 *vs* 9.5 mo; $P = 0.026$)^[60]. Another appealing strategy tested in phase II setting is the combination of pertuzumab and trastuzumab with no chemotherapy agents. Baselga and colleagues reported a 24% ORR and a median PFS of 5.5 mo in 66 patient with previous trastuzumab-based therapy failure, using this combination alone^[61].

Triple positive patients (ER/PR positive and HER-2 positive) benefit from dual blockage: This is a very relevant issue because close to 50% of the tumors that over-expressed HER-2 also have some expression of ER and there is significant cross-talk between them^[62]. Moreover, chemotherapy-free alternatives are always appealing. Two phase III trials have been reported testing the hypothesis that dual blockage is better than anti-hormonal therapy alone. In the TAnDEM study, 207 patients with no brain metastases and no prior chemotherapy received either anastrozole alone or combined with trastuzumab^[63]. The primary efficacy point was PFS which was extended by 2.4 mo with the use of trastuzumab (4.8 *vs* 2.4 mo; $P = 0.0016$). OS was not statistically different but crossover was allowed. Importantly, up to 15% of the patients in the combination arm did not experience relapse for up

to 2 years, suggesting that for at least a small proportion of patients this regimen was quite useful. The second study (EGF 30008 trial) compared letrozole alone or with lapatinib^[64]. No prior therapy for metastatic disease was allowed and 219 of the total 1286 patients randomized were HER-2 positive. In the HER-2 positive subgroup the experimental arm showed a significant extension in PFS (8.2 *vs* 30 mo; $P = 0.019$) but once again no benefit was seen in terms of OS and there were more serious adverse events (8% *vs* 4%) compared with letrozole alone ($P < 0.05$). Lastly, the small eLEcTRA trial was discontinued due to slow enrollment with only 57 patients analyzed^[65]. The cohort was similar to the TAnDEM trial but letrozole was used instead of anastrozole. The authors reported a trend towards improvement in TTP (14.1 *vs* 3.3 mo; $P = 0.23$). Lack of significance, though, could be due to a type 2 error (false negative) based on less than expected accrual. These studies support the use of HER-2 targeted treatments combined with non-steroidal aromatase inhibitors, in post-menopausal patients, as a valid chemotherapy-free option for triple positive patients^[66].

Central nervous system metastases are probably different than the rest: A recent meta-analysis showed a higher risk of brain metastases as initial-only site of recurrence in patients relapsing after receiving adjuvant treatment with trastuzumab *vs* chemotherapy alone^[67]. As expected given its molecular structure, most of the currently available anti-HER-2 drugs - trastuzumab, pertuzumab and ado-trastuzumab - have probably limited access into the central nervous system (CNS). Small molecules have in theory better chances to obtain therapeutic concentrations within the brain sanctuary. The combination of lapatinib and capecitabine has been correlated with a lower rate of CNS relapse compared with capecitabine as a single agent^[68]. Nonetheless, the recent CEREBEL study showed no difference in the rate of CNS relapse as a first-site of disease progression (3.0% *vs* 5.0%; $P = 0.360$) between trastuzumab + capecitabine and lapatinib + capecitabine treatment^[69]. However, the low incidence of CNS metastases might have undermined the ability of the study to show a significant difference. Also, a recently published French phase II trial showed that in patients with previously untreated HER-2 positive diffuse CNS metastases the combination of capecitabine and lapatinib lead to an impressive high response rate (ORR = 66%) when given as initial approach. The regimen was effective in treating both, CNS and extra-CNS disease, and delayed whole brain radiotherapy by a median of 8 mo^[70]. The conclusions from a planned randomized study evaluating capecitabine plus lapatinib plus whole brain radiotherapy are expected soon^[66].

NEW TARGETS

Since this field is quite dynamic and the frontiers are in continuously expansion, it will be appropriate to discuss some of the new strategies that are currently being inves-

tigated for HER-2 positive breast cancer.

Afatinib

Afatinib is an oral small molecule that irreversibly inhibits HER-1, 2 and 4^[71]. A phase II study in trastuzumab-resistant metastatic patients showed 4 out of 35 partial responses^[72]. Adverse events included diarrhea and rash. LUX-Breast 1 is a phase III study of vinorelbine plus trastuzumab or afatinib for metastatic patients who progressed to one chemotherapy regimen containing trastuzumab (NCT01125566)^[73]. A phase II trial is also evaluating afatinib with or without vinorelbine in patients with inflammatory or metastatic breast cancer (NCT01325428).

Neratinib

Neratinib is also an oral, irreversible inhibitor of HER-1, -2 and -4^[74]. A phase II trial evaluated neratinib in 136 HER-2-positive patients^[75]. In pretreated as well as trastuzumab-naïve patients, median PFS were 22.3 and 39.6 wk and ORR were 24% and 56%, respectively. Diarrhea was the most common grade 3/4 adverse effect. Another phase I - II trial combined neratinib plus trastuzumab in 45 metastatic and trastuzumab-resistant patients showing an encouraging 27% ORR^[76]. Finally, a phase I - II trial evaluated neratinib plus vinorelbine in trastuzumab or lapatinib pretreated patients ($n = 77$)^[77]. ORR was 42% in lapatinib-treated and 51% in lapatinib-naïve patients. Open label phase II trials are currently testing neratinib monotherapy in patients with HER-2 positive metastatic brain tumors (NCT01494662). Also a phase III trial (ExteNET) in the adjuvant setting is ongoing (NCT00878709).

MM-11

MM-11 is a bi-specific monoclonal antibody that reversibly targets the HER-2 and -3 heterodimer^[78]. A phase I - II study is currently evaluating its efficacy as single agent in HER-2 positive advanced breast cancer patients who have received prior trastuzumab or lapatinib therapy (NCT00911898)^[79]. Another phase I trial is studying MM-11 plus trastuzumab in HER2-positive, heregulin-positive, advanced and refractory breast cancer (NCT01097460).

HER2-targeted vaccines

Cancer vaccines designed to induce specific anti-HER-2 immunity are being investigated. Different strategies include protein-based vaccines, plasmid DNA-based vaccines, and vaccines that deliver HER-2 in a viral vector. HER-2 peptide-based vaccines have been tested in patients with metastatic HER-2 positive breast cancer^[80,81]. Patients immunized developed delayed-type hypersensitivity reactions and strong CD8⁺ cell responses specific for HER-2^[82]. A dendritic cell based vaccine was also tested in a small group of patients with stage IV breast cancer^[83]. One patient showed a partial response and three had stable disease for ≥ 12 mo. Using a different

strategy, cell-based GM-CSF secreting vaccines were tested in combination with trastuzumab^[84,85].

Pi3k/Akt/mTOR blocking drugs

PI3K/Akt/mTOR is an intracellular signal pathway that is frequently deregulated in breast cancer and mediates primary or secondary resistance to anti-HER-2 agents^[86]. A phase I study tested the combination of everolimus plus weekly paclitaxel and trastuzumab in 33 patients with heavily pretreated metastatic disease^[87]. Encouraging activity was reported, with an overall disease control rate at 6 mo of 74%. There are currently two ongoing phase III trials of everolimus in this setting: BOLERO-1 which assesses the combination of everolimus, trastuzumab, and paclitaxel as first-line therapy and BOLERO-3 which explores the addition of vinorelbine to everolimus plus trastuzumab in patients previously treated. With 569 patients completing the BOLERO-3 study, median PFS was 7.0 vs 5.78 mo in the placebo arm ($P = 0.0067$)^[88].

Histone deacetylase inhibitors

Histones acetylation status regulates the access of transcription factors to DNA and influences gene expression. Histone deacetylase (HDAC) activity reduces acetylation of histones. HDAC inhibitors induce growth arrest and apoptosis of tumor cells. Vorinostat is approved for cutaneous T-cell lymphoma. A phase II trial of vorinostat together with tamoxifen in patients with hormone therapy-resistant breast cancer showed that the combination is reasonably tolerated and exhibits activity in reversing hormone resistance^[89]. Clinical trials combining vorinostat with chemotherapy, EGFR inhibitors and bevacizumab are ongoing.

Heat shock protein 90 pathway

Heat shock protein 90 (Hsp-90) is a molecular chaperone that provides stability and supports the functionality of several proteins. Many of these proteins (*i.e.*, Bcr-Abl, c-Kit and PDGF- α) are pro-oncogenic. Sustained inhibition of Hsp-90 chaperone induces proteosomal degradation of the free proteins. HER-1 and HER-2 require chaperoning by Hsp-90 for their stability^[90]. Clinical data from a phase I trial with the Hsp-90 inhibitor tanespimycin used in combination with trastuzumab as second-line therapy showed evidence of antitumor activity in 63% of patients^[91]. A phase II trial enrolled 31 patients with HER-2 positive metastatic breast cancer whose disease has progressed on trastuzumab. Patients were administered weekly treatment with tanespimycin and trastuzumab. The ORR was 22% and the clinical benefit reached 59%^[92].

Other exploratory anti-HER-2 blocking strategies

Ongoing trials combining anti-HER-2 agents with drugs blocking other signaling pathways hold the promise of further improvement. An auspicious approach seems to be the combination of anti-HER-2 therapy with insulin growth factor receptor (IGFR-1) blocking agents.

IGFR-1 inhibition has been shown to restore sensitivity to trastuzumab in animal models^[93]. Another potential combination is the dual blockade of HER-2 and SRC which was recently shown to work as a central node downstream of multiple trastuzumab-resistance mechanisms^[94]. Finally, HER-3 is another strong activator of PI3K/Akt signaling pathway that has been demonstrated to be up-regulated after HER-2 blockade^[95]. Although still in early phases of development, Rb disruption strategies and the use of CDK-4/6 inhibitors may be clinically useful^[96].

CONCLUSION

We have recently witnessed an unprecedented expansion in the drugs developed to target HER-2 positive breast cancer. Transcendental advances were made and substantial improvements in all relevant outcomes and in all the clinical scenarios for either early or advanced disease were accomplished. However, patients still progress and cure for metastatic disease is still a utopia. We can only envision the arrival of newer and more sophisticated weapons to keep on fighting this lethal disease and we can already start asking ourselves some questions. Would neratinib, for example, soon become available? Could adotrastuzumab be ever used as a first line treatment? Can we make an anti-HER-2 drug with no cardiac effects? We will soon have some answers to these and many other questions, but up to that moment comes the state of the art management of HER-2 positive breast cancer relies on the more than 40 phase III and II clinical trials that were concisely described in the preceding pages.

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WJCO 5th Anniversary Special Issues (2): Breast cancer**Modification in the diet can induce beneficial effects against breast cancer**

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Abstract

The population tends to consume foods that in addition to their nutritional values can offer some benefits to their health. There are many epidemiological evidences and research studies in animal models suggesting that diet plays an important role in breast cancer prevention or progression. This review summarized some of the relevant researches about nutrition and cancer during the last years, especially in breast cancer. The analysis of probiotics and fermented products containing lactic acid bacteria in cancer prevention and/or treatment was especially discussed. It was observed that a balance of fatty acids similar to those of traditional Mediterranean diet, the consumption of fruits and vegetables, dietary fiber intake, vitamin supplementation are, along with the intake of probiotic products, the most extensively

studied by the negative association to breast cancer risk. The consumption of probiotics and fermented products containing lactic acid bacteria was associated to reduce breast cancer risk in some epidemiological studies. The use of animal models showed the modulation of the host's immune response as one of the important effects associated to the benefices observed with most probiotics. However; future assays in human are very important before the medical community can accept the addition of probiotic or fermented milks containing lactic acid bacteria as supplements for cancer patients.

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Key words: Breast cancer; Nutrition; Probiotic; Fermented products

Core tip: The population tends to consume foods that in addition to their nutritional values can offer some benefits to their health. In this sense, there are many epidemiological evidences and research studies suggesting that diet plays an important role in breast cancer prevention or progression. This review summarized some of the relevant researches about nutrition and cancer during the last years, especially in breast cancer. The analysis of probiotics and fermented products containing lactic acid bacteria in cancer prevention and / or treatment was especially discussed.

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INTRODUCTION

The population tends to consume foods that in addition

to their nutritional values can offer some benefits to their health.

The WHO reported that approximately 30% of cancer deaths are due to five behavioral risk factors and diet, such as high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco use, alcohol use^[1]

Breast cancer is a type of tumour in which there are many reports about the influence of nutrition^[2,3].

Lactic acid bacteria (LAB) represent a heterogeneous group of microorganisms that are present in the normal diet of many people and also in the gastrointestinal and urogenital tract of animals, and some of these claimed to be probiotics. Probiotics are defined as live microorganisms which when administered in adequate amounts, confer a health benefit on the host^[4]. These microorganisms and fermented foods containing LAB were growing in their popularity due to increasing numbers of studies proving that certain strains present health promoting properties, among them the prevention or treatment in the early stages of some types of cancers^[5,6].

The use of experimental animal models has a number of advantages in that the environmental conditions and genetics can be either controlled or defined. The value of the models is the insight they can provide into the complex, multi-faceted processes and mechanisms that can result in cancer development. *In vitro* assays are also important to understand the mechanisms of action involved in the LAB or other dietary effects. However, the application of dietary modifications against cancer needs to be ultimately tested in human trials.

This review summarizes some of the relevant researches about nutrition and cancer during the last years, especially in breast cancer. The analysis of probiotics and fermented products containing LAB in cancer prevention and / or treatment will be discussed separately, with emphasis in the possible mechanisms involved.

NUTRITION AND CANCER

Several studies have been demonstrated a relationship (either beneficial or harmful) between diet and development of different types of cancer^[7-9]. High fat diet, fiber consumption and vitamins are among dietary habits more reported by their association with cancer along with probiotic supplements, that will be discussed in a separately section.

Calorie restriction without malnutrition was associated to cancer prevention^[10]. This effect was related to the reduction in the activity of pro-aging pathways, inflammation in the pre-cancerous cells, and to the increase in the apoptosis of damaged cells.

Several epidemiological studies have been shown the relationship between increase consumption of high fat diet and the risk to develop cancer, such as kidney, stomach, lung, esophagus, colon and breast cancer. Colorectal cancer (CRC) is a tumour for which there are many studies that associate obesity with increased risk, especially in men^[11]. The exact mechanisms of this relationship

are still unknown, but metabolic syndrome, insulin resistance^[12], modifications in levels of adipocytokines^[13] seem to be implicated. The role of the microbiota in the maintenance of intestinal homeostasis, and its relationship with intestinal inflammation and colon carcinogenesis was also extensively studied^[14-16].

Dietary fat intake was also related to risk of ovarian cancer. It was suggested that higher intake of omega-3 may be protective, whereas high consumption of trans fat may increase risk of this cancer^[17].

The fiber consumption is another important component of the diet that was associated inversely with cancer risk, such as CRC^[18], nasopharyngeal carcinoma^[19], oesophageal cancer^[20].

The consumption of vitamins and mineral supplements are commonly used to prevent chronic diseases such as cancer. It was demonstrated that vitamin C (ascorbate) was selectively toxic to some types of tumor cells^[21]. Recently, it was reported a case in which the consumption of this vitamin decreased chemotherapy associated side effects^[22]. As regard to vitamin D, Bikle^[23] revised its relationship with different cancers and described that animal and cellular studies supported a role for vitamin D in the prevention and treatment of cancer, but the same conclusion was not arrived from clinical studies.

Folate is essential for DNA synthesis and methylation and its role against cancer is controversial, even against one type of cancer, such as for prostate cancer^[24]. Some studies implicated folates with tumour progression, such as the work reported by Dixon *et al*^[25] showing evidences that folate intake affects ovarian cancer survival. On other hand, the chemopreventive effect of folic acid was evaluated *in vivo* using rat as model for liver carcinogenesis. This effect was observed in association with tributyrin and was related to the potential to inhibit tumour angiogenesis^[26]. A recent review and meta-analysis showed that dietary folate intake was associated with a decreased risk of esophageal and pancreatic cancer, but not gastric cancer^[27].

NUTRITION AND BREAST CANCER

There are many epidemiological evidences and research studies in human and animals suggesting that diet plays an important role in breast cancer prevention or progression^[2,3]. Diet represents one of the most modifiable risk factors for breast cancer^[28]. Changes in the dietary patterns are not only related to less risk but also patients diagnosed and treated for breast cancer who pursue healthier dietary habits can improve their health and survival.

The relationship between obesity and breast cancer was reviewed in many articles because the high incidence and prevalence of both diseases. Overweight and obesity at the time of diagnosis were associated with a worse prognosis in breast cancer patients^[29]. A study in Italy showed that a diet high in glycemic load was associated with increased breast cancer risk^[30].

A systematic review showed that there are some strat-

egies to prevent weight gain that may decrease the risk of breast cancer or improve cancer outcomes in women with breast cancer^[31].

de Lorgeril and Salen suggested that a high omega-3 to omega-6 ratio, such as the case of traditional Mediterranean diet, reduce the risk of cancer, especially breast cancer^[32]. A cohort study of breast cancer survivors showed that intake of marine fatty acids EPA (eicosapentaenoic) plus DHA (Docosahexaenoic) was associated with improved prognosis^[33]. Omega-3 fatty acid, in particular EPA and DHA found principally in oily fish have been demonstrated to exert anti-angiogenic effects inhibiting production of different angiogenic mediators^[34]. The beneficial effect of EPA and DHA intakes was also associated by reducing inflammation through different mechanisms such as the suppression of NF- κ B, and the alteration of the plasma membrane micro-organization (lipid rafts)^[35].

Canola oil has also been associated with a reduced risk of breast cancer. The inhibition of cancer cells *in vitro* and the reduction of tumour volume in rats with chemical induced mammary tumour that consumed canola oil was reported^[36]. It was also suggested that canola oil can be used as prenatal nutritional strategies to reduce breast cancer risk in humans^[37]. This suggestion was based in results obtained *in vivo* using a chemical induced mammary tumour in offspring rats of canola-fed dams. These animals showed significantly decreased tumor volume with increased survival rate comparing to the control group whose mothers received control diet with soybean oil during pregnancy and lactation.

Diets rich in fruits and vegetables are also implicated in breast cancer risk reduction. A meta-analysis including fifteen prospective studies that reported decreased risk of breast cancer associated with fruit and vegetable intake, showed that high intake of fruits, and fruits and vegetables combined can be associated with reduction in risk of breast cancer^[38]. Similar results were obtained in a meta-analysis of prospective studies of blood concentrations of carotenoids and breast cancer risk^[39]. Carotenoid concentrations in blood can be used as biomarkers of fruit and vegetable intake and in this sense; the authors showed that blood concentrations of carotenoids were strongly associated with reduced breast cancer risk. Recently, an inverse association between citrus fruits intake and the risk of breast cancer was suggested^[40].

Dietary fiber intake was also inversely associated with breast cancer risk^[41]. The ingestion of dietary phytoestrogens may increase risk of estrogen receptor alpha (ER α)-positive breast cancer and this effect was associated with their estrogenic effects observed *in vitro* and *in vivo*. The proliferative effect of soy isoflavones was mainly observed in animal models of tumours. However; paradoxically, consumption of phytoestrogens has also been associated with reduced risk of breast cancer^[42-46]. This controversy with regard to the effect of soy isoflavones on breast cancer risk was analyzed and it was demonstrated that soy isoflavone phase II metabolism differs

between humans and rodents, and this should be taken in count to understand the value of the use of these rodents for investigate the effects of isoflavones in humans^[47]. Epidemiologic data indicate that soy intake is associated with a decreased breast cancer risk in Asia. A systematic review among women showed the possible protective effect of isoflavones on breast cancer risk^[48]. It was also demonstrated that soy isoflavone intake was associated with lower risk of recurrence among post-menopausal patients with breast cancer and those who were receiving adjuvant endocrine therapy^[49].

The understandings of the hormonal and non-hormonal mechanisms by which isoflavones can exert the beneficial effects were subject of many researches. The chemical structure of soy isoflavones is similar to that of estrogens. They are therefore considered to be possible selective estrogen receptor modulators (SERMs), which may bind to estrogen receptors and selectively stimulate or inhibit estrogen-like action in various tissues^[50]. It was demonstrated that sera of adult mice consuming soy isoflavone genistein (GEN) or blueberry (BB) polyphenol-containing diet altered mammosphere formation *in vitro* using receptor-positive and estrogen receptor-negative human breast cancer cell lines^[51]. Recently, this group demonstrated that breast cancer prevention by GEN was related to the regulation of mammary adiposity^[52]. The cytotoxic action of GEN against breast cancer cells involved mobilization of endogenous copper ions and generation of reactive oxygen species^[53].

Vitamin supplementation is another strategy, as was explained above, used to reduce cancer risk. With regard breast cancer, there was no found clear evidence of cancer prevention for vitamin supplements^[54]. Folates and folic acid were evaluated in breast cancer patients and also *in vivo* using animal models, and as was explained, the role of folates is controversial. There are epidemiological studies suggesting an inverse association between folate status and the risk of breast cancer^[55,56]. Some studies have also suggested that with alcohol consumption, folate supplementation reduces the risk of breast cancer^[57,58]. The beneficial effect associated to folate intake in some populations was associated to genetic polymorphisms of folate-metabolizing enzyme, methylenetetrahydrofolate reductase (MTHFR)^[59]. A population-based case-control study in Saudi Arabia showed that the MTHFR C677T polymorphism may modify the association between dietary folate intake and breast cancer risk^[60]. Similar results were obtained from the Shanghai Breast Cancer Study^[61] and in a case-control study in the Jiangsu Province of China^[62]. A recent work suggested that intake of natural folates can be inversely associated with breast cancer risk, but this association may vary by race, menopausal status or estrogen receptor status^[63]. The authors also observed an increased risk in European American women with the highest intake of synthetic folate from fortified foods. In this sense, a systematic review analyzed the effect of high folate intake post fortification, especially when folic acid was used, and demonstrated a higher risk of breast can-

cer in these populations^[64]. The authors showed the need to be cautious with high intakes of folic acid, especially in countries with mandatory food fortification, as Chile.

Animal models were used to understand the mechanisms by which folates and folic acid exert their effects, especially in breast cancer patients. Mammary tumors were chemically induced in rats and then, the animals received a diet containing different levels of folic acid^[65]. Folic acid supplementation was associated with significantly higher volume of mammary tumors and increased expression of BAX, PARP, and HER.

Riboflavin intake was also analyzed and an inverse association with breast cancer risk was documented^[66].

Selenium (Se) is an essential micronutrient having high anticancer properties in different animal models^[67,68]. As regard to breast cancer, it was demonstrated, using an animal model, that organic Se supplementation may reduce breast cancer metastasis, while selenite exacerbated it^[69].

Another dietary component (even though is minor in our diet) that was reported as effective against cancer is the inorganic sulfur. It was showed that inorganic sulfur significantly decreased proliferation of MDA-MB-231 human breast^[70]. This effect was due to reduction of ErbB2 and ErbB3 protein and mRNA expression, affecting the he ErbB-Akt pathway. Previously, it was reported that inorganic sulfur reduced cancer cell motility and invasion by inhibiting activity and mRNA expression of matrix metalloproteases (MMP-2 and MMP-9)^[71].

PROBIOTICS AND CANCER

Probiotic microorganisms and fermented foods containing LAB have been growing in popularity due to increasing numbers of studies proving that certain strains present health promoting properties, among them the prevention or treatment in the early stages of some types of cancers^[5,6,72].

The effects of probiotics and fermented products on intestinal disorders have been the most extensively studied considering that these microorganisms enter the organism orally and can positively modulate the intestinal microbiota involved in many of these disorders. The benefits of probiotics on the gut immune system in the prevention of cancer has also been previously described^[73,74]. There are many different mechanisms by which probiotics and fermented products containing viable LAB may lower the risk of colon cancer; among them, the modulation of the intestinal microbiota^[75-80], the inactivation of carcinogenic compound^[81-83], anti-oxidant effects^[84-86], and the modulations of the host's immune response^[87-89]. Recently, the administration of probiotic Dahi containing *Lactobacillus* (*L.*) *acidophilus* LaVK2 and *Bifidobacterium bifidum* BbVK3 alone or in combination of piroxicam showed anti-neoplastic and anti-proliferative activities in a model of DMH-induced CRC in rats^[90].

It was also demonstrated that oral administration of probiotic microorganisms can influence mucosal sites

different to the intestine due to the existence of the common mucosal immune system. In this sense, after intestinal stimulation, both B and T cells can migrate from Peyer's patches to mucosal membranes of the respiratory, gastrointestinal and genito-urinary tract, as well as to exocrine glands such as the lacrimal, salivary, mammary and prostatic glands^[91]. The oral administration of *L. casei* CRL 431 to mice induced an immune stimulation not only at the intestinal level, but also in bronchus and mammary glands^[92].

Beneficial effects of probiotic LAB administration were reported for non-intestinal tumors. The antitumor activity of *L. casei* CRL 431 was studied against a fibrosarcoma induced by methylcholantrene in mice. The administration of the probiotic strain inhibited tumor growth in a dose-dependent form^[93,94], stimulated the immune system with high levels of macrophage activation (the main infiltrative cells in the tumor), high levels of TNF α and with a remarkable decrease in tumor volume.

The effect of LAB or fermented products containing these microorganisms in non-intestinal tumours reported during the last years (2011-2014) were obtained searching the words "probiotic and cancer" in PubMed database.

It was reported the beneficial effect against cervical cancer. A pilot study suggested that probiotic promotes the clearance of HPV-related cytological abnormalities^[95]. Common vaginal lactobacilli exerted cytotoxic effects on cervical tumour cells independently of pH and lactate^[96]. *L. casei* displaying E7 antigen at its surface protected mice against human papillomavirus type 16-induced tumours^[97].

As regard hepatocarcinoma, the administration of probiotic fermented milk containing *L. rhamnosus* GG and, *L. casei* strain Shirota with chlorophyllin reduced liver pre-carcinogenic events in rat AFB1 induced liver carcinogenesis. This effect was attributed to an increased antioxidant status and decreased expression of oncogenes^[98].

The beneficial effects of LAB were also reported in animal models of oral cancer^[99], and skin carcinogenesis^[100].

PROBIOTICS AND BREAST CANCER

Breast cancer is another tumour in which there are reports about the beneficial effects of probiotic administration. Many reports analyzed, as was explained above, the association of soy based products and especially soy isoflavones with breast cancer risk. In this context, soy isoflavone ingestion was studied accompanied with the co-administration of probiotic bacteria, and it was observed that high concentrations of probiotics may alter the metabolism of isoflavones^[101]. Recently, the consumption of beverages containing *L. casei* Shirota and soy isoflavone was inversely associated with the incidence of breast cancer in Japanese women when they were consumed regularly since adolescence^[102]. The cooperative prevention mechanism of soymilk and *L. casei* Shirota was evaluated

Table 1 Examples of breast cancer animal models that have demonstrated the beneficial effects of lactic acid bacteria

Model	Results	Mechanisms	LAB	Ref.
4T1 tumour bearing mice	Significant decrease of tumour growth	Modulation of the host's immune response	<i>L. acidophilus</i> isolated from traditional home-made yogurt and from neonatal stool	[113]
Mice bearing invasive ductal carcinoma	Decrease of tumour growth rate and prolongation of mice survival	Modulation of the host's immune response	<i>L. casei</i> spp. <i>casei</i> ATCC 39392	[114]
4T1 breast cancer bearing mice	Tumor volumes of mice treated with Se nanoparticle-enriched probiotic were decreased and their survival rate increased compared to mice that received probiotic alone or control mice.	Modulation of the host's immune response	<i>L. plantarum</i> strain enriched with selenium nanoparticles	[115] [116]
Swiss mice fed a Westernized chow and FVB strain erbB2 (HER2) mutant mice	Inhibition of mammary neoplasia in both models.	LAB triggered CD4+CD25+ lymphocytes that convey transplantable anti-cancer protection.	<i>L. reuteri</i> ATCC-PTA-6475	
4T1 breast cancer bearing mice	Decrease of tumour growth in mice fed preventively with LAB and also in mice fed probiotic after tumour detection	Modulation of the host's immune response and decrease of tumour angiogenesis	<i>L. casei</i> CRL 431	[117]

LAB: Lactic acid bacteria.

using a rat carcinogenic model. It was observed that soy-milk prevented the development of mammary tumors and that *L. casei* Shirota suppressed tumor growth^[103].

In the West diet, fermented milks are more common as probiotic foods than soy based products. Milks fermented by different LAB and bifidobacteria strains (*B. infantis*, *B. bifidum*, *B. animalis*, *L. acidophilus* and *L. paracasei*) were evaluated *in vitro*, and the inhibition of the growth of a breast cancer cell line was reported^[104]. Other studies performed in humans, showed a negative association between yogurt consumption and breast cancer development^[105]. van't Veer *et al.*^[106] showed similar results in The Netherlands, and suggested that these effects would be related to changes in the intestinal microbiota (which could alter the metabolism of estrogen) and to the modulation on the immune system.

In addition to containing LAB, fermented milks can possess non-bacterial components produced during fermentation that may contribute to their anti-tumor activities^[107]. Thus, cultured dairy products can be proposed to inhibit the growth of many types of cancers, including breast tumors. In this context, milk fermented by *L. helveticus* R389 (a strain with high proteolytic activity) was studied comparatively with the milk fermented by a proteolytic deficient mutant, and both were able to delay tumour growth in an experimental breast cancer model using BALB/c mice^[108,109]. This effect was related to the immunoregulatory capacity of the fermented milks that decreased IL-6 levels, modulating the relationship between immune and endocrine systems. The important increase of IL-10 in mice fed with milk fermented by *L. helveticus* R389 could explain the difference between both fermented milks, attributed principally to the components released into the milk after the fermentation with the proteolytic strain, where the regulation of the immune response was observed in serum, mammary gland and also in the tumour infiltrating immune cells.

Kefir was another fermented product also evaluated in a breast cancer model in mice. Kefir and its cell-free fraction (KF) possess several substances that can exert beneficial effects on the immune system and prevent certain types of cancer^[110]. It was observed that mice receiving 2 d cyclical feeding with whole kefir diminished tumour growth, and the same cyclical feeding with KF showed the most significant delay of the tumour growth^[111]. This effect was related principally to a decrease in IL-6. KF caused not only a decrease of this cytokine but also a regulatory response with increased levels of IL-10 in all the samples studied. The results also demonstrated that the most important effect in this tumour model was due to substances released during milk fermentation (and not the microorganisms themselves)^[112].

Table 1 summarizes the effects reported for different LAB against breast cancer during the last years (2012-2014).

It was reported that *L. acidophilus* isolated from traditional home-made yogurt and also from neonatal stool induced a significant decrease in breast tumour growth pattern using a mouse model^[113]. This effect was associated to the alteration of cytokine production into a Th1 protective pattern.

L. casei spp. *casei* ATCC 39392 was also analyzed in a model of invasive ductal carcinoma in mice, and its administration decreased the growth rate of tumor and prolonged the survival of the animals. This effect was associated to the improvement of the host immune response by inducing Th1 cytokine profile and natural killer cells^[114].

The administration of selenium nanoparticle-enriched *L. plantarum* induced an efficient immune response in 4T1 breast cancer bearing mice. This effect was caused by the elevation of the pro-inflammatory cytokines IFN- γ , TNF- α and IL-2 levels and increased NK cell activity^[115].

The importance of the stimulation of host immune

cells by LAB and their beneficial effect against mammary carcinoma was analyzed using two mice models^[116]. In one model, mice were fed a Westernized chow increasing risk for development of mammary tumors. The other model consisted of FVB strain erbB2 (HER2) mutant mice, genetically susceptible to mammary tumors. Animals received *L. reuteri* ATCC-PTA-6475 in drinking water. It was observed that LAB oral supplementation inhibited features of mammary neoplasia in both models. The protective mechanism was associated to triggered CD4⁺CD25⁺ lymphocytes because when they were isolated and transplanted into other subjects conferred anti-cancer protection in the cell recipient animals.

Recently, our research group evaluated the effect of milk fermented by the probiotic bacterium *L. casei* CRL 431 on a murine breast cancer model. It was observed that the administration of this probiotic fermented milk stimulated the immune response against this breast tumour, avoiding or delaying its growth when it was preventively administrated and also when the administration started after tumour cells injection^[117].

CONCLUSION

There are many epidemiological evidences and research studies in animal models suggesting that diet plays an important role in breast cancer prevention or progression. A balance of fatty acids similar to those of traditional Mediterranean diet, the consumption of fruits and vegetables, dietary fiber intake, vitamin supplementation are, along with probiotic products, the most extensively studied. Although controversial data about isoflavones, epidemiological studies showed that the intake of soy based products in Asia was associated with decrease of breast cancer risk.

Probiotics and fermented products containing LAB have awakened the interest of many researches related to cancer and especially with breast cancer. Some epidemiological studies showed negative association between the consumption of these products and breast cancer development. Animal models were used to understand the possible mechanisms by which probiotic can exert the beneficial effects, and the modulation of the host's immune response was associated to the effects observed with most probiotics.

However, there are not enough human trials where the application of probiotics as biotherapeutics against breast cancer was tested. These assays are very important before the medical community can accept the addition of probiotic or fermented milks containing LAB as supplements for cancer patients.

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WJCO 5th Anniversary Special Issues (2): Breast cancer

Challenges to the early diagnosis and treatment of breast cancer in developing countries

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Abstract

This critical review of the literature assembles and compares available data on breast cancer clinical stage, time intervals to care, and access barriers in different countries. It provides evidence that while more than 70% of breast cancer patients in most high-income countries are diagnosed in stages I and II, only 20%-50% patients in the majority of low- and middle-income countries are diagnosed in these earlier stages. Most studies in the developed world show an association between an advanced clinical stage of breast cancer and delays greater than three months between symptom discovery and treatment start. The evidence assembled in this review shows that the median of this interval is 30-48 d in high-income countries but 3-8 mo in low- and middle-income countries. The longest delays occur between the first medical consultation and the beginning of treatment, known as the provider interval. The little available evidence suggests that access barriers and quality deficiencies in cancer care are determinants of provider delay in low- and middle-income countries. Research on specific access barriers and deficiencies in quality of care for the early diagnosis and treatment of breast cancer is practically non-existent

in these countries, where it is the most needed for the design of cost-effective public policies that strengthen health systems to tackle this expensive and deadly disease.

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Key words: Breast cancer; Early diagnosis; Delays; Time intervals; Clinical stage; Access; Health care delivery

Core tip: This review assembles the available data on breast cancer clinical stage for 10 high-income and 13 low-income countries and the time intervals from symptom discovery to cancer diagnosis and treatment for 33 countries. Most breast cancer patients in low-income countries suffer very long delays and are diagnosed in advanced stages. The scant available evidence for low and middle-income countries suggests that access barriers and quality deficiencies in cancer care are determinants of these delays.

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INTRODUCTION

The World Bank classifies countries according to their gross national income as low income, lower-middle income, higher-middle income and high income. Low- and middle-income countries (LMICs) are also sometimes referred to as “developing” economies, while high-income countries (HICs) are referred to as “developed”^[1]. The term does not imply either that all developing countries

Table 1 Countries with the highest breast cancer incidence and mortality rates^[3]

Country	Incidence rate	Country	Mortality rate
1 Belgium	111.9	1 Fiji	28.4
2 Denmark	105	2 Bahamas	26.3
3 France	104.5	3 Nigeria	25.9
4 The Netherlands	99	4 Pakistan	25.2
5 Bahamas	98.9	5 New Caledonia	24.4
6 Iceland	96.3	6 Armenia	24.2
7 United Kingdom	95	7 Lebanon	24.0
8 Barbados	94.7	8 Trinidad and Tobago	23.5
9 United States	92.9	9 Ethiopia	23.0
10 Ireland	92.3	10 Uruguay	22.7
11 French Polynesia	92.2	11 Barbados	22.1
12 Germany	91.6	12 Serbia	22.0
13 Italy	91.3	13 Jordan	21.8
14 Finland	89.4	14 Syria	21.5
15 Luxembourg	89.1	15 Somalia	20.6
16 New Caledonia	87.6	16 Afghanistan	20.6
17 Australia	86	17 Eritrea	20.5
18 Malta	85.9	18 French Polynesia	20.4
19 New Zealand	85	19 Montenegro	20.2
20 Switzerland	83.1	20 Guyana	20.1
21 Israel	80.5		
22 Sweden	80.4		

Incidence and mortality rates are number of cases and number of deaths, respectively, per 100000 women. Both measures are age-standardized.

are actually in the process of developing or that those in the developed group have necessarily reached some final stage of development^[1]. For global health care, this classification provides a useful framework to assess how the countries' available resources should be allocated to address the most relevant health issues^[2].

Breast cancer is the most frequent cancer in women worldwide, with 1.67 million new cases diagnosed in 2012^[3]. It is also the leading cause of cancer death among women, with approximately 500000 annual deaths^[3]. The highest incidence rates occur in the most developed regions of the world, with 74.1 new cases per 100000 women in comparison to the 31.3 new cases per 100000 observed in less-developed regions^[3]. Nevertheless, the mortality rates are actually higher in developing countries. Table 1 presents the countries with the highest breast cancer incidence rates (above 80 per 100000 women) and those with the highest mortality rates (above 20 per 100000 women) in 2012. As shown, the majority of countries with the top incidence rates are high-income countries (HICs), while the majority of those with the highest mortality rates are low- and middle-income countries (LMICs).

Cancer survival data are extremely scarce for developing countries, but the few data available are in line with the observed incidence/mortality differences. The 5-year survival rates for breast cancer are much worse for low- and low-middle income countries such as Gambia (12%), Algeria (38.8%), India (52%) and Brazil (58.4%) in comparison to HICs such as the United States of America (83.9%), Sweden (82.0%), Japan (81.6%) and Australia (80.7%)^[4,5].

The higher breast cancer mortality rates in LMICs are thought to be due to diagnosis in advanced stages and

access barriers to medical care^[6]. The limited data available for developing countries have made it difficult to determine how many more cases of advanced breast cancer are actually diagnosed in LMICs than in HICs. Even more rare are data from LMICs on time to care and access barriers. The purpose of this review was to assemble and compare the available data on the clinical stage, time intervals and access barriers across different countries to identify the main challenges in the early treatment of breast cancer in developing countries.

A critical review of the literature was conducted of quantitative studies published in English, Spanish, or Portuguese in the last 15 years that reported breast cancer clinical stage, time intervals and/or access and quality barriers associated with delayed cancer care. The PubMed and SciELO electronic databases were searched for "breast cancer" combined with each of the following terms: "clinical stages", "survival", "delay", "time intervals", "help seeking behavior", "access", "barriers"; plus one of the subsequent terms: "developing countries", "limited resource", "low income" or "middle income". For data on clinical stage, Google searches were also performed, using the terms "breast cancer" and "clinical stages". Additionally, references from relevant studies were used to trace other studies. The search was updated to December 2013. All articles relevant to clinical stage, time intervals and access and quality barriers were included, as they are scarce, particularly those performed in developing countries, which were the most relevant to this analysis.

This review presents information on clinical stage, which was collected from 20 studies or registries providing data for 10 HICs and 13 LMICs. Evidence on the time intervals to care is summarized for 33 studies that

gathered data for 10 HICs and 23 LMICs. Finally, the data from 26 studies on access barriers to care are presented, of which only three studies took place in LMICs.

ADVANCED CLINICAL STAGE OF BREAST CANCER IN LMICs

The clinical stage at breast cancer diagnosis remains one of the most important prognostic factors of survival^[7]. The most accepted classification is the TNM staging system developed by the American Joint Committee on Cancer (AJCC)^[8]. The estimated 3-year survival rates for high-income countries such as Canada, Sweden, Norway, Denmark and the United Kingdom are between 99.3 and 100.0% for patients diagnosed in stage I, 91.5% to 96.4% for stage II, between 69.0% and 83.0% for stage III, and 27.4% to 41.8% for distantly spread disease (stage IV)^[9]. Another staging classification that is sometimes used is that proposed by the United States National Cancer Institute of Surveillance, Epidemiology, and End Results (SEER) Program. This system considers three stages: (1) localized, for tumors confined to the breast with no extension to the lymph nodes (equivalent to TNM stages I and II A); (2) regional, when breast cancer has disseminated to the regional lymph nodes (equivalent to stages II B, III A, III B and III C); and (3) distant, when cancer has spread to distant organs (TNM stage IV)^[10]. The reported 5-year survival rates for 317340 patients who were diagnosed between 2003 and 2009 in the United States SEER regions were 98.6% for localized stage cancer patients, 84.4% for regional stage patients and 24.3% for distant stage patients^[10].

Table 2 summarizes the clinical stage data reported for different countries. As shown, while the majority of breast cancers are diagnosed in localized stages in HICs, most are detected in regionally spread stages in LMICs. In HICs, more than 70% of breast cancer patients are diagnosed in stages I and II; Sweden and Norway have proportions above 90%. In contrast, in LMICs, only between 20 and 60% of patients are diagnosed in these earlier stages, while between 30 and 80% are diagnosed in stages III and IV. The exceptions in the table are Porto Alegre in Brazil and white women in South Africa, who behave similar to women in developed countries, with 70% and 68% of breast cancer cases detected in stages I and II, respectively. The data presented for the different regions or subpopulations in Brazil, South Africa and India reveal tremendous disparities within each of these countries. Similar differences have been reported in the United States, the United Kingdom and other developed countries and have been shown to be a result of socioeconomic disparities, as will be discussed in detail later on. These inequities are revealed in this table only for these particular cases because the data available for developing countries come from country-regions or even hospitals, while the data for most HICs were gathered through national registries and thus constitute a single measure for the entire population.

The question remains as to why cancer patients are di-

agnosed in such advanced stages in developing countries. Research on this matter is scarce. Most study findings in the developed world show an association of advanced clinical stage of breast cancer with delays greater than three months between symptom discovery and treatment start (total delay)^[11-13]. Additionally, delays greater than three months are associated with reduced survival^[12,13]. A reasonable explanation of the relationship between delay and survival is that delay influences disease progression, which in turn affects survival. This hypothesis is supported by studies in which the association between delay and survival disappears once clinical stage is controlled for^[12,14].

TIME INTERVALS FOR BREAST CANCER CARE

Traditionally, breast cancer total delay has been defined as more than three months between symptom discovery and the beginning of cancer treatment and has been classified in two types: patient delay and provider delay^[15-18]. Patient delay refers to the lengthening of the interval between the discovery of symptoms and the first medical consultation, and the most accepted threshold to establish it is three months. Provider delay is that which takes place between the first medical consultation and the beginning of definitive treatment, and the threshold used to define it is highly variable between studies. Table 3 summarizes the data for the total, patient and provider intervals reported in different countries. The median lengths of the intervals are reported when available and, in the absence of medians, some mean intervals and/or percentages of delays greater than three months are reported.

Diverse classifications and names of the provider interval have been used. The most commonly used are the diagnosis and treatment interval classifications. The diagnosis interval is that from the first medical consultation to the confirmation of a cancer diagnosis. The treatment interval is the time between diagnosis and the beginning of oncologic treatment. Two other classifications have also been used: (1) the doctor (from first consultation with the primary physician to the first investigation of cancer) and system (from the first investigation to the beginning of cancer treatment) intervals; and (2) the referral (from the first medical consultation with the primary physician to the patient's referral to the specialist) and specialist care (from the patient's referral to the beginning of cancer treatment) intervals^[19]. These two last classifications (doctor/system, and referral/specialist) are rarely used, although the names are commonly used interchangeably in reference to the provider interval. They have been properly used only in health systems with well-organized primary and secondary care services, such as those of the United Kingdom and Denmark. They would be extremely difficult to measure in the context of fragmented health services or a lack of registries and electronic medical records, as is the case for the majority of developing countries. For the sake of clarity, despite the delay

Table 2 Clinical stage of breast cancer patients by country-summary from the literature

	Year(s)	TNM staging system				SEER staging system		
		I	II	III	IV	Localized	Regional	Distant
High-income Countries:								
Australia ^[9]	2000-2007	-	-	-	-	55.9	38.1	6.0
Canada ^[9]	2000-2007	41.0	38.1	13.3	7.6	-	-	-
Denmark ^[9]	2000-2007	29.3	47.2	15.8	7.7	-	-	-
Germany (Saarland) ^[11]	1996-1998	-	-	-	-	52.0	44.0	4.0
Northern Ireland ^[91]	2006	30.4	43.6	19.6	6.4	-	-	-
Norway ^[9]	2000-2007	43.4	47.1	3.8	5.7	-	-	-
Saudi Arabia ^[92]	2004	-	-	-	-	27.8	56.2	16.0
Sweden ^[9]	2000-2007	45.2	46.5	5.3	3.0	-	-	-
United Kingdom ^[9]	2000-2007	40.0	45.4	9.2	5.4	-	-	-
United States ^[10]	2002-2008	-	-	-	-	62.3	32.6	5.1
Low and middle-income countries:								
Brazil								
Goiás ^[93]	2002-2009	14.7	36.1	27.9	21.3	-	-	-
Porto Alegre ^[94]	1975-1997	16.0	54.0	19.0	11.0	-	-	-
Sao Paulo ^[94]	1979-1989	11.0	22.0	53.0	14.0	-	-	-
Colombia (Bogota) ^[95]	2006-2007	-	-	-	-	26.4	68.2	5.4
Egypt (South Cancer Inst.) ^[96]	2001-2008	11.0	39.0	25.0	25.0	-	-	-
Egypt (Gharbiah) ^[97]	1999-2008	-	-	-	-	25.2	60.3	14.5
India ^[98]								
Mumbai	1995	7.8	57.4	28.4	5.9	-	-	-
Trivandrum	1996	4.4	42.3	40.5	12.8	-	-	-
Chennai		1.0	23.0	52.0	24.0	-	-	-
Iraq (Kurdistan) ^[99]	2006-2008	4.9	53.3	31.8	9.9	-	-	-
Jordan ^[100]	2009	29.0	30.0	23.0	10.0	-	-	-
Libya ^[22]	2008-2009	9.0	25.5	54.0	11.5	-	-	-
Malaysia (East Coast and Kuala Lumpur) ^[26]	2005-2007	5.2	38.7	44.8	11.3	-	-	-
Mexico								
INCAN ^[101] -uninsured pop.	2007	10.2	36.4	40.9	12.5	-	-	-
IMSS ^[102] -insured pop.	2002	13.8	39.6	33.9	12.7	-	-	-
Nigeria (Lagos) ^[103]	2009-2010	5.5	15.4	62.7	16.4	-	-	-
Peru (Lima) ^[94]	1985-1997	9.0	42.0	33.0	16.0	-	-	-
South Africa ^[104]								
Whites	1970-1997	30.8	38.0	18.8	11.9	-	-	-
Blacks		5.4	16.9	41.6	36.1	-	-	-
Thailand ^[36]	2009	12.0	38.0	41.0	9.0	-	-	-

Data are population-based, except for the following countries where data is hospital-based: Brazil, Colombia, Egypt, India, Iraq, Libya, Malaysia, Mexico, Nigeria, Peru, South Africa and Thailand. All percentages were corrected to exclude Non-Staged cancers.

nomenclature used in each study, the terms presented in Table 3 are those that correspond to the definition that was used. When this was not possible, only the definition is shown and not the term used by the researchers.

To further complicate things, a wide range of methods has been used to measure time points and intervals, with the majority of existing studies lacking methodological rigor^[20,21]. As a result, research findings are not easily comparable between studies and countries. Nevertheless, to obtain a rough idea of the differences in intervals of care between developing and developed countries, data from all the retrieved studies were included.

Among HICs, the median total intervals range between 30 and 48 d, and more than 60% of patients begin treatment less than 3 mo after symptom discovery (Table 3). In comparison, the median total intervals for LMICs are between 5.5 mo (Malaysia) and 8 mo (Brazil), and for countries with available data (Brazil, Libya, Mexico and Malaysia), it is striking that fewer than 30% of patients start treatment in less than three months after abnormal screening or

symptom discovery^[22-27].

The median patient interval is between 7 and 16 d for HICs and between 10 d and 3 mo for LMICs. The lengthiest median patient intervals have been reported for Iran (3 mo), Egypt (2.7 mo) and Malaysia (2 mo)^[26,28,29]. Among countries that report mean instead of median intervals, including Eastern European countries, India and Ethiopia, the average patient interval is between 24 d (Hungary) and 1.5 mo (India) for all except Ethiopia, which reports a striking 18-mo patient interval mean.

Finally, available provider intervals or subintervals are also presented in Table 3. It is hard to compare these because of the diverse definitions used. The full provider interval is only reported for one HIC, Germany, with a median duration of 15 d. In contrast, the median provider intervals in LMICs, which are available only for Brazil, Colombia, Mexico and Turkey, range between 2.6 mo and 6.5 mo. The median diagnosis intervals for the HICs of France and the United States are 7 and 32 d, respectively, while that for the LMIC Brazil is 6.5 mo^[24,30,31]. Notably,

Table 3 Time intervals for breast cancer care-findings from the literature

Country (region)	Year	n	Total interval			Patient interval		Provider/system intervals			
			Definition	Median/ mean ¹	> 3 mo (%)	Median/ mean ¹	> 3 mo (%)	Reported interval	Definition	Median/ mean ¹	> 3 mo (%)
High-income countries											
Canada (Quebec) ^[105]	1992-1998	29606	-	-	-	-	-	Treatment	1 st diagnostic study to surgery.	42 d	17.1
Canada ^[106]	1996	4465	-	-	-	-	-	Diagnosis	Abnormal screening to diagnosis	31 d	-
France ^[30]	2003	1494	1 st abnormal screening to treatment start	34 d	-	-	-	Diagnosis	Abnormal screening to diagnosis	7 d	-
								Treatment	Diagnosis to treatment start	27 d	-
Germany (Saarland) ^[11,107,108]	1996-1998	380	Symptom discovery or abnormal screening to diagnosis	-	26.1	16 d	17.4	Provider	1 st consultation to treatment start	15 d	11.0
Italy (Campania) ^[56]	1998-1999	644	Symptom discovery to surgery	-	35.0	-	20.0	-	1 st medical consultation to hospital admission	-	11.0
Italy (Campania and Apulia) ^[109]	2004-2006	959	-	-	-	-	-	Diagnosis	1 st consultation to diagnosis	-	60.0
Nether-lands ^[110]	1996-2002	1503	-	-	-	-	-	Diagnosis	Screening to diagnosis	-	6.5
North Ireland ^[91]	2006	759	-	-	-	-	-	Treatment	Diagnosis to treatment start	15 d	-
Scotland ^[111]	2005-2007	1250	-	-	-	7 d	-	Referral	1 st consultation to referral	1 d	-
								Specialist	Referral by GP to 1 st consultation by specialist	18 d	-
Spain (Catalonia) ^[112]	2001-2002	266	-	-	-	-	-	Treatment	Diagnosis to treatment start	35 d	-
United Kingdom ^[113]	1999-2000	25627	Symptom discovery to diagnosis	30 d	-	9 d	-	-	GP referral to diagnosis	11 d	-
United States ^[31]	1991-1995	1659	Abnormal screening to treatment start	48 d	22.9	-	-	Diagnosis	1 st abnormal screening to diagnosis	32 d	-
								Treatment	Diagnosis to treatment start.	10 d	-
United States ^[54]	1995-2005	246957	-	-	-	-	-	Treatment	Diagnosis to treatment start	23 d	-
United States (Califor- nia) ^[114]	2003-2005	921 low income women	Symptom discovery to biopsy		39.0	-	-	-	-	-	-
United States (North Carolina) ^[115]	2000-2002	1786	-	-	-	-	-	Treatment	Diagnosis to treatment start	22 d	-
Middle-income countries											
Brazil (Brasilia) ^[25]	2010	250	Symptom discovery to treatment	7.5 mo	88.8	-	29.9	Provider	1 st consultation to treatment start	-	77.6
Brazil (Rio) ^[24]	2004	104	Symptom discovery to diagnosis	8 mo	-	1 mo	-	Diagnosis	1 st consultation to diagnosis	6.5 mo	80.0

Bulgaria ^[23]	2011	448	Symptom discovery to treatment	3.9 mo ¹	-	1.2 mo ¹	-	Provider	1 st consultation to treatment start	3.1 mo ¹	-
Colombia ^[95,116]	2006-2007	852	-	-	-	-	20.3	Provider	1 st consultation to treatment start	4.5 mo	31.0
Croatia ^[23]	2011	167	Symptom discovery to treatment	3.4 mo ¹	-	1.2 mo ¹	-	Provider	1 st consultation to treatment start	2.6 mo ¹	-
Egypt ^[29]	2010	163	-	-	-	2.7 mo	-	-	1 st consultation to hospital arrival	18 d	-
Ethiopia ^[117]	2008	69	-	-	-	18 mo ¹	-	-	-	-	-
Haiti ^[118]	2012	90	-	-	-	1 wk	42.0	-	-	-	-
Hungary ^[23]	2011	167	Symptom discovery to treatment	4.0 mo ¹	-	24 d ¹	-	Provider	1 st consultation to treatment start	3.6 mo ¹	-
India ^[23]	2011	207	Symptom discovery to treatment	7.4 mo ¹	-	1.5 mo ¹	-	Provider	1 st consultation to treatment start	6.2 mo ¹	-
Iran ^[28]	2000-2001	200	-	-	-	3 mo	42.5	-	-	-	-
Latvia ^[23]	2011	111	Symptom discovery to treatment	4.4 mo ¹	-	1.5 mo ¹	-	Provider	1 st consultation to treatment start	3.3 mo ¹	-
Libya ^[22]	2008-2009	200	Symptom discovery to diagnosis	-	70.0	-	54.5	-	-	-	-
Lithuania ^[23]	2011	368	Symptom discovery to treatment	3.0 mo ¹	-	1.2 mo ¹	-	Provider	1 st consultation to treatment start	2.1 mo ¹	-
Malaysia ^[26]	2005-2007	328	Symptom discovery to diagnosis	5.5 mo	72.6	2 mo	43.3	-	-	-	-
Mexico ^[34]	2008	32	Symptom discovery to treatment start	7.5 mo	-	10 d	-	Diagnosis	1 st consultation to diagnosis	2.8 mo	-
Mexico ^[27]	2010-2011	384	Abnormal mammogram or symptom discovery to treatment start	7.8 mo	90.0	11 d	20.6	Provider	1 st consultation to treatment start	4.7 mo	73.7
Nigeria ^[103]	2009-2010	201	-	-	-	-	81.0	-	-	-	-
Poland ^[23]	2011	557	Symptom discovery to treatment	2.9 mo ¹	-	25 d ¹	-	Provider	1 st consultation to treatment start	2.4 mo ¹	-
Romania ^[23]	2011	271	Symptom discovery to treatment	6.4 mo ¹	-	1.5 mo ¹	-	Provider	1 st consultation to treatment start	7.4 mo ¹	-
Russia ^[23]	2011	718	Symptom discovery to treatment	3.9 mo ¹	-	1.2 mo ¹	-	Provider	1 st consultation to treatment start	3.1 mo ¹	-
Slovakia ^[23]	2011	154	Symptom discovery to treatment	3.3 mo ¹	-	1.0 mo ¹	-	Provider	1 st consultation to treatment start	2.7 mo ¹	-
Serbia ^[23]	2011	663	Symptom discovery to treatment	3.2 mo ¹	-	1.1 mo ¹	-	Provider	1 st consultation to treatment start	2.3 mo ¹	-
Thailand ^[35]	1994-1996	94	-	-	-	1 mo	26.6	Provider	1 st medical consultation to hospital admission	1 mo	24.4
Thailand ^[36]	2009	109	-	-	-	12 d	17.0	Provider	1 st consultation to treatment start	21 d	42.0
Turkey ^[23]	2011	694	Symptom discovery to treatment	3.4 mo ¹	-	1.2 mo ¹	-	Provider	1 st consultation to treatment start	2.6 mo ¹	-

¹These correspond to mean intervals, instead of medians. Patient interval is not defined in the table because studies coincide in the accepted definition: symptom discovery or abnormal screening to first medical consultation.

the median patient interval in LMICs is between 1.4 and 12.9 times longer than that observed in HICs, and the diagnosis interval is between 3.8 and 27.9 times longer. The patient interval prolongation is primarily influenced by the patients' help-seeking behavior, which varies according to different socioeconomic and cultural factors. In turn, the delayed provider intervals most likely reflect access barriers and quality deficiencies in cancer care in the LMIC health systems, as has been observed in some of the few available studies^[32-36].

ACCESS BARRIERS AND QUALITY OF CARE DEFICIENCIES ASSOCIATED WITH DELAYED BREAST CANCER TREATMENT

Access to health care is a multidimensional concept that has

been defined as the “degree of fit” between a patient's socioeconomic characteristics, the health system, and health services organization^[37], and it includes both financial and non-financial dimensions^[38-41]. Five different components of access have been described: affordability, acceptability, accessibility, accommodation, and availability^[37]. Availability refers to the adequacy of the supply of health providers, facilities and services in relation to the patients' health needs. Accessibility is the relationship between the geographical location of services and that of patients (*e.g.*, transportation resources, travel time, distance and cost). Accommodation is the relationship between the manner in which the supply resources are organized to accept patients (*e.g.*, operation hours, appointment systems, telephone services), the patients' ability to accommodate these factors, and the patients' perceptions of their appropriateness. Affordability is the relationship between

Table 4 Studies of access or quality of care barriers related to provider delay

Access or quality barriers	Studies		
	Country	Year of publication	Sample size
Low socioeconomic status	England ^[47]	2005	19760
	Canada ^[48]	2007	696
Ethnic minorities	United States ^[31]	2000	1659
	United States ^[51]	2004	831
	United States ^[54]	2011	246957
Lack of health insurance	United States ^[54]	2011	246957
Patient's old age	United States ^[54]	2011	246957
Patient's young age	England ^[55]	1999	36222
	Italy ^[56]	2001	644
	Scotland ^[57]	2004	1069
	Scotland ^[58]	2004	5283
Travel time to hospital	England ^[47]	2005	19760
	Thailand ^[36]	2013	180
Distance from hospital	Thailand ^[36]	2013	180
Long waiting times to get medical appointments	Mexico ^[33]	2011	125
Consulting 3 or more different health services before arrival to a cancer center	Mexico ^[33]	2011	125
Type of first health service contacted	Thailand ^[35]	2000	94
Medical specialty of first provider consulted	Italy ^[56]	2001	644
Medical errors in initial diagnosis, screening interpretation or pathology review	United States ^[31]	2000	1659
	England ^[64]	2000	1004
	Thailand ^[35]	2000	94
	United States ^[65]	2002	454
	Scotland ^[58]	2004	5283
	Netherlands ^[119]	2004	153969
	Canada ^[48]	2007	696
	Mexico ^[33]	2011	125

the prices of services and the patients' ability to pay and/or existing health insurance. Finally, acceptability refers to the patients' beliefs, perceptions and attitudes in regard to the characteristics of health personnel and facilities (*e.g.*, doctor's gender or ethnicity, clinic type), as well as the health personnel's attitudes about the acceptable personal characteristics of the patients.

Table 4 summarizes different factors related to access or quality of care deficiencies that have been associated with breast cancer provider delay. As shown, there is little research on this matter, and the vast majority of studies have taken place in developed countries. Furthermore, the predominating focus has been to quantify associations between the patients' socio-demographic characteristics and delays, without exploration of specific access and quality of care issues that could explain these relationships.

Socioeconomic status (SES) has long been linked to morbidity, mortality, illness behavior, health services utilization and access to care^[42-44]. SES differences in health are embedded in the larger problem of health disparities associated with social disadvantage^[44]. As SES decreases, breast cancer clinical stage has been shown to increase and 5-year survival rates to decline^[45,46]. These associations have been confirmed for several measures of SES, including income, education and occupation. SES has a direct impact on the access dimension of affordability^[37]. Therefore, a plausible explanation for the disparities of breast cancer clinical stage and survival is that people

with low SES suffer longer provider delays than people with high SES, as documented^[47,48], most likely because they face access barriers to health care that remain to be identified and are most likely specific to each health system.

The relationship between ethnicity and provider delay may also be mediated by lower socioeconomic status and reduced access to medical care. Black people in the United States have poorer breast cancer survival rates than whites (79.1% *vs* 91.7%), and these gaps persist within clinical stages^[10]. These ethnic disparities in breast clinical stage have been shown to dissolve when controlling for socioeconomic position^[49,50]. Additionally, the relationship between ethnicity and provider delay has been shown to disappear when poverty and insurance status are controlled for^[51]. Moreover, a study that examined the influence of ethnicity, socioeconomic position and gender on an individual's perception of the need for and urgency or seeking health care found that Black respondents and respondents from lower socio-economic groups were at least as likely to report immediate health care seeking as White respondents and those from higher socio-economic groups^[52]. These findings suggest that the ethnicity differences observed in provider delay are very likely due to socioeconomic disparities that influence access to care.

Access to health insurance is also related to socioeconomic position and has long been known to be one of the most relevant enabling factors for health care utilization^[39,53]. Therefore, it is not surprising that lack of health

insurance is related to provider delay for breast cancer care^[54]. This might be particularly important in countries with fragmented systems, where the uninsured population has access to only certain types of health services (availability and accommodation) and/or has to pay out-of-pocket for each consultation, medical study and treatment (affordability).

The relationship between age and delay is very interesting. Older age has been found to be associated with patient delay in several studies^[11,17], while younger age has been linked with provider delay^[47,55-58]. Several mechanisms have been proposed to explain the association between older age and patient delay. Studies conducted in developed countries have suggested that older women may attribute early breast cancer symptoms to other comorbid conditions or to normal aging^[11,16]. Likewise, older women may be more fatalistic, thinking they have lived long enough^[16]. Study findings have also confirmed that delay in these older patients could be a consequence of negative attitudes toward seeing their general practitioner and fears about the consequences of the diagnosis and treatment of cancer^[59]. The relationship between older age and provider delay has been less studied, and the plausible mechanisms of this relationship have not been explained^[54]. Nevertheless, some of the mechanisms discussed for patient delay might also occur after the first medical consultation has taken place, when the patient might decide to postpone studies and/or the beginning of treatment. Another possible mechanism includes the tendency for older people to be affected simultaneously by other chronic conditions in addition to cancer, such as hypertension or diabetes. In these cases, the physician might postpone cancer treatment until the other comorbidities are stable. Yet another mechanism that is particularly relevant for developing countries is that older women may face more access barriers to health care because of unemployment and its consequences on the lack of health insurance and socioeconomic problems.

The relationship between young patient age and provider delay is most likely a consequence of medical errors. The majority of studies that have found a significant association between young age and delay have failed to explore the mechanisms behind this relationship^[47,60]. Some studies, however, have suggested that young age increases the difficulty of a medical diagnosis^[58]. The sensitivity of mammography has been found to be significantly lower in young women than older women (68 vs 91 percent), and tumors have been found to be more ill-defined for palpation because of background mammary density or a diffuse growth pattern^[61]. Additionally, the suspicion of a cancer diagnosis may be less common among young patients than their older counterparts^[55]. The presentation of breast cancer is highly unlikely in women younger than 40 years, with an estimated risk for a 30-year-old woman of 0.44 to develop a breast cancer in the next 10 years in comparison with a risk of 3.84 for a 70-year-old woman^[10]. To further complicate things, breast benign conditions such as fibroadenoma and cysts

are very common in young women^[62,63].

Travel time to the hospital, distance from the patient's home to the hospital, long waiting times for medical appointments and the consultation of 3 or more different health services before arriving at a cancer hospital reflect different dimensions of access to care: accommodation, availability and affordability^[37]. The study of these types of specific access barriers is scarce and much needed in developing countries where delays for cancer treatment and other life-threatening conditions are very common. For each country's health system, and even each health service within countries and country regions, specific access barriers need to be identified in order to address them and improve time to care.

Finally, the associations found between provider delay and type of first health service contacted, the medical specialty of the first provider that was consulted and medical errors all reflect differences in the quality of care that patients receive. Medical errors in relation to provider delay have been studied in terms of the primary care physician's failure to suspect cancer at the initial consultation^[33,35], false-negative interpretations of mammography^[31,48,64,65] and false-negative biopsy interpretations^[48,64]. The relationships reported between the specialty of the first doctor consulted and provider delay as well as that of the primary care physician's failure to suspect cancer highlight the relevance of the role of the first medical professional consulted. This is very pertinent for developing countries, where highly specialized human resources are scarce and the first contact for the majority of the population is a general physician, that is, a recently graduated medical doctor (NOT a specialist in General Medicine). The majority of these doctors have never seen breast cancer and are typically not familiar with breast cancer screening and diagnostic guidelines.

IMPLICATIONS FOR PUBLIC POLICY IN LMICs

As the limited available data for LMICs presented here show, breast cancer is being treated in very advanced stages after long intervals of time. This is most likely because patients in these countries face significant access barriers to quality health care. The situation may be even worse for countries in which there are no data available. As Indraneel Mittra well points out in his interesting discussion about the global applicability of cancer screening recommendations, "the real unresolved problem of cancer control in developing countries is how to make accessible to the population at large the minimum level of cancer care that will reduce mortality and suffering^[66]". A common proposed solution is to enhance early detection through mammography screening. However, as I will argue, this is most likely not the right path to follow for LMICs.

Organized population-based mammography programs have been adopted as the gold standard of early detection in the majority of HICs. Many LMICs are

trying to follow this example, even if they lack the infrastructure and human and financial resources to implement programs of this magnitude. Therefore, they are typically ending up with opportunistic screening mammography programs that are not only inequitable^[67], more expensive and less effective than organized screening^[68,69] but also make it harder to assure test quality and access to adequate diagnosis and treatment^[70].

In recent years, the benefit of screening mammography has been seriously questioned^[71-74]. There is evidence from several HICs that most of the reductions in breast cancer mortality that have occurred since the national mammographic screening programs began are not attributable to mammographic screening but to improved adjuvant therapy^[75-81]. A recent Cochrane Systematic Review showed no effect of screening on either cancer mortality after 10 years or on all-cause mortality after 13 years^[74]. Additionally, over-diagnosis and consequent over-treatment have been reported to occur in approximately 30% of screen-detected breast cancers^[82,83].

If the benefit of screening mammography is questionable in HICs, it should be more so in LMICs. The World Health Organization has suggested that for a mammography screening program to be effective in the reduction of mortality, it needs to cover at least 70% of the population at risk^[84], which is a very difficult coverage to reach, even for HICs. Furthermore, for HICs, it has been estimated that for every 2000 women 50 years and older screened throughout 10 years, one breast cancer death will be avoided, and 10 healthy women who would not have been diagnosed if there had not been screening will be treated unnecessarily; more than 200 women will experience distress because of false-positive findings, and approximately half of them will undergo an unnecessary biopsy^[74,85]. These estimations were calculated considering HIC incidence rates and under the assumption that screening reduces breast cancer mortality in 15% of patients and has a 30% rate of over-diagnosis and unnecessary treatment. Considering that the incidence of breast cancer in LMICs is much lower and that the peak incidence occurs at a younger age, the benefits of screening mammography in LMICs are likely to be lower than in HICs, while the costs required to establish an organized screening program are most likely unaffordable for many LMICs^[70]. Some screening mammography pilot programs in LMICs have actually been shown to be ineffective and unsustainable on a larger scale because of a lack of resources^[86,87].

In the context I have presented here for breast cancer care in LMICs, with most breast cancer cases diagnosed at advanced stages and long times to diagnosis and treatment due to access barriers and substandard quality of care, the benefit of a screening program is even more questionable. Screening is useless if access to adequate diagnosis and treatment cannot be assured. The Breast Health Global Initiative Guidelines recommend that a population-based screening mammography program should not be implemented until access to the basic can-

cer diagnosis and treatment resources is guaranteed^[88].

A more cost-effective strategy could be early diagnosis or down-staging, which has been endorsed for LMICs by the World Health Organization and the Breast Health Global Initiative^[84,86,89]. The early diagnosis approach consists of the promotion of the awareness of early signs and symptoms among the public, the education of first-line health professionals and improved referral procedures to facilitate the prompt and adequate diagnosis and treatment of breast cancer in early stages.

A successful example of a down-staging program was performed in Malaysia^[90]. The program consisted of training 400 first-line health personnel in hospitals and rural clinics to improve their skills in early detection and of raising public awareness through visual information and sensitization by trained health personnel. After four years of program implementation, late-stage (III and IV) breast cancer cases were reduced from 60% to 35%^[90].

Although there is still not sufficient evidence regarding the benefits of down-staging programs, the World Health Organization and the Breast Health Global Initiative Guidelines recommend them as the most basic breast cancer early detection strategy to implement and strengthen in low-resource settings before moving on to consider mammography screening^[84,86,89]. After reviewing the evidence of advanced clinical stage and prolonged times to treatment in LMICs, it is evident that much more than just screening remains to be done to improve breast cancer mortality rates. There are serious problems in access to health services, the strength of the first level of care for the early detection of symptomatic patients, the regulation of establishments where breast imaging tests are performed, and the faulty or absent delineation of referral pathways to cancer care. Programs directed at improving these problems, which are widespread in LMICs, are likely to be much more cost-effective and have an impact in a shorter term than attempting to establish population-wide mammography screening programs in low-resource settings.

CONCLUSION

This review assembled sufficient evidence to argue that the lower breast cancer survival rates observed for LMICs in comparison to HICs are due to diagnosis in much more advanced stages. Although there is scant information on the length of care intervals, which are incomparable in many cases, the presented data provide sufficient evidence to state that breast cancer patients in LMICs suffer long diagnosis and treatment delays, and this is most likely why they present in such advanced stages. In contrast to what has usually been assumed, the greatest delays in LMICs are not attributable to patients delaying care. The longest delays appear to occur after the first medical consultation has taken place, and they are likely the result of access barriers and substandard quality of care. Research on access barriers and quality of care for the diagnosis and treatment of breast cancer is practi-

cally non-existent for LMICs, where it is most needed. To strengthen the capacity of each country's health system(s) and health services for the early diagnosis and treatment of cancer, specific barriers need to be identified throughout the entire cancer care trajectory. Such knowledge could enable individualized designs of public policies and programs for each country, region, city or even health facility that are likely to be more effective and affordable for LMICs than attempting to implement expensive and complex screening mammography programs, which are currently proving to be more harmful than beneficial, even in HICs.

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WJCO 5th Anniversary Special Issues (2): Breast Cancer

Immediate nipple-areola-sparing mastectomy reconstruction: An update on oncological and reconstruction techniques

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Abstract

Nipple-sparing mastectomy (NSM) is a safe technique in patients who are candidates for conservation breast surgery. However, there is worry concerning its oncological safety and surgical outcome in terms of post-operative complications. The authors reviewed the literature to evaluate the oncological safety, patient selection, surgical techniques, and also to identify the factors influencing postoperative outcome and complication rates. Patient selection and safety related to NSM are based on oncological and anatomical parameters. Among the main criteria, the oncological aspects include the clinical stage of breast cancer, tumor characteristics and location including small, peripherally located tumors, without multicentricity, or for prophylactic mastectomy. Surgical success depends on coordinated planning with the oncological surgeon and

careful preoperative and intraoperative management. In general, the NSM reconstruction is related to autologous and alloplastic techniques and sometimes include contra-lateral breast surgery. Choice of reconstructive technique following NSM requires accurate consideration of various patient related factors, including: breast volume, degree of ptosis, areola size, clinical factors, and surgeon's experience. In addition, tumor related factors include dimension, location and proximity to the nipple-areola complex. Regardless of the fact that there is no unanimity concerning the appropriate technique, the criteria are determined by the surgeon's experience and the anatomical aspects of the breast. The positive aspects of the technique utilized should include low interference with the oncological treatment, reproducibility, and long-term results. Selected patients can have safe outcomes and therefore this may be a feasible option for early breast cancer management. However, available data demonstrates that NSM can be safely performed for breast cancer treatment in selected cases. Additional studies and longer follow-up are necessary to define consistent selection criteria for NSM.

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Key words: Breast reconstruction; Skin-sparing mastectomy; Nipple-sparing mastectomy; Outcome; Complications; Silicone breast implants; Tissue expanders; Oncoplastic surgery

Core tip: In selected patients, nipple-sparing mastectomy (NSM) has allowed an adequate oncologic control with satisfactory aesthetic outcome. In addition, utilizing the native breast skin optimizes the aesthetic outcome of the reconstructed breast and minimizes post-mastectomy deformity. The satisfactory results are due to a close collaboration with the oncological surgical

team in terms of incision selection and mastectomy flap dissection. In general, choice of reconstructive procedure requires careful consideration of various patient related factors, including: breast volume, degree of ptosis, areolar size, patient preference and expectation, and surgeon experience. With careful patient selection and well-planned surgical technique, NSM can provide satisfactory outcomes with acceptable complication rates. However, available data demonstrate that NSM can be safely performed for breast cancer treatment in selected cases. Although NSM reduces the psychological trauma associated with nipple-areola complex resection, the oncologic safety as well as functional and aesthetic outcomes needs additional investigation. Thus, additional clinical studies and longer follow-up are necessary to define consistent selection criteria for NSM.

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INTRODUCTION

Early breast cancer treatment has advanced greatly in recent years. The introduction of skin-sparing mastectomy (SSM) technique has improved the aesthetic outcome of oncological breast surgery and immediate reconstruction^[1]. In fact, breast reconstruction following mastectomy can result in a prominent scars and a paddle of skin that is of a different color. Thus, the SSM involves en-bloc resection of the glandular tissue, nipple-areola complex (NAC), and the skin overlying superficial tumours^[2-5]. Simultaneously, the native breast skin envelope and infra-mammary fold are preserved therefore facilitating the reconstruction procedure. Utilizing the breast skin envelope optimizes the contour of the breast, resulting in a satisfactory aesthetic outcome and minimizing scarring and post-mastectomy deformity^[6-9].

Recently, an argumentation has advanced about the opportunity of extending conservation of the skin to include the NAC^[10-29]. In fact, although breast reconstruction following SSM may offer aesthetic advantages over mastectomy, removal of the NAC significantly impacts on the aesthetic outcome. Some surgical techniques have been developed to repair the NAC, including local skin flaps, skin grafts, and nipple-sharing procedures^[30,31]. However, different surgical stages are usually necessary to achieve an acceptable aesthetic result and sometimes with an unpredictable outcome^[30-32]. In one clinical series, Jabor *et al.*^[32] evaluated the satisfaction following NAC reconstruction and observed that almost 36% of patients mentioned dissatisfaction.

First described by Freeman in the 1960s as a subcutaneous mastectomy with NAC sparing, the author indicat-

ed the technique for benign diseases, however he did not report the procedure for oncological objectives or as a risk-reduction alternative^[10,11]. Recently, there has been an increase in clinical experience studies of NSM for breast cancer prophylaxis or early cancer treatment, evidencing revived interest in this surgical procedure^[12-30,33-44]. In fact, there is evidence that NSM provides aesthetic advantages, with reduced need for further surgery and NAC reconstruction^[15,17,20-22,29,33-39,44-48]. However, it is important to emphasize that most of these clinical series do not have sufficient follow-up, thus definite conclusions based on the present data is precipitated. In addition, to date there have been no controlled clinical trials evaluating the oncological effectiveness of nipple-sparing mastectomy (NSM) *vs* traditional SSM surgery. In spite of the controversies involving risk of local relapse, some current clinical studies have shown that the NSM is a safe procedure for selected cases^[11,14-16,18,23-27,29,30,33-39,44,47,48].

LITERATURE SEARCH/DATA EXTRACTION

Two independent reviewers have evaluated titles and abstracts without language restrictions to assess eligibility in terms of outcome measures and study design. A literature search was carried out up to October 2013 to identify studies of breast cancer patients submitted to NSM and determine if any technique of immediate reconstruction was recorded. In an attempt to minimize the omission of potentially relevant clinical studies, we also reviewed the reference lists of included studies and relevant reviews for additional eligible articles. Potential studies were identified by searches of MEDLINE and PubMed databases using the terms “Nipple-Areola Sparing Mastectomy”, “Total Skin-Sparing Mastectomy”, “Subcutaneous Mastectomy” and “Immediate Reconstruction”. Studies identified were screened for those that focused on techniques, surgical and oncological outcomes after NSM reconstruction and references of each study were further investigated to include all relevant published data. All types of reconstruction techniques were included (tissue expander, implant, autologous tissue, and combination of methods) and compared.

A total of 440 potential articles were identified during the primary evaluation. After appraisal of the inclusion criteria, 265 articles were identified for potential inclusion and reviewed in detail. A total of 150 articles were excluded, leaving 109 articles to form the basis of this review.

ONCOLOGICAL ASPECTS

Oncological safety/patient selection

The main criteria include the clinical stage of breast cancer and tumor aspects^[11,15,27,37-39]. From the oncological point of view, the NAC is resected because of the traditional concept that the adjacent ducts may contain tumor cells and the possibility of local recurrence^[40,41].

Table 1 Occult neoplastic involvement of the nipple areola complex

Ref.	Year	n	Nipple areola complex involvement (%)
Santini <i>et al</i> ^[45]	1989	1291	12
Menon <i>et al</i> ^[46]	1989	33	58
Verma <i>et al</i> ^[47]	1997	26	0
Vyas <i>et al</i> ^[42]	1998	140	16
Laronga <i>et al</i> ^[38]	1999	246	5.6
Simmons <i>et al</i> ^[43]	2002	217	10.6
Loewen <i>et al</i> ^[48]	2008	302	10
Rusby <i>et al</i> ^[53]	2008	130	24.6
Banerjee <i>et al</i> ^[49]	2008	219	20
Voltura <i>et al</i> ^[50]	2008	34	5.9
Pirozzi <i>et al</i> ^[51]	2010	50	28
Reynolds <i>et al</i> ^[52]	2011	29	7
Wang <i>et al</i> ^[54]	2012	787	7

In addition, some clinical series observed that nipple involvement in mastectomy specimens ranges from 0% to 58%^[12,38,42-54] (Table 1). One might surmise that this wide range is chiefly due to divergences in techniques used for pathology tests of the breast specimens, differences in technique and subgroup of patient populations. In fact, early anatomical studies proposed by Sappey described a centripetal lymphatic drainage toward the areolar plexus, thus justifying the rationale of NAC resection^[15,44]. Contrarily, recent anatomical studies demonstrated a lymphatic drainage to the deep pectoral plexus^[44,55,56].

Concerning the clinical aspects, recent studies have noted that the risk of tumor involvement of the NAC has been magnified^[38,41-43]. Thus, some clinical series have demonstrated that the NSM is a safe technique for some group of patients^[11,14-16,18,19,23-27,29,30,33-39,44,57]. In fact, some studies have considered NSM safe in patients with peripherally located tumors, small, without multicentricity, or for risk reduction^[24]. Although there is no unanimity regarding the selection criteria, the major part of studies include tumor size up to 3 cm, lack of clinical involvement of the NAC and tumor to nipple distance greater than to 2 cm. In addition, patients with clinical axillary node involvement; whose tumors are centrally located; who have inflammatory breast cancer, or Paget disease are not candidates for NSM.

In a clinical experience of 286 SSM specimens, Laronga *et al*^[38] observed that 5.6% were found to contain tumor in the NAC and did not define significant differences between groups regarding tumor size and histological subtype. However, sub-areolar tumor location and multicentricity were important risk factors for NAC involvement. Based on these findings, the authors observed that in patients with negative axilla and tumors situated on the periphery, the probability of an occult tumor is less than 2%. Similarly, Vyas *et al*^[42] in a clinical series of 140 mastectomies analyzed whether NAC correlated with areola-tumor distance, tumor size, nodal status and lymphatic embolization. In this sample, the authors also observed tumour size and nodal positivity as a potential risk factor for NAC involvement. Correspondingly, Simmons *et al*^[43]

analyzed 217 mastectomy specimens and evaluated tumor involvement of the NAC. Concerning the NAC involvement, the overall frequency was 10.6% and comparisons of patients with tumors < 2 cm with tumors ≥ 2 cm did not present a significant difference. The authors observed that only 6.7% of small tumors with up to two positive lymph nodes only had NAC involvement. For tumors located in central quadrants, the NAC was involved in 27.3% of cases. Contrarily, for those located in any of the four quadrants, the NAC was compromised in only 6.4% of cases. Gerber *et al*^[57] in a series of 112 NSMs, evaluated patients whose tumors were more than 2 cm from the NAC. The frozen sections of the subareolar tissue were negative for tumor in 54.5% of cases, thus enabling NAC preservation. During the follow-up, 5.4% local recurrences (LR) occurred in patients who underwent SSM compared with 8.2% of 134 patients who had undergone conventional mastectomy during the same follow-up. Regolo *et al*^[19] in a clinical study of 219 mastectomies observed that 20% of NACs were compromised by tumor, consisting of 9.4% of stage 1-2 tumors and 30% of stage III tumors. Concerning the tumor location, the NAC was compromised in 2.5% of peripheral tumors and in 68% of central quadrants. The authors failed to observe any cases of local relapse in patients undergoing NSM after an average of 16 mo follow-up (Table 2).

Caruso *et al*^[16] indicated NSM in patients with tumors that were peripherally situated. Their study included 50 patients with a 12% overall recurrence rate. Similarly, Sacchini *et al*^[18] evaluated patients who had NSM with reconstruction for either risk reduction, treatment of cancer, or both. With a median follow-up of 24 mo, two breast cancer patients and two patients who had NSM for prophylaxis presented a local recurrence outside of the NAC. Based on this clinical experience, the authors concluded that the risk of local relapse is low and the procedure is feasible in the risk-reducing and breast cancer-treatment.

Munhoz *et al*^[33] evaluated 158 consecutive patients submitted to NSM. In almost 35% of patients the procedure was indicated for cancer prophylaxis including high-risk lesions, prophylactic, familial history and carriers of the *BRC11* or *BRC42* mutation. In the remaining breast cancer patients, almost 75% of tumors measured 2 cm or less (T1) and the majority were stage 0 and I. Similarly as observed by other authors, the present study also included a few stage III breast carcinomas; however in the preoperative period these patients were staged as earlier-stage carcinoma^[9,58]. Additionally, the authors excluded patients with NAC infiltration, NAC bleeding or with the tumor at less than 5 cm from the NAC. Considering these parameters, the authors believe that NSM is feasible with low local recurrence. With a mean follow-up of 65.6 mo, local recurrence rate was 3.7% and the incidence of distant metastases was 1.8%.

In a comprehensive review, Tokin *et al*^[24] observed that the local recurrence following NSM was between 0%-20%, with studies varying widely in inclusion criteria and follow-up period. Boneti *et al*^[26] reported in a series

Table 2 Clinical outcome and local recurrences following nipple-sparing mastectomy

Ref.	Year	n	Stage	Follow-up (mo)	Nipple areola complex recurrence	Local recurrence
Gerber <i>et al</i> ^[25]	2009	61	0- I	59	1.6	5.4
Garcia-Etienne <i>et al</i> ^[15]	2006	42	0- I	10.5	0	0
Bistoni <i>et al</i> ^[106]	2006	10	0- I	36	0	0
Voltura <i>et al</i> ^[50]	2008	51	0-III	18	0	5.9
Crowe <i>et al</i> ^[14]	2004	54	0-II	41	0	0
Petit <i>et al</i> ^[104]	2005	579	0-I	19	0	0.9
Sacchini <i>et al</i> ^[18]	2006	192	0-III	24.6	0	3
Paepke <i>et al</i> ^[103]	2009	109	0-III	34	0	1.83
Babiera <i>et al</i> ^[107]	2010	54	0-III	15	0	0
Benediktsson <i>et al</i> ^[105]	2008	216	0-III	156	0	8.5
Munhoz <i>et al</i> ^[33]	2013	158	0- II	65.6	0	3.7

of 281 NSM with 25.3 mo mean follow-up, a 4.6% local recurrence rate. Jensen *et al*^[27] published results from 149 patients without local recurrences at a mean 5-years follow-up.

In a recent review, Mallon *et al*^[59] quantified the incidence of occult NAC cancer and identified the factors influencing occult nipple malignancy, local recurrence rates, and complication rates. According to the authors, the overall nipple (0.8%) and flap (3.4%) recurrence rates were similar to those reported after mastectomy and conservative breast surgery. However, care must be taken to distinguish that follow-up periods for NSM clinical studies are briefer than those for mastectomy and partial mastectomy. For definitive conclusions, a longer and similar follow-up is necessary, as the greater part of recurrences occur within 5 years.

Therefore, it would appear oncologically safe to perform NSM, provided the tumor is not close to the NAC, small, peripherally located, without multicentricity and a frozen section protocol is performed. Although various clinical series including SSM and NSM aided in the selection of patients for NSM using tumor to NAC distance values, the ideal tumor to NAC distance has yet to be clarified, since the total number of patients analyzed in these clinical series is insufficient and requires validation^[41,59]. Additionally, patients must be informed that NAC resection may still be necessary if residual tumor is identified on frozen sections of the subareolar tissue or definitive histology.

Timing: One stage x two stage approach

NSM may be planned in one setting with immediate reconstruction (one-stage approach)^[39,57,60], or in two settings with partial glandular resection or NAC autotomization followed by additional breast tissue resection and total reconstruction weeks to months afterwards (two-stages approach)^[30,34,39,61-65].

Preoperative planning should include the breast ptosis and volume and mostly addressing singular reconstructive requirements, enabling each patient to receive an individual “custom-made” planning. In addition, an in-depth discussion concerning alternatives for NSM reconstruction should be undertaken with the patients and her family, including the risks and positive aspects of one vs

two-stages approaches.

One-stage approach: With one-stage approach both procedures (breast cancer treatment/risk reduction and reconstruction procedures) are associated in one operative setting. Additionally, the emotional benefit of having begun reconstruction at the time of NSM procedure may decrease the impact of the loss of the breast. In fact, Sahin *et al*^[60] in a series of 21 bilateral prophylactic NSM due to higher risk for cancer indicated the one stage approach and simultaneous breast reconstruction using submuscular silicone implants. According to the authors, better projection and shape may be achieved with serial expansion of the submuscular pocket, but this has to be weighed against the morbidity associated with two surgical procedures. In their clinical experience, a one-stage procedure using high-profile implants resulted in very good projection while avoiding the morbidity of a second surgery.

Other centers indicated both approaches according to the quality and the width of the remaining breast skin flap. Chen *et al*^[30] in a series of 115 NSM evaluated the risks and benefits of the procedure associated with immediate breast reconstruction. In all patients, reconstruction with tissue expander or silicone implant was performed immediately following the NSM. Of the 66 patients, 58 underwent tissue expansion followed by implant placement in a two-stage reconstruction (87.9%) and eight patients underwent one-stage reconstruction (12.1%). According to the authors, nineteen patients had wound-healing problems. Full and partial necrosis of the NAC was not associated to initial expander volume but was more prevalent in thin flaps and larger breasts.

Although NSM and immediate implant reconstruction can be accomplished in a single stage, this is not the first option in some cancer centers^[30]. In fact, Chen *et al*^[30] emphasized that with two the stage approach it is possible to have a better control over the NSM skin flap. First, some aspects relating to implant asymmetry can be treated at the time of the second stage. Second, by limiting the volume of the expander such that the skin flap is not redundant but also not under tension, the risk of necrosis is reduced. Finally, patients usually desire a volume change, and starting the reconstruction with a two stage

approach allows the surgeon to customize the outcome to patient preference.

In spite of these aspects, for some group of patients the one-stage approach can be advantageous. In fact, patients with small breasts, without ptosis and cardiovascular clinical diseases are the best candidates for one-stage NSM. Caruso *et al.*^[16] considered NSM in patients with small to moderate-sized breasts with moderate to minimal ptosis and a healthy breast skin. Similarly, in a systematic review Endara *et al.*^[39] examined current trends with NSM, including selection criteria, incision choice, and reconstructive techniques. In the major part of the cases, NSM requires no skin resection, however with increasing breast volume (> 500 g) or breast ptosis, higher rates of NAC or breast skin flap necrosis are expected. In addition, low BMI and minimal ptosis were consistently used to screen patients for NSM in these studies.

Conversely, with the one-stage approach the surgical time can be lengthened and potential complications of the NSM (*e.g.*, skin/NAC necrosis, dehiscence, infection) can adversely influence the postoperative outcome. In addition, the procedure can be compromised by positive margins, especially in the sub-areolar region. In fact, Mallon *et al.*^[59] in a recent comprehensive review demonstrated that the greater part of the NSM studies performed biopsy of the retroareolar tissue separately from the mastectomy specimen. Concerning the technique, some studies used frozen section analysis, however, this technique has a false-negative rate as high as 8.7% according to the present review. Therefore many cancer centers await definitive pathologic evaluation of sub-areolar specimens before deciding on NAC resection. Thus, it is advocated that all patients submitted to one stage therapeutic NSM have a retroareolar sampling. In addition, these patients must be informed that the NAC may need to be ultimately resected if result of the retroareolar biopsy is compromised.

Two-stage approach: With two-stage approach, the surgical process is less extensive than NSM and immediate reconstruction in one operative setting. Some patients are so distressed by their cancer diagnosis, that they are not able to cooperate in reconstructive decisions. Additionally, some potential complications of the NSM and reconstruction techniques (*e.g.*, skin necrosis, dehiscence, infection) can unfavorably defer the adjuvant therapy. However, while the rationale for this approach is reasonable, the addition of a different surgical stage may introduce possibilities for complications^[65].

First proposed by Palmieri *et al.*^[66], the two-stage concept of delayed NSM had the objective of complete removal of all breast tissue, including the lactiferous ducts. According to the authors, the first stage involves NAC autonomization by performing a periareolar incision to detach the ductus from the nipple. The second stage is then performed 2-3 wk later. The authors observed one case of NAC necrosis that occurred during the NAC autonomization, delaying the NSM for 6 wk to allow

complete revascularization with a satisfactory outcome. Similarly, Jensen *et al.*^[67] indicated the two-stage approach with NAC surgical delay in 20 patients who were at high risk for NAC necrosis following NSM. The authors performed the delay technique 7-21 d prior to NSM mastectomy. Sub-areolar biopsy was performed at the time of the delay procedure and if the biopsy revealed malignancy, the NAC was removed at the time of NSM. All of the NAC survived and in 2 patients the subareolar biopsy was positive and 3 NAC were removed.

Another important point is related to the possibility of another stage to improve the aesthetic outcome^[30,34]. In fact, Blechman *et al.*^[34] in a series of 55 NSM performed in 29 consecutive patients evaluated the technical aspects and outcome. After tissue expansion the implant volume can be selected during the second stage without causing flap tension. Also, this strategy provides an opportunity to refine the breast contour such as by fat grafting.

In the greater part of the clinical series, NSM are related to patients with relatively small, minimally ptotic breasts or for risk reduction^[14,39,61,62]. However, the NSM reconstruction of large and/or ptotic breasts poses a more troublesome challenge than the NSM of small sized breasts because of an excessively large skin flap^[33]. In addition, the Wise-pattern skin excision best addresses this excess skin but is associated with a high incidence of flap necrosis with subsequent reconstruction failure^[22,33]. Munhoz *et al.*^[33] in a series of 158 patients submitted to NSM observed a significantly higher incidence of complications in the obese and larger specimen group. This aspect can be partially explained by a decreased perfusion of the relatively large skin flaps that result from SSM in much larger breasts. According to the authors, after adjusting for other risk factors (BMI, weight of breast specimen), the probability of complications tends to be higher for the Wise pattern with superior pedicle incision approaches.

Although large breasts and severe ptosis may represent a contraindication for NSM, surgical strategies based on the two-stage concept were planned to correct the ptosis followed by NSM in a second stage. Introduced by Spear *et al.*^[61] the NSM staged procedure includes patients with large or ptotic breasts and candidates to NAC preservation. In fact, the authors observed that although there are breasts that are too large to be considered for a NSM, it is possible to extend the indications by using the two stage approach and reducing the breast volume and ptosis previously. Thus, the main objective in these sub-group of patients is to preserve the oncological objective of the NSM (therapeutic or risk reduction) while expanding the aesthetic outcome and minimizing complications. For this objective, some authors divided the one-stage Wise-pattern skin excision into a two-stage procedure^[61,63,64]. In the first stage, the mastopexy or reduction mammoplasty is performed, keeping periareolar dermis preserved to maintain the adequate NAC blood supply at the time of the future definitive NSM. At the time of the

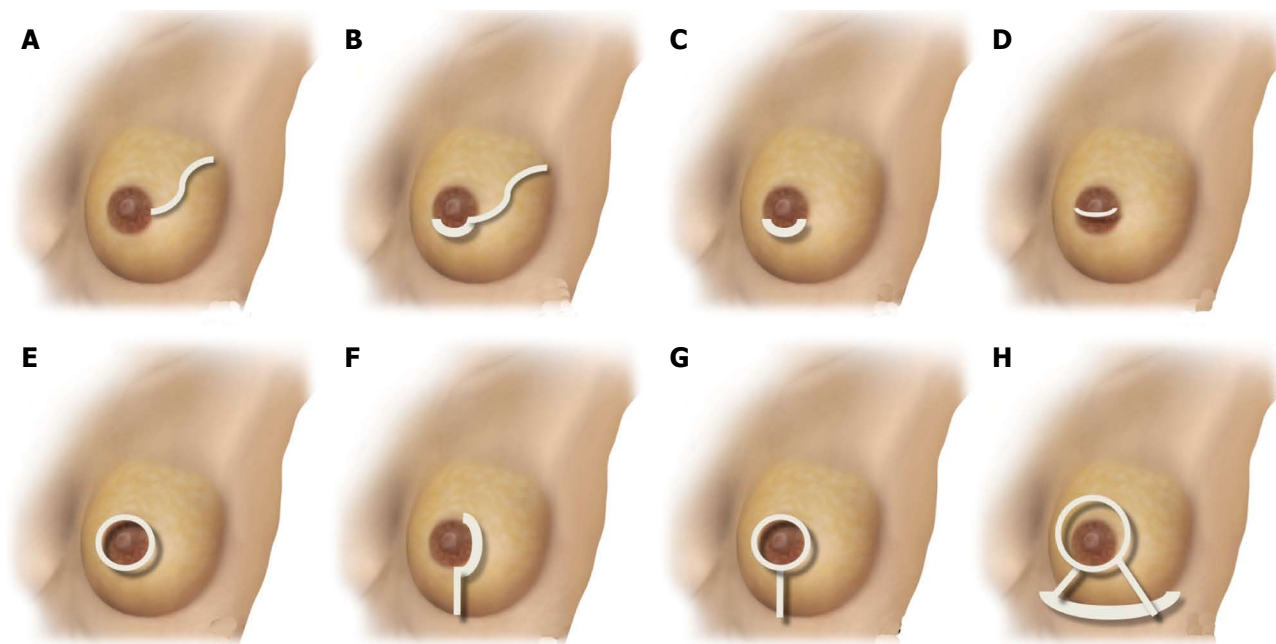


Figure 1 Schematic representation of nipple-sparing mastectomy incisions. A: Radial lateral incision; B: Periareolar with lateral extension; C: Hemi-periareolar (superior and inferior); D: Transareolar; E: Circumareolar (periareolar total); F: Periareolar with vertical extension; G: Circumareolar (periareolar total) with vertical extension; H: Wise-pattern mastectomy.

second stage, care must be taken to guarantee consistent flap thickness in order to avoid damage to the skin flap blood supply.

Liu *et al.*^[63] in a series of 12 patients achieved successful outcome using the two staged Wise-pattern excision. In the first stage, the NSM and reconstruction were performed using a vertical excision. In the second stage, the redundant skin at the inframammary fold was excised, tightening the breast skin envelope vertically. According to the authors, the addition of the two staged incisions recreates the Wise pattern, breaking up the T point into two straightforward primary closures. Similarly, Spear *et al.*^[61] reported a successful two-stage NSM in 15 patients (24 breasts). All patients underwent NSM after mastopexy or reduction (71% prophylactic and 29% therapeutic) with an average follow-up of 13 mo. Four of the 24 operated breasts (17%) presented a complication. Besides the satisfactory outcome, it is important to emphasize that although the two-stage NSM is acceptable in the prophylactic group, patient selection is somewhat more complex in the group with breast cancer. Thus, the two-staged procedure must be correctly planned so that it does not significantly delay the oncological treatment in this patient population. Yacoumettis^[64] in a retrospective study of 52 patients evaluated the results of bilateral subcutaneous mastectomy for breast cancer prophylaxis. All reconstructions were completed in two-stages with tissue expanders followed by textured gel filled silicone implants. According to the authors and during the average follow-up of 7.2 years, no cases of invasive cancer were observed, and the aesthetic outcome was considered satisfactory.

Thus, the two-stage concept can be, in theory, advan-

tageous when compared to the one stage NSM. However, as we observed in any procedure this approach can present some limitations. The main negative aspects are related to some technical difficulties, *i.e.*, scar tissue and fibrosis. Additionally, the procedure can be time consuming and demanding additional costs, which can represent some limitations to the insurance coverage and resource implications for community hospitals.

Incision selection

Numerous incisions have been described by a variety of designs incorporating a periareolar approach, or other variations in the shape around the NAC^[11-16,18-22,30,33-37,39,59,61,63,66-68]. Although the incision types vary with configuration, the impasse of the access incision with no complications has drawn attention in the great part of the studies^[20,25-28,30,33] (Figure 1).

A critical survey shows that the procedure is normally performed by numerous approaches, but the greater part more than one type of incision is performed^[11-16,18-22,30,33-37,39,59,61,63,66-69]. In fact, Endara *et al.*^[39] analyzed 48 NSM studies, of which 41 described details related to the type of NSM incision. A total of 15 diverse approaches were described and the greater part of the studies (70%) more than one type of incision was indicated. According to the authors, the most common incision described were radial, followed by periareolar, inframammary, mastopexy, and transareolar (Figures 2-4).

The radial incision is one of the most performed techniques for NSM. Endara *et al.*^[39] reported that this incision represented almost 46% of all incisions performed. Stoler *et al.*^[20] in a series of 82 NSM for risk reduction and cancer treatment described that the most

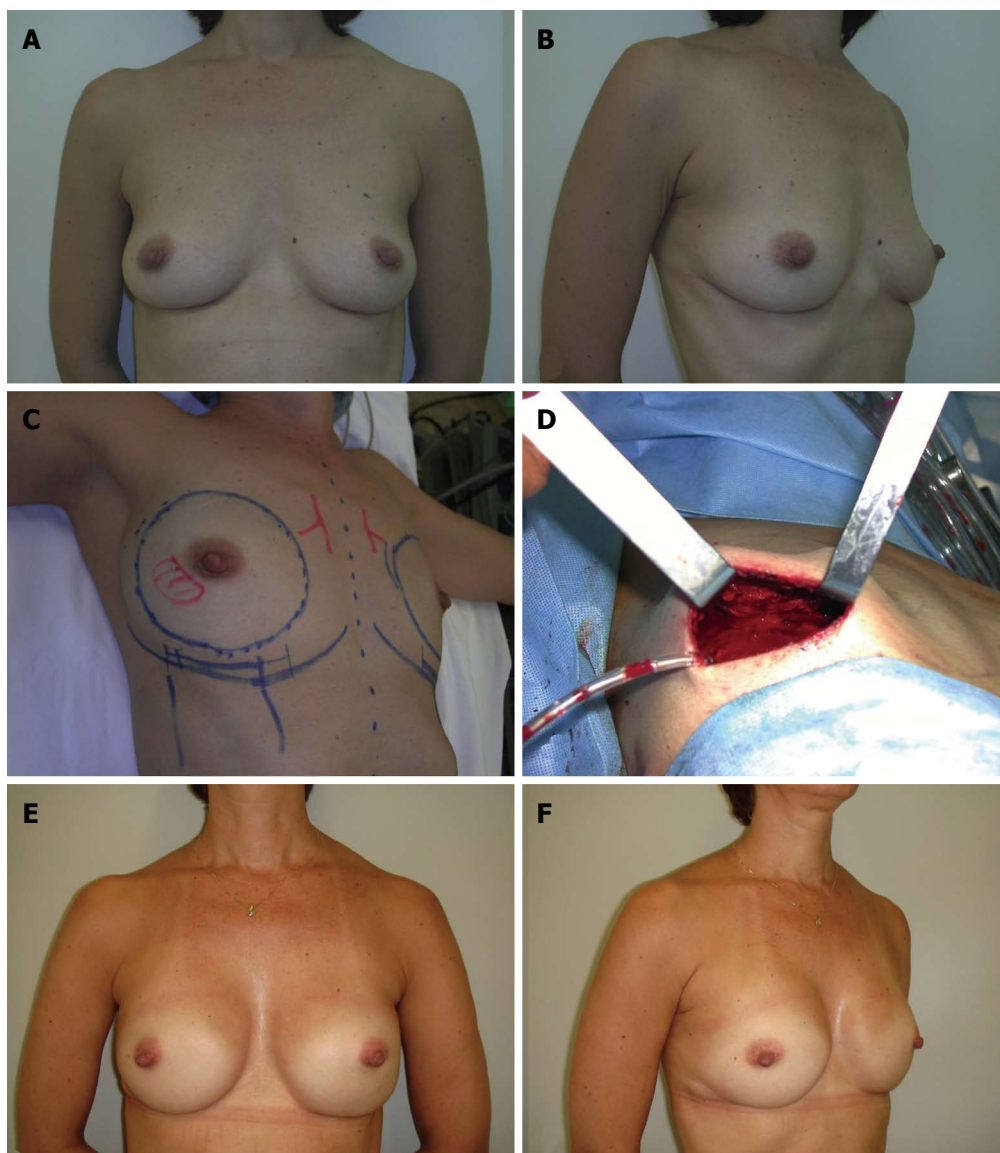


Figure 2 Nipple-sparing mastectomy/inframammary incision. A and B: A 44-year-old patient with an invasive ductal carcinoma in the right breast (1.4 cm) and a familial history of breast cancer; C and D: Nipple-sparing mastectomy preoperative planning was based on a bilateral through a inframammary approach and immediate reconstruction with biodimensional implant-expander (Allergan 150 SH, 285 cm³). Intraoperative frozen sections demonstrated nipple-areola complex free of tumor; E and F: Five years postoperative appearance with a very good outcome.

common incisions utilized were related to the radial incision and a lateral incision beginning from outside the NAC. According to the authors, this incision allowed an adequate exposure to all regions including the axillary tail and the internal thoracic vessels for free flap anastomosis. Colwell *et al*^[70] performed an inferolateral approach with the incision located in the lateral quadrant. Similarly, Chung and Sacchini evaluated NSM incisions, which the greater part associated the periareolar to the radial incisions^[65]. The same group reported NSM through different incisions and the periareolar incision with lateral extension was used in 42% of cases^[11]. The authors mentioned a satisfactory exposure as advantages of the use of the radial extensions. Compared to other incisions, complications were observed in 67% of cases with an inferior lateral incision (inframammary fold extended

laterally). Wijayanayagam *et al*^[71] in a series of 64 NSMs performed in 43 patients evaluated the technical aspects and surgical outcome. Using different types of incisions, the authors observed that the radial incision provided the best approach and had the greatest likelihood of maintaining viable NAC without necrosis, which was observed in almost 97% of the sample. Despite the benefits, some authors do not advocate this approach due to aesthetic disadvantages. In fact, this technique creates a scar that is especially visible in the oblique and profile views^[60].

The periareolar incisions are the second most performed techniques for NSM. In fact, Endara *et al*^[39] reported that the periareolar approach represent almost 27% of all incisions performed for NSM. The main benefits are related to scar camouflage with a more satisfactory outcome. Despite its advantages, the periareo-

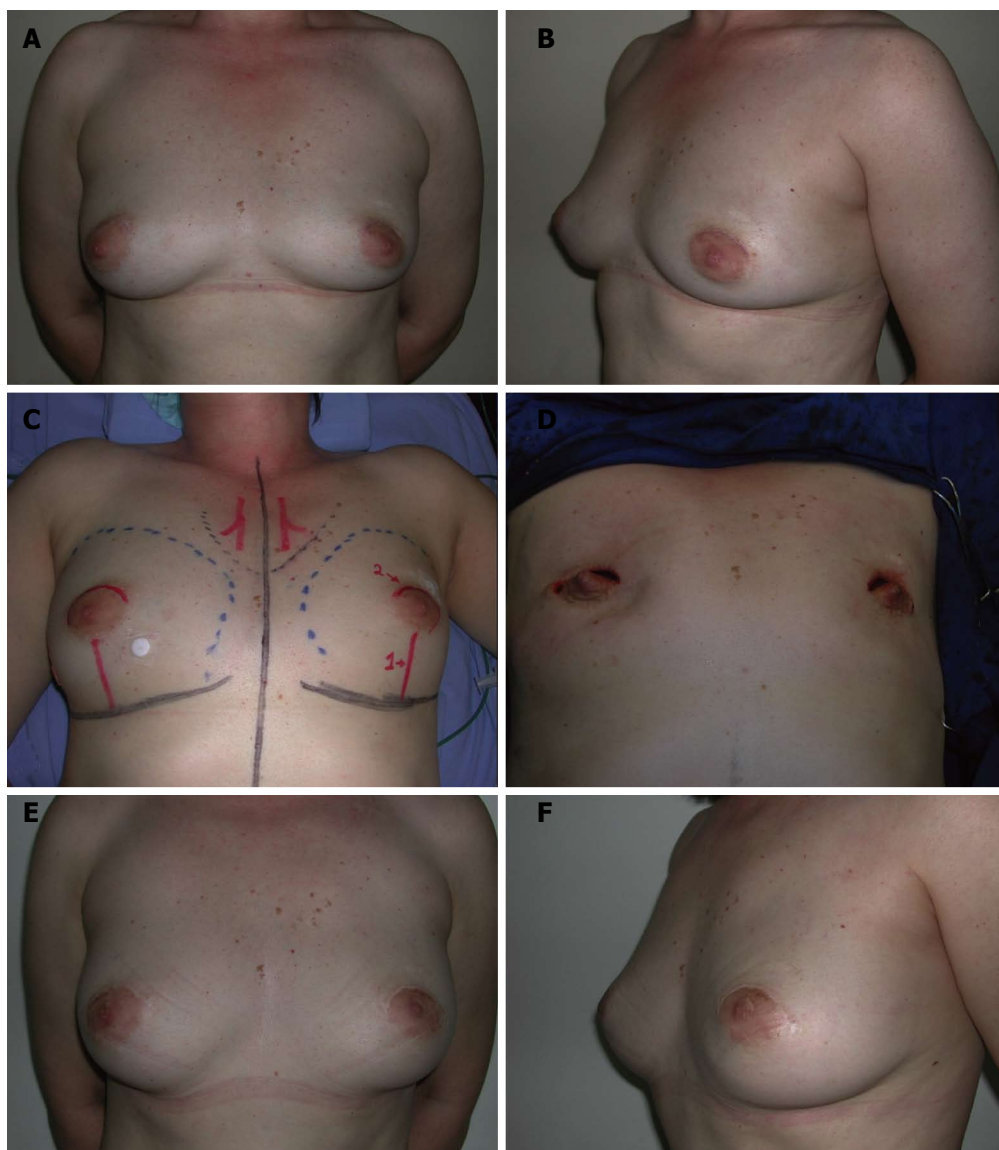


Figure 3 Nipple-sparing mastectomy/superior periareolar incision. A and B: A 52-year-old patient with *in situ* multifocal carcinoma in the right breast (4.8 cm) and atypical hyperplasia in the left breast; C and D: The patient underwent a bilateral nipple-sparing mastectomy through a superior periareolar incision and sentinel lymph node biopsy; E and F: The oncological procedure was immediately followed by a bilateral pedicled transverse rectus abdominis myocutaneous flap reconstruction. Four years postoperative appearance with a very good outcome.

lar incision is not adequate for all patients candidate to NSM. In fact, the more suitable indication is in patients with small breasts with an adequate areola diameter. A limited exposure and difficulty in breast flaps dissection are commonly observed in small areola patients and inexperienced breast surgeon. For patients with large areola diameter without breast ptosis, a hemicircumareolar incision is usually indicated. Another important indication is the presence of a marked color transition between the NAC and the breast skin and small/medium volume breasts (cup size A-B). Sahin *et al*^[60], in a series of prophylactic NSM usually indicated the periareolar incision for small-breasted patients. According to the authors, the NSM and the reconstruction are performed through this incision, extending circumareolar or semicircular in the lower half of the NAC. Rivolin *et al*^[35] in a series of

22 patients submitted to NSM evaluated the benefits of the periareolar approach associated with mastopexy for patients with ptotic breasts. All patients in the periareolar group were submitted to a one-stage reconstruction, while a two-stage approach was selected in 20% of patients. The complication rate was higher in the periareolar group, although the difference did not reach significance. Despite the satisfactory outcome, the mastopexy technique was inadequate if repositioning the NAC was more than 3 cm or in sufficiently large reductions to reduce excess skin. In women with larger and more ptotic breasts, Chen *et al*^[30] advocated the omega-type elliptical incision. Similar to the periareolar incision with lateral extension, the omega-type approach gave the surgeon wide access to the breast regions and axilla.

Besides the limited exposure, the periareolar incision

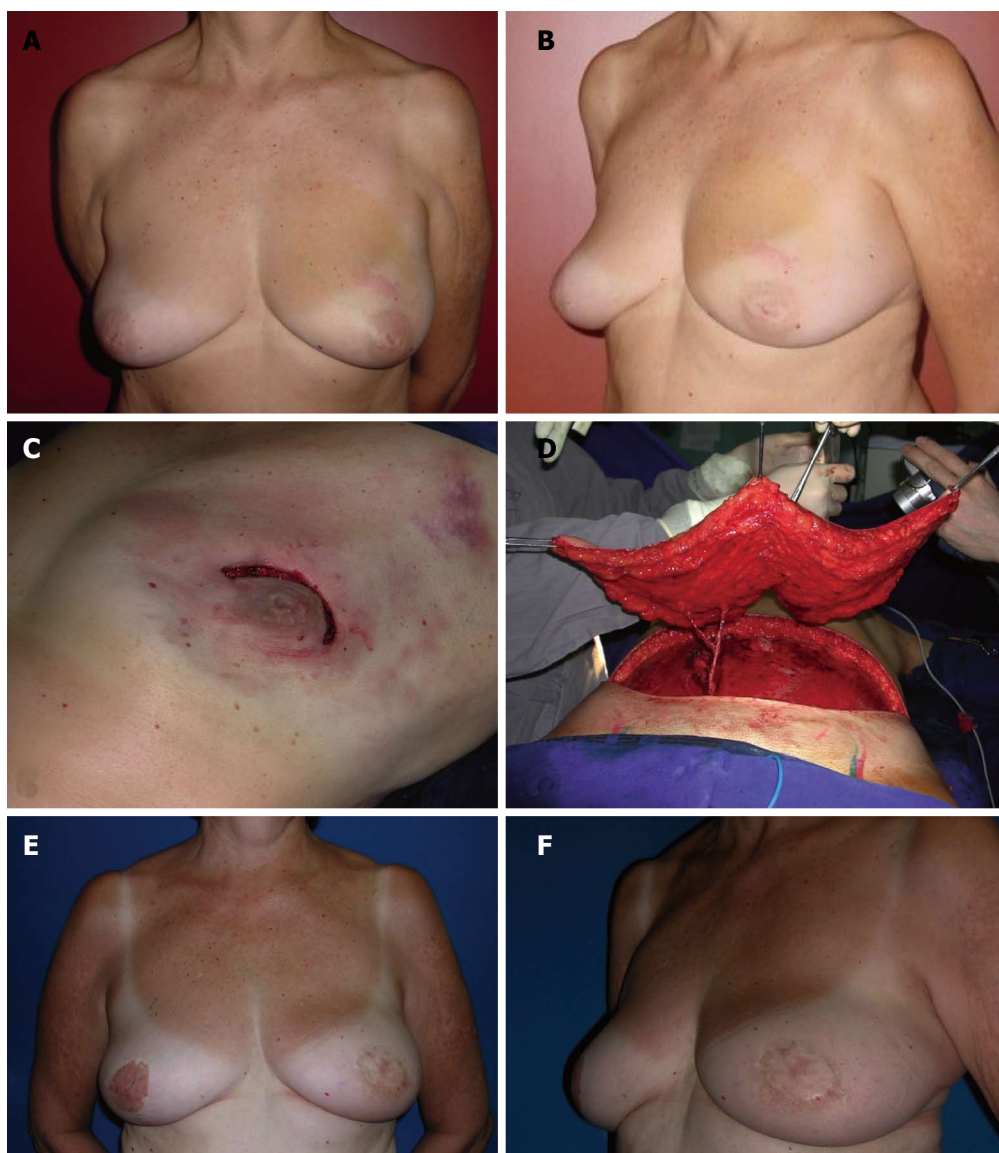


Figure 4 Nipple-sparing mastectomy/superior periareolar incision. A and B: A 56-year-old patient with invasive ductal carcinoma of the left breast (2.3 cm); C and D: The patient underwent a left nipple-sparing mastectomy with a superior periareolar incision and sentinel lymph node biopsy. The oncological procedure was immediately followed by a free deep inferior epigastric perforator flap reconstruction; E and F: Five years postoperative appearance with a very good outcome. The superior periareolar incision was converted to a total circumareolar incision in order to achieve a better symmetry during the second stage of reconstruction.

can result in an impairment to blood supply, which can induce NAC necrosis. In fact, Regolo *et al*^[19], in a series of 32 NSM utilizing the periareolar incision observed a high rate of complications of the NAC (60%). Consequently, Munhoz *et al*^[21] developed an approach to improve the surgical exposure based on total circumareolar incision. This technique was based on the double concentric periareolar incision to resect the glandular tissue, while maintaining the vascularization of the NAC through the subdermal vascular plexus. In addition, the authors advocated de-epithelializing the whole periareolar incision to allow for triple-layer closure of the wound. Therefore, no part of the suture lines present only one layer, thus lessening the risk of breast implant exposure.

The inframammary incision is the third most performed approach for NSM. According to Endara *et al*^[39]

the inframammary technique represents almost 20% of all incisions performed for NSM. Blechman *et al*^[34] in a clinical series of 55 NSM through a lateral inframammary incision performed in 29 consecutive patients evaluated the technical aspects and outcome. The authors indicated the lateral IMF approach for a variety of breast volumes, and were able to place different volumes of implants. According to the authors, the benefits are related to hiding the scar and the incision is the furthest from the NAC and thus it is the least likely to threaten its vascular stability. In addition, rotating the IMF incision laterally facilitates easier access to the sentinel lymph node biopsy. Contrarily, Chen *et al*^[30] in their review of a series of 115 NSMs evaluated the risks and benefits of the procedure associated with immediate breast reconstruction. The IMF approach was indicated for patients with smaller

breasts. Stoller *et al*^[20] observed that the inframammary fold incision was uncertain. According to the authors, surgical access to the recipient vessels may be problematic, making this incision more adequate to implant reconstruction. In addition, they reported an inaccurate dissection around the NAC and in the upper quadrants. Similarly, Wijayanayagam *et al*^[71] in a series of 64 NSMs performed in 43 women observed that the inframammary incision provided a large exposure. However, they were concerned about the ability to access the upper quadrants in patients with large breasts and limited this incision to patients with very small breasts. Thus, the authors recommended using an incision of at least 10 cm because the larger incision enables easier eversion of the skin for improved visualization of sub-areolar region. Saliban *et al*^[30] analyzed 118 NSMs in 80 consecutive patients and observed that patients with different breast sizes underwent inframammary approach, except those patients who had very large breasts or those who requested a breast lift.

Contrarily, some authors avoid the inframammary fold incision due to the technical limitation to dissect the upper pole breast tissue and inadequate resection^[20,30,33,36,60,70]. In fact, Chen *et al*^[30] observed that although the inframammary incision allows a better final position of the scar, the resection of glandular tissue superiorly could be more challenging. Additionally, in some cases the authors believe that it is difficult to place the incision on the right position once the final implant volume is decided at the end of the surgery^[33]. Besides these limitations, some authors believe that the inframammary incision could impair the inframammary blood supply^[36,72]. Proano and Perbeck compared skin blood supply in patients having either an inframammary approach or a lateral lazy S incision using laser Doppler and fluoresceinometry^[72]. In a series of 69 patients, they observed a significant reduction in flow to an area of skin 2 cm below the NAC in the group submitted to inframammary approach.

The mastoplasty incision has been previously described for planning SSM/NSM in ptotic breasts^[1]. Classified by Carlson *et al*^[5,6] as a Type IV, it involves breasts that require a reduction of the skin flap and offers a wide exposure^[22,33,39,73-76]. According to Endara *et al*^[39] the mastoplasty approach represents almost 4% of all incisions performed for NSM. The main benefits are related to a better surgical access in patients with large breast and moderate/severe ptosis. Another potential advantage is related to reduction of the skin envelope and the dead space between the skin and the implant. Rusby observed that a limited volume of fluid collecting between skin flaps and reconstruction allows the preserved skin to redrape over the breast mound to a variable and uncontrolled extent^[75]. In fact, by reducing the skin flap, such that it is closer to the breast mound size, movement is reduced.

Munhoz *et al*^[33] reported that almost 35% of the patients were submitted to the mastoplasty incision. The superior pedicle and inferior pedicle techniques were indicated for moderate ptosis and severe ptosis cases respec-

tively. In spite of the main benefits, this technique has some limitations since the lateral and medial skin flaps that close down to the inframammary fold may become ischemic, and implant exposure can be observed^[33,74,75]. Another negative aspect is related to the relative lack of space in the inferior and medial aspects of the submuscular pocket. It is possible to release the inferior aspect of the pectoralis muscle, however a subcutaneous pocket could become an implant exposed, in the situation of an ischemic NSM flap^[22]. According to Toth and Lappert^[1], this aspect is critical and not rare if the general surgeon during dissection needs to leave very thin poorly vascularized NSM flaps. Thus, the technique requires close collaboration between the oncologic and reconstructive surgeons. In higher risk patients or severe breast ptosis, Munhoz *et al*^[33] preferred the inferior pedicle technique since the well-vascularized pedicle provides a stable soft-tissue cover for the implant, which protects against exposure. Similarly, Nava *et al*^[22] in a series of 28 patients with ptotic breasts proposed a combined flap technique to reconstruct by use of anatomical silicone implants. After preoperative planning, a large area in the lower half of the breast was deepithelialized according to the conventional Wise pattern.

Skin flap and NAC complications

In spite of the NSM advantages, the outcome is not always predictable. Surgical concerns are related to increased complications such as wound healing problems or ischemic necrosis^[19,24-29,33]. In fact, one of the most problematic complications of NSM is skin flap and NAC necrosis, which can lead to unsatisfactory aesthetic result (Table 3) (Figure 5).

Early reports on the NSM technique described high rates of complications^[18,28,30,57]. Gerber *et al*^[57] in one of the first clinical series of NSM evaluated the NAC outcome in 61 patients. The authors observed that 9.8% of patients presented partial nipple necrosis with no cases of total necrosis. Komorowski *et al*^[28] observed a 7.9% incidence of total nipple necrosis and a 5.3% of partial nipple loss. In 2006, Sacchini *et al*^[18] observed necrosis of the nipple in 11% of the sample and it was judged minimal in 59% of patients. Munhoz *et al*^[33] identified patient and breast related factors that increased complication rates. Concerning the NAC outcome, the majority of NAC demonstrated some degree of immediate ischemia manifested by coolness. However, the NAC skin survived in almost 95% of cases and partially survived in 4.4%. In these cases, the NAC developed epidermolysis/partial-thickness necrosis and most of these healed conservatively.

Previous studies have reported some risk of skin flap/NAC necrosis^[20,24-30,33,36,39]. Although comparing NAC necrosis rates between different populations, techniques and experiences can be challenging, most studies report rates from 0 to 19.5%^[23,25]. As techniques have improved, the rates of local complications have been reduced to satisfactory levels^[19-22,24-27,33]. Some authors advo-

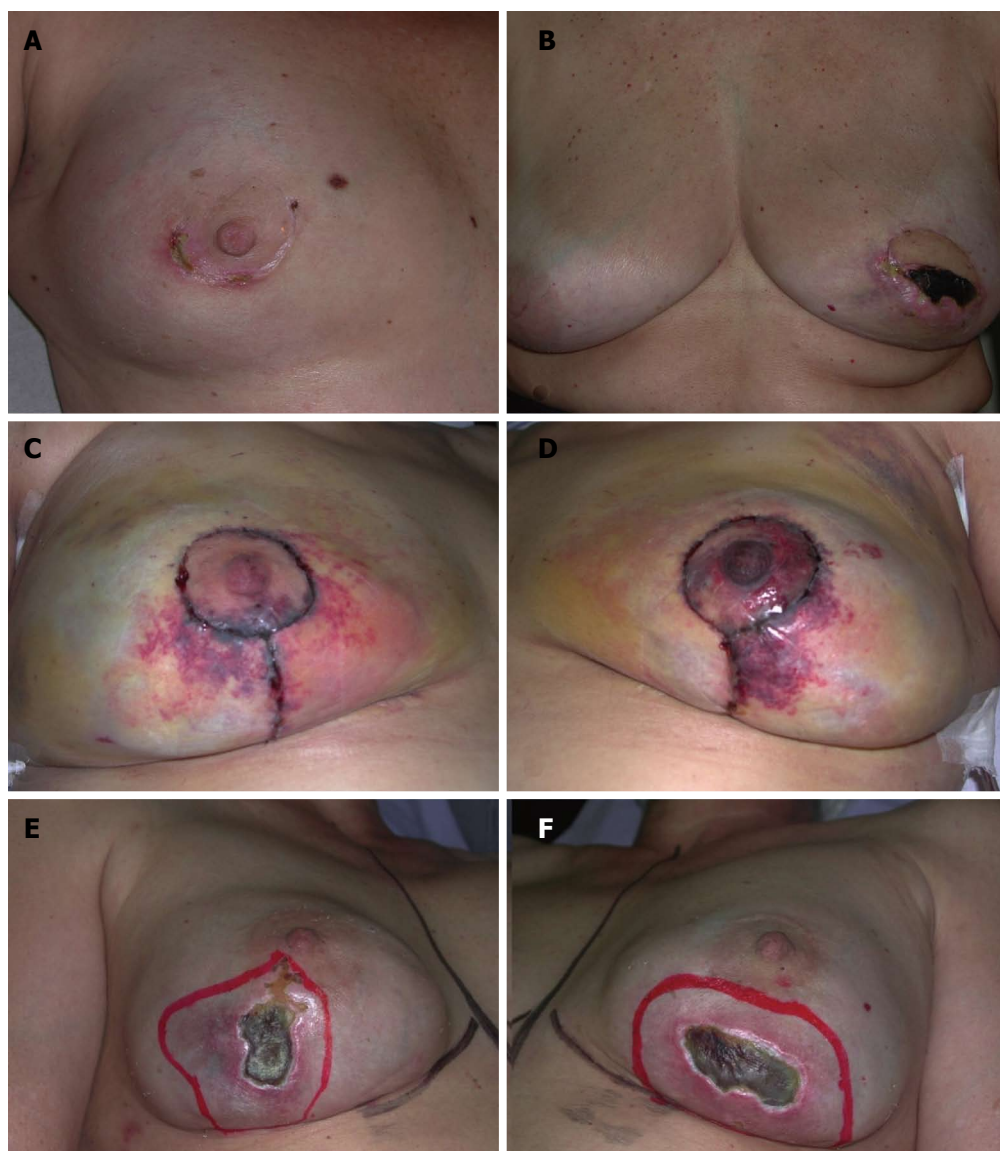


Figure 5 Local complications following nipple-sparing mastectomy. A: Inferior periareolar incision with partial wound dehiscence; B: Superior periareolar incision with partial nipple areola complex necrosis; C and D: Wise pattern incision with partial mastectomy and nipple areola complex necrosis; E and F: Inframammary incision with partial mastectomy necrosis.

cate the use of lateral incisions, avoidance of periareolar incisions which require more skin traction, limiting dissection beyond the lateral aspect of the anterior axillary line and over the sternum to preserve blood supply to the skin flap, and the use of scissors to avoid thermal lesion^[11,44]. In addition, the option of the adequate surgical approach is critical and depends on previous scars, tumor location, breast volume, degree of ptosis and NAC anatomy. Although large studies are necessary to evaluate the best incision type, reduced NAC necrosis have been described with radial areolar incisions^[20,36,39,71,76].

In a recent review, Endara *et al.*^[39] evaluated the incision type and outcome following NSM. Based on 48 clinical studies in a pooled analysis, the authors reported similar rates of NAC necrosis between radial and inframammary incisions (8.83% and 9.09%, respectively) but an increased rate of necrosis following periareolar

approaches (17.81%). In this review, the transareolar incision presented the highest incidence of nipple necrosis (81.82%). Based on the results of this review, the preferred incision is either the inframammary fold or the radial with a lateral extension.

Contrarily, Munhoz *et al.*^[33] observed that the type of incision was not significantly predictive of complications in univariate analysis. However, after adjusting for other risk factors (BMI and weight of specimen), the probability of complications tends to be higher for hemipariareolar and Wise-pattern superior pedicle incision. In addition, they observed a lower incidence of NAC necrosis with the double circle incision technique. This aspect is probably due to the full access along the inferior border of NAC, which seems to allow adequate blood supply to the NAC. The authors believed that besides the limited access, the hemipariareolar technique can

Table 3 Clinical outcome and vascular related complications following nipple-sparing mastectomy

Ref.	Year	n	Nipple necrosis	
			Total (n)	Partial (n)
Petit <i>et al</i> ^[109]	2009	1001	35	55
Nahabedian <i>et al</i> ^[17]	2006	11	0	1
Spear <i>et al</i> ^[108]	2011	49	3	3
Voltura <i>et al</i> ^[50]	2008	51	0	0
De Alcantara Filho <i>et al</i> ^[23]	2011	341	0	1
Wijayanayagam <i>et al</i> ^[71]	2008	64	3	10
Jensen <i>et al</i> ^[27]	2011	127	0	8
Paepke <i>et al</i> ^[103]	2009	109	1	23
Babiera <i>et al</i> ^[107]	2010	54	0	4
Munhoz <i>et al</i> ^[33]	2013	158	1	8

potentially result in vascular impairment to blood supply due to traction, which can induce partial necrosis. In fact, Regolo *et al*^[19] in a series utilizing the periareolar incision observed a high rate of necrotic complication of the NAC, which they abandoned in favor of a lateral incision. Similarly, Stoller *et al*^[20] reported no cases of nipple necrosis when using a lateral or radial incision and Chung *et al*^[65] found adequate postoperative NAC viability by using a periareolar and lateral skin incision or an inframammary approach.

Some authors suggest that clinical co-morbidities are relevant risk factors for complications^[5,6,28,33,77-82]. Komorowski *et al*^[28] analyzed such factors and concluded that age below 45 years is associated with a reduced risk of necrosis. Contrarily, Munhoz *et al*^[33] did not observe age as a significant factor for NAC necrosis. However, in an univariate analysis the authors showed a significantly higher incidence of complications in the obese, hypertensive and larger specimen group. In fact, the deleterious effect of obesity on breast reconstruction was previously studied^[77,78,80]. One might suppose that increased BMI may predispose the flap necrosis due to the compromised sub-dermal plexus brought about by the increased surface of the flap^[83]. In addition, obese patients are likely to have additional complications due to associated vascular disease. Similarly as observed by Wooderman *et al*^[79], the authors observed that specimen weight more than the mean weight seems to be associated with statistically significant odds ratios to develop complications^[33]. This aspect can be partially explained by a decreased perfusion of the relatively large skin flaps that result from SSM in much larger breasts.

RECONSTRUCTIVE ASPECTS

In general, the NSM reconstruction are related to autologous and alloplastic techniques and sometimes include contra-lateral breast surgery. Various reconstructive techniques have been described, and aesthetic outcomes of NSM reconstruction continue to be met with variable satisfaction rates. Choice of reconstructive technique requires consideration of numerous patient related factors, including: breast volume, degree of ptosis, areola size, pa-

tient preference and expectation, clinical factors, smoking and surgeon experience. In addition, tumor related factors include size, location and proximity to the skin and NAC. Regardless of the fact that there is no unanimity regarding the best procedure, the criteria are determined by the surgeon's experience and the anatomical aspects of the breast. The main advantages of the technique utilized should include low interference with the oncological treatment, reproducibility and long-term outcome.

During a NSM, the NAC is preserved and incisions are located in more aesthetically regions. The breast volume, consisting of the breast tissue and fat, is entirely removed and reconstruction of the breast skin is not necessary. Thus, the objectives are to repair contour, volume and position.

In a recent systematic review, Endara *et al*^[39] examined current trends with NSM, including the reconstructive options. Based on 48 clinical studies that met the inclusion criteria, yielding 6615 NSM, the authors observed that 2373 (45.5%) were two-stage expander to implant, 2126 (40.7%) were one-stage direct to implant, and 719 (13.8%) were autologous tissue.

Autologous reconstructions involve pedicle flaps such as the latissimus dorsi myocutaneous (LDMF) or transverse rectus abdominis myocutaneous (TRAM) flaps. Although these techniques presents positive aspects some limitations have arisen regarding the muscle resection^[83-88]. Thus, alternatively free tissue transfer including the deep inferior epigastric perforator (DIEP), pedicled thoracodorsal perforator flap (TAP), free TRAM or the gluteal artery perforator (GAP) flaps can be indicated with a lower donor site morbidity. In fact, the DIEP flap diverges from abdominal myocutaneous flaps with maintenance of well-vascularized tissue and total abdominal muscular and aponeurotic layer preservation^[89-94]. Mosahebi *et al*^[83] in a series of 61 NSM reconstructions compared alloplastic and autologous tissue in terms of aesthetic outcome and satisfaction survey. According to the authors, all three reconstruction methods (implant, LDMF and DIEP) achieved good evaluation scores. However, in patients who had adjuvant radiotherapy, tonometry demonstrated that the breast remained softer in DIEP flap reconstruction.

In spite of the positive aspects, the outcome following SSM and NSM reconstruction with autologous tissue is not frequently predictable^[39,95-97]. Utilizing autologous tissue and particularly free flaps require special considerations in terms of recipient vessels and a monitoring skin flap. Preoperatively the plastic surgeon should evaluate the incision approach, the recipient vessels and the width of the remaining skin flaps for adequate skin preservation. Munhoz *et al*^[8] in a series of SSM DIEP reconstructions utilized five different incision approaches. According to the authors, the criteria decision was based on the breast anatomy (volume, ptosis and areola), the biopsy incision and the tumor location. The periareolar incision was the second most common incision selected and the restricted surgical exposure and difficulty in DIEP

anastomosis were the main negative technical aspects. Thus, a correct selection of a suitable recipient pedicle is decisive for a successful outcome^[90,92-94]. In NSM, the use of internal thoracic recipient vessels can be troublesome since the surgical exposure is restricted^[8,95]. Thus, longer instruments and the use of endoscopic lighting are necessary^[95]. In this situation, some authors advocated the peri-areolar approach with a lateral extension to obtain a better exposure^[94,95]. In addition, some authors advocate that use of the internal thoracic vessels may result in a higher rate of NAC necrosis compared with using the thoracodorsal vessels^[39,95]. Yang *et al.*^[95] in a series of 92 NSM free flap reconstructions utilized the internal mammary vessels if the mastectomy flap did not restrict the access. The authors observed that the thoracodorsal vessels were indicated in 59 cases, and internal mammary vessels in 33 cases including 4 cases with perforators of the internal thoracic vessels. In a selected group of patients, the internal thoracic branches can be used as an alternative to the internal mammary pedicle. The main advantages are sparing the internal mammary vessels and decreasing the operative time by limited dissection. However, Munhoz *et al.*^[92] reported that the internal thoracic branches are potentially available in only 55% of patients, therefore, this should not be the first option as recipient site.

Although autologous tissue presents advantages, it is not adequate in all cases especially in those without donor areas. In these cases, alloplastic techniques are usually indicated, and involve two-stage approach with tissue expanders followed by silicone gel implant replacement or one-stage reconstruction with conventional silicone implants. Although NSM reconstruction can be performed in a single stage, this is not the standard practice in several cancer centers^[39]. Enthusiasts of single-stage reconstruction promote lower costs, however supporters of the two-stage approach advocate a second operation to improve symmetry and the unpredictability of the NSM flaps. Chen *et al.*^[30] evaluated reconstruction with tissue expander or silicone implant performed immediately following the 115 NSM. Of the 66 patients, 58 patients underwent tissue expansion followed by subsequent implant placement in a two-stage reconstruction (87.9%) and eight patients underwent one-stage reconstruction (12.1%). The authors advocate that with two-stage reconstruction, it is possible to achieve the maximum control over the skin flap and by limiting the volume of the tissue expander such that the skin envelope is not redundant, the risk of ischemia is reduced. In addition, future aspects relating to NAC position, asymmetry and implant asymmetry can be managed at the time of the replacement of the tissue expander with a silicone implant. Starting the reconstruction with a tissue expander allows the reconstructive surgeon to customize the results to patient preference. Endara *et al.*^[39] has demonstrated that the incidence of NAC necrosis is little increased with one-stage approach (4.50% x 3.90%), however the overall complication rates were higher in the two-stage group (52.4% x 18.6%). The authors emphasized that there is

no ideal algorithm for reconstruction and the decision to proceed with reconstruction and the technique should be made by the surgeon based on assessment of skin flap viability.

The introduction of biodimensional implant-expander system (BIES) has proved increasingly popular over the last years^[9,21,33,97-102]. Designed with the objective of combining the advantages of the silicone gel implant and tissue expander into one system, it may present a superior breast form compared with what might be achieved using unshaped implants or expanders. The system design permits postoperative adjustments in implant volume and contra-lateral symmetry^[9,97-102].

In spite of the positive aspects, complications can be expected and are best avoided by placing the BIES under a submuscular pocket. Regardless of the good results observed with total muscular coverage, in some patients this technique is not free of unpredictable outcome^[9,21,74,101,102]. Total muscular coverage can limit lower pole expansion and can result in a high-riding device^[74,102]. Mahdi *et al.*^[99] in a series of BEIS reconstructions, observed that some patients failed to develop adequate lower pole projection and 35% required inferior muscular release to obtain a satisfactory result. Munhoz *et al.*^[33] advocated performing only minimal immediate expansion of the skin flaps in order to avoid tissue tension^[29,33]. In fact, in a series of patients submitted to NSM reconstruction, Peled *et al.*^[29] observed that NAC necrosis greatly decreased after the technical refinements of incision selection and performing implant reconstruction in a two-stage fashion.

Another option for implant coverage in NSM reconstruction is the use of acellular dermal matrices (ADM). Boneti *et al.*^[26] in a series of 281 NSM reconstructions utilized the alloplastic tissue situated in a partial muscular pocket, with ADM bridging the lateral and inferior edge of the muscle and the chest wall. The authors observed an overall complication rate of 7.1% (20 of 281) and the most frequent complication was breast skin flap ischemia. Spear *et al.*^[61] described a successful two staged NSM in 15 patients (24 breasts) utilizing the ADM. According to the authors, four of the 24 operated breasts (17%) experienced a complication, in that 2 patients (8%) developed flap necrosis and two patients developed partial NAC necrosis. Endara *et al.*^[39] in a systematic review could not assess the impact of acellular dermal matrix on reconstructive outcomes following NSM. According to the authors, the studies either did not report acellular dermal matrix use, or did indeed place acellular dermal matrix for all cases in only three studies, totaling NSM reconstructions. Given the insufficient number of patients comparison of complication rates between the two groups was not possible.

CONCLUSION

NSM is not a new concept but is becoming increasingly accepted by breast surgeons. In selected patients, this approach has allowed an adequate oncologic control with

satisfactory aesthetic outcome. Although NSM requires more intraoperative care, the concept can optimize the aesthetic result in early-stage breast cancer patients.

The satisfactory outcome are due to a close collaboration with the oncological surgical team in terms of incision selection flexibility and skin flap dissection. Alternately, care must be taken during the oncological procedure with meticulous surgical technique and gentle handling of tissues to avoid complications. In general, choice of reconstructive procedure requires careful consideration of various patient related factors, including: breast volume, degree of ptosis, areolar size, patient preference and expectation, clinical factors, and surgeon experience. Regardless of the fact that there is no consensus concerning the best technique, the criteria are determined by the surgeon's experience and the anatomical aspects of the breast. Probably, all these objectives are not achieved by any single procedure and each technique has advantages and limitations. With careful patient selection and well-planned surgical technique, NSM can provide satisfactory outcomes with acceptable complication rates. However, available data demonstrate that NSM can be safely performed for breast cancer treatment in selected cases. Additional studies and longer follow-up are necessary to define consistent selection criteria for NSM.

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Pattern response of dendritic cells in the tumor microenvironment and breast cancer

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phenotype which limits the activity of effector T cells and supports tumor growth and progression. Various factors and signaling pathways have been implicated in the immunosuppressive functioning of DCs in cancer, and researchers are working on resolving processes that can circumvent tumor escape and developing viable therapeutic interventions to prevent or reverse the expression of immunosuppressive DCs in the tumor microenvironment. A better understanding of the pattern of DC response in patients with BC is fundamental to the development of specific therapeutic approaches to enable DCs to function properly. Various studies examining DCs immunotherapy have demonstrated its great potential for inducing immune responses to specific antigens and thereby reversing immunosuppression and related to clinical response in patients with BC. DC-based immunotherapy research has led to immense scientific advances, both in our understanding of the anti-tumor immune response and for the treatment of these patients.

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Abstract

Breast cancer (BC) is the most common malignant neoplasm and the cause of death by cancer among women worldwide. Its development, including malignancy grade and patient prognosis, is influenced by various mutations that occur in the tumor cell and by the immune system's status, which has a direct influence on the tumor microenvironment and, consequently, on interactions with non-tumor cells involved in the immunological response. Among the immune response cells, dendritic cells (DCs) play a key role in the induction and maintenance of anti-tumor responses owing to their unique abilities for antigen cross-presentation and promotion of the activation of specific lymphocytes that target neoplastic cells. However, the tumor microenvironment can polarize DCs, transforming them into immunosuppressive regulatory DCs, a tolerogenic

Key words: Breast cancer; Dendritic cells; Tumor microenvironment

Core tip: Breast cancer is a worldwide major public health problem, and dendritic cells are of crucial importance for activating an effective antitumor immune response. Deepening our understanding of the tumor microenvironment can enable the development of new therapies that will make it possible to induce an efficacious antitumor response. Given the search for effective means with which to induce such a response *via* dendritic cell (DC) immunotherapy, the study of the mechanisms involved in the DC pattern of response in the tumor microenvironment is important.

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INTRODUCTION

Breast cancer (BC) is the most common cancer among women and the second most prevalent form of terminal cancer in this population^[1]. Cancer is characterized by accelerated and uncontrolled proliferation of normal cells due to specific genetic mutations that alter the cell cycle^[2,3]. These genetic changes can culminate in the expression of mutant proteins, that in theory, should be recognized as foreign by the immune system^[4]. Within the immune system, dendritic cells (DCs) play a distinct role wherein they are essential both to innate and to acquired immunity^[5]. They have the unique ability for antigen cross-presentation for T helper lymphocytes (CD4⁺) and cytotoxic T lymphocytes (CD8⁺)^[6] in secondary lymphoid organs, a process that activates a sophisticated and specific immunological response^[7].

Although they perform the functions of antigen capture and processing in peripheral tissues, DCs are initially immature. They mature when interactions with Toll like Receptors (TLRs) or inflammatory cytokines convert immature DCs into mature DCs that present a specific antigen to naïve T cells, thereby activating them^[8]. DCs with differing maturation stimuli upregulate the expression of the cell surface and intracellular molecules required for their journey to secondary lymphoid tissues. However, the activation and maturation of DCs depend on the local microenvironment and can be blocked or polarized by a combination of specific local factors, resulting in the formation of DCs with tolerogenic or immunosuppressive activities^[9].

Various types of cells and factors present in tumor microenvironment generate a range of stimuli for DCs, that affect all aspects of their biology and thus control their functionality and longevity^[10]. It's known that immunotherapy with CDs is currently being studied and used as therapy for a large variety of tumors, solid or not. We have chosen to study the mechanisms involved in the pattern of response of the CDs in the tumor microenvironment, specifically in the BC because it is considered major public health problem. The knowledge of these cells response will be the basis for developing an effective immunotherapy. In this work, we provide an up-to-date review of the pattern of DC response in the BC tumor microenvironment and the use of immunotherapy with a DC vaccine.

ROLE OF DCs IN TUMOR IMMUNITY

DCs are key regulators of immunity by B and T lymphocytes because of their superior ability to capture, process and present tumor-associated antigens^[11]. They

are derived from a hematopoietic line with cell subsets that differ in terms of their morphology, phenotype and function depending on the particular conditions and the tissue in which they reside. However, the various DC subsets generally have the ability to stimulate T cells^[12]. DCs have different co-stimulatory molecules, such as B7, intercellular adhesion molecule 1 (ICAM-1), lymphocyte function-associated antigen-1 (LFA-1), lymphocyte function-associated antigen-3 (LFA -3) and CD40 on their cell surfaces. As a set, these proteins generate a chain of secondary signals essential to reinforcing the T cell activation, with the resultant immune response initiation, programming and regulation being directed specifically at the tumor^[13].

The DC maturation process determines the presentation of processed tumor antigens and the subsequent activation of CD4⁺ and CD8⁺ T cells^[14]. Their migration from tissues to the lymph nodes is fundamental to this process, since it provides the period during which expression levels of co-stimulatory molecules as class II MHC molecules, adhesion molecules and the C-C motif chemokine receptor (CCR7) are increased. This process is accompanied by a reduction in CCR6^[15,16], which coincides with increase in the expression of particular C-C motif chemokine ligands (CCLs)-namely CCL19 (a.k.a. EBI1 ligand chemokine) and CCL21 (a.k.a. secondary lymphoid-tissue chemokine)-within the lymph nodes as well as increased CCR7 binding^[17]. The expression of molecules on the DC membrane and the migration of DCs to T-cell populated areas in the lymph nodes, where drainage occurs, during this process are fundamental to specific immune response by the T lymphocytes^[7,18]. Nevertheless, depending on microenvironment conditions, which involve the cells present in the tissue as well as the presence of mediators, DCs can present alternative phenotypes that are implicated directly in an immunogenic to tolerogenic change of function^[19].

PROGRESSION OF BC, ITS RELATIONSHIP WITH THE TUMOR MICROENVIRONMENT AND THE PROCESS OF DC MATURATION AND ACTIVATION

If found in an early stages, BC has a good cure rate, though the metastatic or recurrent form of the disease has a poor prognosis. BC consists of a heterogeneous group of neoplasms derived from the ductal epithelium. The majority of BCs (80%) are ductal (including medullary, papillary, tubular and mucinous subtypes), and the remaining 20% are lobular cancers. The normal mammary gland consists of a layer of luminal epithelial cells, myoepithelial cells and a continuous basal membrane. The stroma contains fibroblasts, immune system cells (*e.g.*, macrophages, DCs and lymphocytes) and vasculature surrounded by the extracellular matrix and adipocytes, which maintain the structure of normal tissue^[20].

The evolution of malignant breast tumors beings with

epithelial hyperproliferation and progressing to *in situ*, invasive ductal and metastatic carcinomas^[21]. In *in situ* ductal carcinoma, epigenetically and phenotypically changed myoepithelial cells, which are surrounded by a basal membrane that is still mostly continuous, are incapable of contributing to the polarization and organization of normal cellular processes. In invasive ductal carcinoma, there has been a loss in the continuity of the basal membrane, loss of function of the myoepithelial cells and an invasion of the epithelial cells into the stroma and vasculature. During conversion from *in situ* to invasive carcinoma, there is an increase in the production of extracellular proteases that degrade the stromal matrix and cells^[22]. Concomitantly, tumor cells in the stroma produce proteolytic enzymes; while chemokines and cytokines continue to attract leukocytes, modulate tumor remodeling, and facilitate tumor cell invasion of distant organs, leading to metastasis. Infiltrating fibroblasts and leukocytes are induced to increase the secretion of growth factors, cytokines, chemokines and matrix metalloproteinases (MMPs) which produce an immune-evasion mechanism that enables the tumor to progress and achieve angiogenesis^[23].

We now know that the tumor microenvironment is the main regulator of carcinogenesis and is responsible for the course of tumor progression, including how tumors respond to various types of treatment^[24,25]. The development and progression of a tumor to a malignant phenotype is highly dependent on interactions between the tumor cells and normal cells present in the tumor microenvironment^[23,26]. There are extensive changes in genetic expression in all cell types during BC progression. Genetic changes detected in cancerous epithelial cells have been tied to the overexpression of particular C-X-C motif ligand (CXCL) chemokines, namely CXCL14 by myoepithelial cells and CXCL12 by myofibroblasts. These chemokines bind receptors in cancerous epithelial cells, resulting in increases in their proliferation, migration, and invasion, thus favoring tumor remodeling, as well as their ability to migrate to and induce metastasis in distant organs. Chemokines and cytokines produced by tumor cells and infiltrating cells continue to attract more leukocytes, further facilitating tumor progression^[27]. Abnormal matrix remodeling in BC tumor progression is attributable to MMPs, which can also activate chemokines, cytokines, adhesion molecules, and growth factors that support tumor progression. Angiogenic factors are activated by MMPs 1, 2, 9 and 14^[28].

The tumor microenvironment promotes BC initiation, growth, migration, metastasis, and therapeutic resistance. The mediators and enzymes produced can induce important genetic changes in tumor-associated cells, such as fibroblasts, endothelial cells, adipocytes, and leukocytes. These cells are critical components of the tumor stroma and can damage the microenvironment, enabling malignancy as shown in Figure 1^[25]. Tumor occurrence has been shown previously to be associated with DNA methylation and histone modifications in fibroblasts^[29]. We also know that germinal mutations in *BRC A1* and

BRC A2 confer increased risk of BC and ovarian cancer and reduced risk of other types of cancer; *TP53* and *PTEN* mutations are also found in BC cells^[30-32]. Mature adipocytes influence tumor behavior by producing hormones, growth factors, and cytokines, as well as a heterogeneous group of molecules known as adipokines, which can change the phenotype of the epithelial cell, increasing its mobility. Interestingly, these molecules provide a link between obesity and BC risk^[33,34].

The endothelial cells that form tumor neovessels are distinct from normal endothelial progenitor cells^[35,36]. Under the influence of proteins in the tumor microenvironment, such as macrophage colony-stimulating factor (M-CSF), monocytes can differentiate into endothelial cells that provide angiogenesis within the tumor^[37]. This process, which can also be mediated by components of the extracellular matrix (*e.g.*, fibronectin), leads to the phenomenon of monocyte-epithelial cell differentiation^[38].

Tumor-associated macrophages are a major component of the leukocyte infiltrate; when activated, they exercise a tumoricidal action^[39]. However, the presence of tumor-associated vascular leukocytes has been shown to be linked directly with tumor growth through increased expression of the cytokine tumor necrosis factor (TNF)-alpha^[38]. Release of TNF-alpha in the tumor microenvironment promotes the differentiation of tumor-associated monocytes towards a myeloid-epithelial pro-angiogenic phenotype *via* positive regulation of the fibronectin receptor $\alpha 5\beta 1$.

In a study of tumors obtained from patients with invasive BC^[40], we found that differences in T and B lymphocytes, in terms of peritumor/intratumor infiltrate, were related to tumor size. Specifically, small tumors (≤ 2 cm) had relatively lower levels of intratumor B lymphocytes, whereas large tumors (2-6 cm) had relatively low levels of peritumor T lymphocytes. We know that tumor-infiltrating B cells can subserve an antibody response to breast tumors. In a study analyzing the presence of B lymphocytes (CD20⁺) in 1470 invasive breast carcinomas, Mahmoud and colleagues documented that B lymphocytes were diffuse in areas at a marginal distance from the carcinoma (average distance, 12 cells) compared to their more dense presence within the carcinoma and in stromal compartments adjacent to the tumor. Total B-cell counts correlated with a higher tumor grade and a basal phenotype, as well as with estrogen receptor (ER)/progesterone receptor (PR) negativity^[41].

Regulatory T lymphocytes (Tregs) and cytokines have also been implicated in the immune cell infiltration of tumors. Invasive BC tumors have been reported to exhibit elevated levels of intratumoral Tregs, with ER/PR negativity and HER2 overexpression being associated with an unfavorable prognosis^[42]. Furthermore, enrichment of Tregs in invasive ductal carcinoma of the breast correlates with upregulation of interleukin (IL)-17A expression and augmented invasive ability^[43].

In a letter to the editor about an article entitled "cancer stage and local immune response," the researcher^[44]

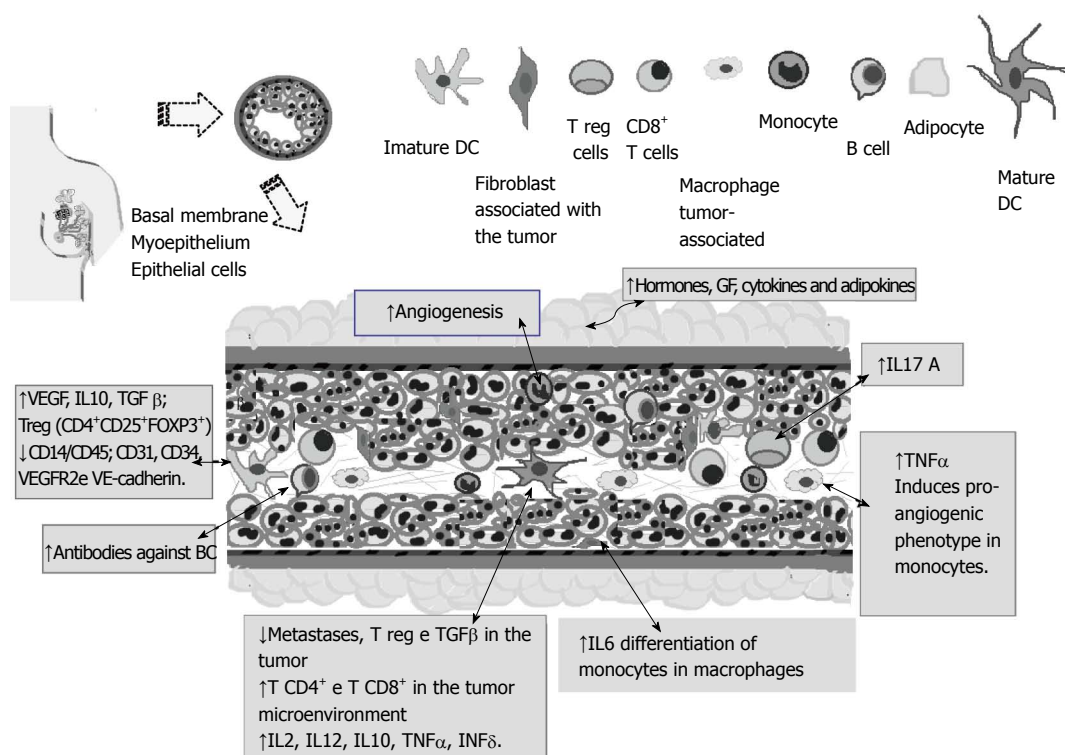


Figure 1 Changes to the tumor microenvironment seen in a mammary gland with breast cancer, consisting of a layer of luminal epithelial cells surrounded by myoepithelial cells, bordered by a continuous basal membrane, with adipocytes external to the mammary gland. In the stroma, there are tumor-associated fibroblasts immune cells surrounded by the extracellular matrix that maintains the tissue structure. In ductal carcinoma, the myoepithelial cells are changed epigenetically and phenotypically. The number of fibroblasts, monocytes and macrophages associated with the tumor are increased in the stroma, elevating secretion of growth factors, cytokines and chemokines, which promotes tumor progression. Dendritic cells (DCs) have plastic characteristics, showing distinct phenotypes depending on their mature state.

Murta reiterated and defended the idea that the BC stroma and peri- or intratumoral lymphocyte infiltrates are important to the development of and prognosis for BC and that immunotherapy for breast cancer is still under investigation. Moreover, the tumor microenvironment has innumerable escape mechanisms and ways to favor tumor progression, even using the cells themselves and mediators of the immune response in its favor.

INFLUENCE OF THE TUMOR MICROENVIRONMENT ON DENDRITIC CELLS

DCs can change their phenotype in response to tumor microenvironmental factors and contribute to angiogenesis. This cellular plasticity characteristic seems to be exploited by tumors that repress DC maturation, thereby inhibiting specific antitumor immune responses^[45]. Tumors can induce the generation and accumulation of immunosuppressive cells (*e.g.*, Tregs) in the tumor microenvironment, improving tumor's ability to evade immunological defenses^[42]. Tumor-associated cytokines such as vascular endothelial growth factor (VEGF), IL-10, and prostaglandin E₂ (PGE₂) can affect the phenotype of DCs^[46]. Liu *et al.*^[47] placed DCs in co-culture with isolated Lewis lung carcinoma cells to simulate the tumor micro-

environment. The co-cultured DCs were induced to differentiate into regulatory DCs with a CD11c^{low} CD11b^{high} Ia^{low} phenotype and elevated expression of IL-10, nitric oxide, VEGF, and arginase I. These regulatory DCs inhibited T-cell proliferation both *in vitro* and *in vivo*, with PGE₂ being the major inducer of arginase I in the regulatory DCs^[47]. Corroborating this idea, several studies have shown that tumor-associated DCs can induce Treg expansion and become not only incapable of inducing specific immune responses but also immunosuppressive^[45,48,49]. Furthermore, BC tumors have been reported to promote the differentiation of DCs into a phenotype that expresses IL-10 and tumor growth factor (TGF-β), which, in turn, induce the expansion of Tregs (CD4⁺CD25⁺FOXP3⁺)^[50].

Tumor-associated DCs can produce pro-angiogenic factors in the tumor microenvironment. Rapid tumor growth factors are associated with the infiltration of immature DCs that promote angiogenesis and tumor growth, whereas mature DCs are known to suppress angiogenesis^[51]. BC cell-secreted IL-6 determines whether monocytes in the tumor stroma will differentiate into DCs or macrophages. *In vitro*, activated monocytes placed in contact with fibroblasts (common in tumor stroma) induce the fibroblasts to release IL-6. IL-6 regulates the expression of M-CSF receptors in functional monocytes and enables them to become reactive to autocrine M-CSF.

Interaction between IL-6 and M-CSF favors monocyte differentiation into macrophages, rather than DCs. Like fibroblasts, Hs578T BC cells modulate monocyte differentiation into macrophages *vs* DCs depending on the presence of IL-6^[52].

DCs are a specialized group of antigen-presenting cells with extraordinary functional plasticity. They have the potential to stimulate or suppress immunity, depending on the sequence and combination of microenvironmental stimuli present^[12]. DCs cultivated *in vitro* in the presence of tumor factors can have some characteristics of epithelial cells, such as low-density lipoprotein absorption, leptin binding^[53], and even suffer a process of endothelialization characterized by loss of CD14/CD45, loss of the endothelial markers CD31 and CD34, and loss of the von Willebrand factor, vascular endothelial growth factor receptor 2 (VEGFR-2), and vascular endothelial (VE)-cadherin^[54].

By means of a literature review, Curiel^[55] defends the need of a reduction in the function of Treg cells in the microenvironment tumor recurrence in patients with cancer, either individually or in combination with other therapies. According to this study the reduction may be clinically beneficial and potentially effective as an immunotherapy against cancer. In his work, he highlights other studies in which the function of Treg cells can be decreased, as an indirect effect, after the use of other immunotherapeutic agents, as the use of Denileukin diftitox (DAB389IL-2), a fusion protein of interleukin 2 (IL-2) and diphtheria toxin^[56], and doses of diftitox denileukin, in combination with a recombinant vaccine poxviral can increase the immune responses in an antigen-specific normal murine model. The authors noted that Treg cells in spleen, peripheral blood and bone marrow of the animals were variously reduced after a single intraperitoneal injection of denileukin diftitox, evident reduction in 24-h effect after around 10 d. Similarly, in another study^[57] examined the effects of a single dose of low dose cyclophosphamide (CTX) on immunogenicity of DC vaccines in animals with tumors of the colon and melanoma. As a result there was an increase in IFN- γ produced by lymphocytes in the spleen and significantly reduce CD4⁺ CD25⁺ FoxP3⁺ Treg cells, thus establishing a proposal for an immunotherapy strategy by combining low dose CTX with DC.

In the study^[58] an antiangiogenic therapy was used in clinical trials of patients with BC through Sunitinib and Bevacizumab and demonstrated that there is a significant induction of hypoxia in the tumor microenvironment, with increased of stem cell cancer population.

MATURE DCs AND THE TUMOR MICROENVIRONMENT IN BC

As shown in Figure 1, the BC tumor microenvironment is highly immunosuppressive due to the presence of growth factors (*e.g.*, VEGF) and cytokines (*e.g.*, IL-10), and the infiltration of Tregs and immature DCs. Infiltration

of mature DCs into primary tumor lesions is associated with prolonged patient survival and reduced risk of metastasis^[59]. These findings reinforce the importance of DCs in antitumor immunological oversight, including the migration of DCs into regional lymph nodes where they present specific tumor antigens to naïve T cells.

In 2008, Dieu-Nosjean and colleagues reported a study^[60] that indicated that the density of tumor-infiltrating lymphocytes (CD4⁺, T-bet⁺ Th1 T cells, in particular) was reduced in tumors that had weak infiltration by mature DCs. The authors interpreted the findings as indicating that the density of mature DCs was a better predictor of clinical evolution than the other parameters tested.

The therapeutic potential of mature-DC vaccines remains of great interest. Research probing the benefits of DC immunotherapy has demonstrated safety with few adverse secondary effects as well as interesting results in terms of efficacy for stimulating immune cells^[61,62]. Current strategies to curtail immune evasion by tumors include a combination of DC vaccination with Treg depletion by way of administration of anti-CD25 antibodies^[63], and blockade of endothelin receptors in endothelial cells to facilitate the infiltration of cytotoxic T cells into the tumor microenvironment^[64]. Stimulation of an immune response by DC immunotherapy has been evidenced by increases in the percentages of IL-2-, TNF-alpha-, and IL-10-expressing T-helper (CD4⁺) cells. A similar result was observed for IL-2 expression by cytotoxic T (CD8⁺) cells. The percentage of total T (CD3⁺) cells remained elevated during immunotherapy. Counts of Treg (CD25⁺/FOXP3⁺) cells remained significantly lower than the pre-treatment baseline count throughout the treatment period^[65].

Vaccines made from DCs pulsed with autologous tumor lysates induced secretion of Th1 cytokines and an increase in natural killer cells and CD8⁺ IFN-gamma⁺ T lymphocytes in the peripheral blood of BC patients. This finding suggests that a vaccine of DCs pulsed with tumor lysates may be an effective source of tumor antigens, capable of inducing effective immune responses^[66]. In another study, a vaccine of DCs pulsed with HER-2 was reported to be well-tolerated and to attenuate expression of HER-2/neu, enabling an immune response to invasive cancer to be mounted^[67]. Finally, in a clinical trial, an antitumor DC vaccine administered together with IL-2 was found to induce specific cellular immunity in patients with BC that was accompanied by a reduction in TGF-beta levels, an increase in IL-12 secretion, and a reduction in CD4⁺ CD25⁺ T cells^[68]. Based on these promising findings, we believe that DC-based therapies designed to disrupt a tumor-promoting microenvironment should be investigated with the aim of developing more powerful DC vaccines that can generate an intense and long-lasting anti-BC immune response^[69].

In conclusion, growing evidence shows that the BC tumor microenvironment is immunosuppressive, at least in part through modification of DCs toward a Treg phenotype, *i.e.*, mature DCs are not functional in the tumor

microenvironment in patients with BC. DC maturation is critical for enabling specific molecular identification of BC antigens and DC vaccine immunotherapies have the potential to induce specific immunity against tumor antigens. Importantly, changes in immunological parameters that favor a specific antigen immune response, together with a reduction in immunosuppression, correlate with a positive clinical response in patients treated with a DC vaccine. Clarification of the microtumor environment, the mediators involved, interactions with immune response cells, and immune-evasive mechanisms may lead to new forms of immunotherapy.

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WJCO 5th Anniversary Special Issues (2): Breast cancer

Triiodothyronine and breast cancer

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Abstract

The thyroid hormones (THs), triiodothyronine (T3) and thyroxine (T4), are essential for survival; they are involved in the processes of development, growth, and metabolism. In addition to hyperthyroidism or hypothyroidism, THs are involved in other diseases. The role of THs in the development and differentiation of mammary epithelium is well established; however, their specific role in the pathogenesis of breast cancer (BC) is controversial. Steroid hormones affect many human cancers and the abnormal responsiveness of the mammary epithelial cells to estradiol (E2) in particular is known to be an important cause for the development and progression of BC. The proliferative effect of T3 has been demonstrated in various types of cancer. In BC cell lines, T3 may foster the conditions for tumor proliferation and increase the effect of cell proliferation by E2; thus, T3 may play a role in the development

and progression of BC. Studies show that T3 has effects similar to E2 in BC cell lines. Despite controversy regarding the relationship between thyroid disturbances and the incidence of BC, studies show that thyroid status may influence the development of tumor, proliferation and metastasis.

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Key words: Thyroid hormone; Triiodothyronine; Breast cancer; Mammary gland and metabolism

Core tip: Breast cancer (BC) is a malignant tumor occurring much more frequently in women than in men; worldwide, the incidence of BC has increased markedly in recent years. It is estimated that 1.7 million women will be diagnosed with BC in 2020, marking an increase of 26%, compared to the current incidence: 1.35 million new cases annually. Countless environmental risk factors, pathological conditions, and physiological agents, as well as thyroid hormones (THs), have been involved in the development of BC. Various lines of evidence suggest tumor-promoting effects of THs. The literature contains controversial reports regarding the relationship between thyroid diseases and BC; furthermore, studies reporting both an excess of and a lack of THs may affect breast development and progression to cancer. Epidemiologically, many studies suggest that hyperthyroidism is a factor in the development of BC. Furthermore, experimental studies have shown that high levels of THs reduce the interval of multiplication of BC cell lines. Therefore, the influence of THs on BC is unclear. However, the majority of BC research suggests a relationship, primarily, when the molecular aspects of these hormones are considered in the progression of this type of tumor.

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INTRODUCTION

Thyroid hormones (THs), 3,5,3',-triiodothyronine (T3) and thyroxine (T4), play critical roles in the differentiation, growth, metabolism, and physiological function of nearly all mammalian tissues^[1,2]; in addition, they are required for amphibian metamorphosis^[3]. Multiple biological effects of THs depend on intracellular levels of T3, which binds to the thyroid hormone receptor (TR) and is for the most part generated in peripheral tissues by outer-ring deiodination of T4^[4]. TH is a key metabolic regulator that coordinates short-term and long-term energy needs^[5]. Significant metabolic changes are seen with variations in human thyroid status^[6].

Hypothalamus-secreted thyrotropin-releasing hormone (TRH) and thyroid stimulating hormone (TSH), secreted by the anterior pituitary (hypophysis), act in the thyroid gland where THs^[7,8] are produced (Figure 1A). TRH and TSH are negatively regulated by T4 and T3 (negative feedback), acting directly on the TSH receptor (TSH-R), expressed on the thyroid follicular cell basolateral membrane^[9].

THs regulate a wide range of genes after activation from the prohormone (T4), to the active form (T3)^[10]. The signaling pathway is complex and highly regulated because in cells and tissue there is an expression of TH transporters, multiple TR isoforms, as well as interactions with corepressors and coactivators^[11,11] (Figure 1B). The functional TR complex consists of a heterodimer with retinoid X receptor (RXR) that binds to a TH response element (TRE) to modulate gene expression. Liganded TR stimulates genes that are positively regulated by triiodothyronine (T3), whereas unliganded TR binds to a TRE to repress those genes. The repressive actions of unliganded TR act in metabolic regulation, particularly in antagonizing the action of other nuclear receptors^[12].

TH action has been substantially altered by recent clinical observations of thyroid signaling defects in hormone resistance syndromes and in a broad range of conditions, including profound mental retardation, obesity, metabolic disorders, and a number of cancers^[13]. However an uncontrolled study of 3,3'-diiodothyronine (3,3'-T2), iodothyronines as T3 and T4, administration to humans for 4 weeks by an unspecified route was associated with increased metabolic rate and reduced body weight^[14], no specific role of 3,3'-T2 in humans has been demonstrated^[15]. Animal studies, however, suggest that the 3,5-diiodothyronine and 3,3'-T2 increase metabolic rate^[15,16], by acting at the mitochondrial level to increase hepatic cytochrome oxidase activity^[17] and supraphysiological dose of T3 causes genotoxicity and potentiates oxidative stress^[18].

T3 can influence the mammary gland; this involves

the activation of TR present in the mammary gland inducing differentiation and lobular growth in an estrogen-like manner. However, there is controversy regarding the relationship between thyroid disorders and breast cancer (BC) incidence^[19].

In this chapter, the influence of T3 on BC will be reviewed. The physiological role of THs in the normal breast will be discussed.

MAMMARY GLAND DEVELOPMENT

Mammary glands are composed of conjunctive tissue and adipose tissue; the latter may vary according to the size of the breast. The development of these glands begins in the embryo phase *via* the extension of ectoderm tissue^[20]. This extension is due to allometric growth, which represents the relationship between growth of the ectoderm and the metabolic profile of the epithelial cells^[21]. The maintenance and regulation of breast epithelial cells are also controlled by the complex interaction of various hormones including estrogen, progesterone, glucocorticoids, insulin and prolactin^[22]. At the onset of puberty, some hormones in the ovary influence the maturation of the mammary glands. They act on the glands by filling a system of branches and lateral ducts surrounding the layer of fat. During pregnancy, mammary glands produce milk due to the high level of estrogen secreted by the placenta. This milk is stored in alveolar secretory units to be supplied later for breastfeeding^[21].

During lactation, lipoprotein lipase activity decreases in the adipose tissue and increases in the breast tissue. This indicates an increase in the capture of fatty acids in this tissue^[23]. Therefore, the quantity of milk produced is also influenced by the hormone levels; as a result, the physiological process of the epithelial cells is modified^[22].

Regarding cell proliferation, studies conducted on human breast tissues report that cell multiplication in mammary epithelium is constant following the complete development of the mammary glands. This mitotic state of proliferation during the luteal phase of the ovarian cycle may coincide with the increase in secretion of estrogen and progesterone^[22].

Thus, at this phase, the epithelial cells of the mammary glands may be stimulated by the presence of progesterone or in its synergy with estrogen. After several days of peak cell proliferation, and at the end of the luteal phase, these cells undergo apoptosis. This apoptotic peak coincides with the decrease in estrogen and progesterone secretion from the ovaries; furthermore, a low level of apoptosis occurs in the entire mammary lobule^[22].

Based on the foregoing, it is noteworthy that breast tissue constantly undergoes marked physiological cell renovation. This process is due to a response to the levels of estrogen and progesterone secreted by the ovaries. It is also evident that the proliferation and cell replacement of the mammary epithelial cells, after complete development of the breast, plays an important role in the maintenance and tissue function of the normal breast^[17].

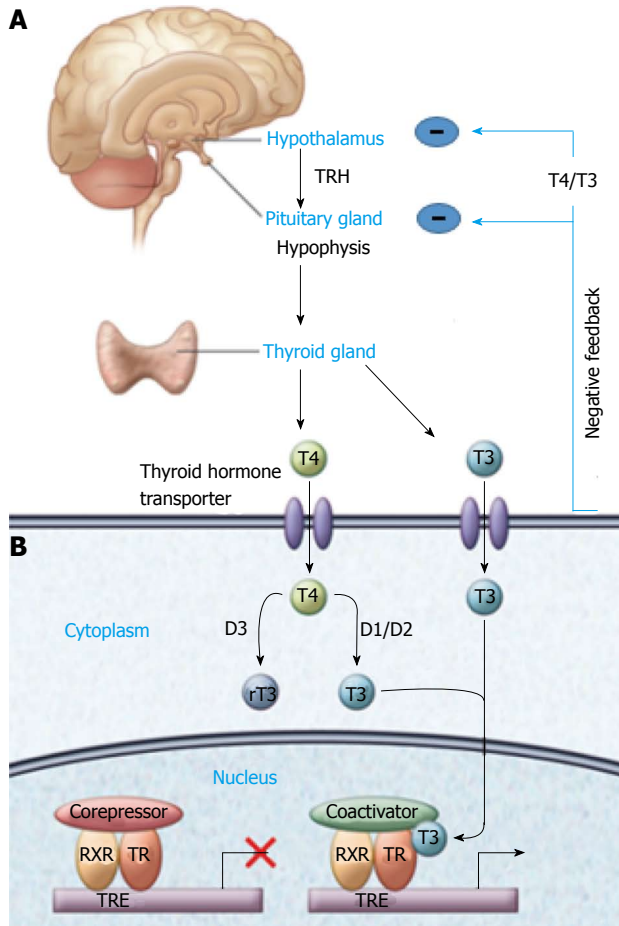


Figure 1 Production and action of thyroid hormone. The key components required for thyroid hormone action are shown, demonstrated by a range of clinical observations. (1) Thyroid hormones (T4 and T3) are produced by the thyroid gland and are regulated by thyroid stimulating hormone (TSH) produced by the hypophysis, which is stimulated by thyrotropin-releasing hormone (TRH). Once released, T4 and T3 exert a negative feedback mechanism on the production of TRH and TSH; and (2) The effects of T4 *in vivo* are mediated via T3. T4 is converted to T3 in target tissues by deiodinases 1 and 2 (D1 and D2). Deiodinase 3 (D3) converts T3 to the inactive rT3. Unliganded TR heterodimerizes with RXR and binds to a TRE and then to a corepressor, such as a nuclear receptor corepressor (NcoR); thus, repressing gene expression. T3 binding to the ligand-binding domain results in movement of the carboxyterminal helix 12, disruption of corepressor binding, and promotion of coactivator binding, which then leads to recruitment of polymerase III and the onset of gene transcription. Adapted from Ref.[13].

TRIIODOTHYRONINE EFFECT IN THE MAMMARY GLAND

The hormones that regulate breast development have been known since the 1930s^[24] and the involvement of THs in the development and differentiation of normal breast tissue has been established^[25-27]. Breast growth and development require the coordinated action of many hormones, such as prolactin, estrogen, progesterone, adrenal steroids, insulin, growth hormone, and THs^[28,29]. THs exert a wide variety of biological effects in vertebrate animals; however, their most noteworthy actions may be found in the regulation of cell development and differentiation^[30]. From the onset of embryologic devel-

opment, the mammary glands in women go through a process of ductal development, which supports the creation of alveolar structures during pregnancy, before the onset of lactogenesis. This development includes various stages of proliferation and morphogenesis, which are largely driven by simultaneous changes in the main hormones and growth factors in various reproductive states. Ductal extension is driven by estrogen, growth hormone, growth factor similar to insulin I, and epidermal growth factor; however, ductal branching and alveolar budding are influenced by additional factors, such as progesterone, prolactin, and THs^[27].

THs are not essential for the development of the breast ducts; however, they appear to stimulate the development of the lobules of these glands^[25]. It is also thought that, in states of excess or lack of this hormone, the process may be negatively affected^[31]. In 1986, Vonderhaar *et al.*^[26] verified that THs influence the development of epithelial cells of the mammary glands in rats. In regard to cell differentiation, the responsiveness of the breast tissue to prolactin in the rat increases *via* the activation of prolactin receptors. In rabbit breast tissue, THs stimulate the synthesis of casein induced by prolactin^[32].

BREAST CANCER

This cancer is characterized by a multiphasic process in which a series of genetic and epigenetic changes take place in sequence, leading to the cells' loss of control of the proliferation, differentiation, apoptosis, and repair of DNA^[33]. Worldwide, BC is the most common malignant neoplasm in women^[34]. It is characterized by epithelial cells and by the tendency to metastasize to distant locations. Most of the tumors are adenocarcinomas, derived from the terminal ductal lobular unit^[35].

In Brazil, the estimated incidence in 2012 was 53 thousand new cases, according to the National Cancer Institute^[36]. The estimated incidence in the United States for 2011 was 231 thousand cases of BC^[37]. The etiology of BC is complex and involves endogenous and exogenous factors that make up the risk factors of the disease^[38]. These factors are associated with the onset and development of the tumor; furthermore, they confer a significant histopathologic, genetic, and prognostic variability to this disease^[39,40].

Endogenous factors include: family history; an excessive number of ovulatory cycles due to early menarche and/or late menopause; high-density breasts (primarily in post-menopausal women); and genetic mutations (such as BRCA1 and BRCA2). Some examples of exogenous factors are the use of oral contraceptives, hormone replacement therapy, diet, physical exercise, and socioeconomic factors^[39,40].

Family history is one of the main risk factors for the development of BC. However, 80%-95% of cases do not have any familial relationships; they are sporadic, resulting from mutations, epigenetic changes, or even the polymorphism of genes involved in metabolism. BC can also

arise from genes that codify components of the paths of hormonal signaling and growth factors or from DNA repair genes. Despite the foregoing causes, BC is primarily related to the exposure of breast tissue to estrogen^[41-43].

Epidemiologic studies have shown a possible association between thyroid dysfunction and BC. Postmenopausal BC patients have been reported to have a significant increase in THs, suggesting that an imbalance between estradiol (E2) and T3 fosters the development of BC^[44-46]. Conversely, hyperthyroidism has been associated with a reduction in the incidence of BC^[47]. The literature describes an increase in survival in hyperthyroid patients with BC, suggesting that hyperthyroidism may protect against the development of BC by increasing steroid hormone binding globulin and by reducing estrogen stimulation of the breast tissue^[48].

Despite advances in the knowledge of the pathogenesis and molecular aspects of the disease, little is known about the mechanism whereby T3 modulates its receptor in tumor tissue. Studies are contradictory regarding the type of thyroid pathology and how this influence may take place^[48,49].

TRIIODOTHYRONINE AND BREAST CANCER

Experimental studies have shown that THs influence both the differentiation of normal breast cells^[44] and the proliferation of BC cells^[44,50,51]. The proliferative effect of T3 has been demonstrated in various types of cancer. In BC cell lines, T3 may foster tumor proliferation and increase the effect of cell proliferation by E2; thus, T3 may play a role in the development and progression of BC^[52,53]. Apart from this, THs appear to have a stimulating effect on the angiogenesis of certain types of cancer^[54,55]. TH levels are related to the risk of BC. As shown by Tosovic *et al.*^[56], high levels of T3 in postmenopausal women positively correlate with the risk of BC in a dose-response manner. In the case of hyperthyroidism, this correlation was found in postmenopausal women but not in premenopausal woman who had BC^[44]. These postmenopausal women with BC had a significant increase in their thyroid/estradiol ratio, suggesting that an imbalance between E2 and T3 fosters the development of BC^[44]. Conversely, hyperthyroidism has been associated with a reduction in the incidence of BC^[47].

Many *in vitro* and *in vivo* studies have related THs to human cancer since Beatson^[57] described the use of thyroid extracts for treating metastatic BC in 1896. Abundant data indicate that the thyroid status affects the formation of a tumor, its growth, and metastasis in both laboratory animals and humans^[45]. However, the relationship between thyroid status and the pathogenesis of human BC is currently not elucidated^[45].

EXOGENOUS TRIIODOTHYRONINE THERAPY AND BREAST CANCER RISK

The relationship between THs and BC is a controversial

topic. The topic was first addressed in 1896 when Beatson used thyroid extract as a treatment for BC^[57]; however, some studies reported that THs increased the risk of BC^[57,58]. In addition, other studies reported an increase in survival of BC patients who had high TH levels^[48]. Some studies that evaluated thyroid pathology reported that BC rarely occurred in hyperthyroid women^[58], while others reported that primary hyperthyroidism is associated with a reduced incidence of primary BC^[47].

TH replacement therapy primarily entails administering doses of levothyroxine sodium ranging from 1.6 to 1.8 µcg/kg per day^[59]. An inhibitory effect of iodine on BC was suggested to be attributable to its antioxidant role^[60-62]; however, some *in vitro* experiments have shown a proliferative effect of triiodothyronine on BC cell lines^[63,64].

A recently conducted meta-analysis found no statistically significant association between TH replacement and BC risk^[19]. One of these studies demonstrated primary hyperthyroidism as a strong protective factor against BC after adjusting for clinical parameters including TH replacement therapy^[47].

The literature contains a few studies that show a correlation between TH replacement therapy and BC, despite a tendency towards a non-statistical association between the two. Laboratory studies have demonstrated the ability of triiodothyronines to induce BC proliferation in an estrogen receptor-dependent manner, possibly through crosstalk between the THs and estrogen pathways^[50,63,65]. Others THs should be studied in relation to BC, as 3,3'-T2, because Jonklaas *et al.*^[66] found in their study that the concentrations of 3,3'-T2, T3 and T4 were higher in patients with thyroid cancer, patients who had undergone thyroidectomy, and those who were taking levothyroxine (LT4), than patients without.

A large prospective study should be conducted on women who are undergoing TH replacement therapy to clarify any association with BC.

CONCLUSION

The relationship between THs and the mammary gland can be viewed from various aspects, such as from the influence of these hormones in differentiation and lobular growth in a manner similar to estrogen and even with research that identifies its participation in the development of BC. Thus, it is apparent that controversy is present in the literature in regard to the relationship between thyroid diseases and BC, with research showing that both an excess of and a lack of THs may affect breast development and progression to cancer.

Many epidemiologic studies suggest that hyperthyroidism is a factor that leads to BC. However, experimental studies have shown that high TH levels can reduce the time of multiplication of BC cell lines, pointing, inclusively, to a possible crossing of pathways between the molecular action of THs and estrogen. Research that attempts to determine a relationship between TH replacement therapy and BC found no relationship between the two factors. Thus, a relationship between THs and BC is

not clear. However, a significant amount of research on this topic indicates that there is a relationship, principally when molecular aspects of these hormones are considered in the progression of this type of tumor.

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Preventing breast cancer in LMICs *via* screening and/or early detection: The real and the surreal

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Abstract

To review the present status of breast cancer (BC) screening/early detection in low- and middle-income countries (LMICs) and identify the way forward, an open focused search for articles was undertaken in PubMed, Google Scholar and Google, and using a snowball technique, further articles were obtained from the reference list of initial search results. In addition, a query was put up on ResearchGate to obtain more references and find out the general opinion of experts on the topic. Experts were also personally contacted for their opinion. Breast cancer (BC) is the most common cancer in women in the world. The rise in incidence is highest in LMICs where the incidence has often been much lower than high-income countries. In spite of more women dying of cancer than pregnancy or childbirth related causes in LMICs, most of the focus and resources are devoted to maternal health. Also, the majority of women in LMICs present at late stages to a hospital to initiate treatment. A number of trials have been conducted in various LMICs regarding the use of clinical breast examination and mammography in various combinations to understand the best ways of implementing a population level screening/early detection of BC; nevertheless, more research in this area is badly needed for different LMIC specific contexts. No-

tably, very few LMICs have national level programs for BC prevention *via* screening/early detection and even stage reduction is not on the public health agenda. This is in addition to other barriers such as lack of awareness among women regarding BC and the presence of stigma, inappropriate attitudes and lack of following proper screening behavior, such as conducting breast self-examinations. The above is mixed with the apathy and lack of awareness of policy makers regarding the fact that BC prevention is much more cost-effective and humane than BC treatment. Implementation of population level programs for screening/early detection of BC, along with use of ways to improve awareness of women regarding BC, can prove critical in stemming the increasing burden of BC in LMICs. Use of newer modalities such as ultrasonography which is more suited to LMIC populations and use of mHealth for awareness creation and increasing screening compliance are much needed extra additions to the overall agenda of LMICs in preventing BC.

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Key words: Breast cancer; Screening; Early detection; Mammography; Clinical breast examination; Breast self examination; Ultrasonography; Awareness; Developing countries; Low- and middle-income countries

Core tip: Implementation of population level breast cancer (BC) screening/early detection programs will prove to be most cost-effective for low- and middle-income countries (LMICs). Accompanying awareness creation regarding BC among women, more research and change in policy are also necessary to reduce the burden of BC in LMICs.

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INTRODUCTION

Breast cancer (BC) is the most common cancer among women in all parts of the world, be it high income countries (HICs) or low- and middle-income countries (LMICs), accounting for 1/10th of all malignancies detected in both men and women^[1]. HICs and LMICs have been defined by the World Bank as having per capita incomes of \$12616 or more and \$12615 or less respectively^[2]. In general, there is a 10-fold difference in BC incidence across the world, with HICs having a higher incidence compared to LMICs^[3]. Moreover, BC is also the primary cause of cancer death among women worldwide, accounting for about 375000 deaths in 2000^[1]. Mortality due to BC is higher in LMICs than HICs, mostly due to lack of timely detection and treatment^[3]. However, the incidence of BC is rising in all parts of the world, whether in HICs or LMICs^[3], with higher rates of increase observed in LMICs^[4].

Screening and early detection of BC has been well established in HICs due to concerted efforts through many decades. As a consequence of mammographic screening for women aged 50-69, a decrease in BC mortality has been clearly depicted^[5,6]. This is based on observations in the United Kingdom, Northern Europe and Australia of an increasing incidence of early stage and *in situ* BCs after implementation of screening programs, followed by decline in advanced BC and mortality^[7-10]. The estimates are that 10 years after screening began in the United Kingdom, about one-third of the overall 21% reduction in BC mortality was directly due to screening^[11].

The situation in LMICs is in contrast to that of HICs. Although BC has become a health priority for most LMICs due to increasing incidence, lack of early detection and adequate treatment^[12], BC control strategies are hardly in place^[13], resulting in most women presenting with late stage disease when very little can be done^[14]. It is increasingly being realized that detecting BC early and efficiently must be the cornerstone of preventing morbidity and mortality due to BC in LMICs^[15]. In spite of this, the evidence base for implementing early detection/screening of BC in LMICs is extremely thin^[16]. This is unacceptable given the rising populations and the demographic and epidemiological shifts seen in LMICs. It is apparent that along with such changes, an accompanying increase in cancer burden is to be expected in the coming decades in LMICs^[17]. If we were to limit the impact of rising cancer burden in LMICs, it is of utmost importance that there be adequate application of existing knowledge regarding cancer prevention as well as generation of new evidence^[17]. This is also imperative in the light of strong evidence that demonstrates that diagnosing BC early can reduce BC mortality rates, mainly through initiation of appropriate and adequate treatment in the disease's natu-

ral history^[18-20].

As a part of this review, an attempt has been made to consolidate the evidence that exists regarding prevention of BC in LMICs in terms of screening and/or early detection, along with exploring ways to implement the existing evidence and identifying loopholes in research and implementation. The immediate need for prevention of BC in LMICs cannot be emphasized enough.

LITERATURE SEARCH

For the purposes of this focused review, an open search for articles was undertaken in MEDLINE (www.pubmed.com) or the PubMed database, Google Scholar and Google search using keywords like BC, prevention, control, screening, early detection, low- and middle-income countries, LMICs, developing countries, mammography, clinical breast examination (CBE), self breast examination (BSE), ultrasonography and ultrasound with their corresponding MeSH terms in combination with OR, AND where applicable. In addition, the reference list of the articles obtained from the preliminary search was used to further obtain relevant articles and so on *via* a snowball technique. The search was limited to English literature and there were no time limitations for the search. Apart from the search, a question was also put up on ResearchGate^[21] related to this topic, namely "What are the options for early detection and/or screening for BC in low- and middle-income countries (LMICs)?", to find out the opinion of various experts in the field, from both HICs and LMICs. The content of the responses was used to further obtain articles to be included in the review and also to understand the prevalent opinion regarding screening options for BC in LMICs. In addition, experts in the field were also contacted for more references and opinions.

STATUS OF BREAST CANCER PREVENTION IN LMICs-THE REAL

BC, the most common neoplasm in women worldwide, is on a fast and steady rise in LMICs^[1,22]. Mortality caused by BC is also rising quickly^[1,22]. This trend of increasing incidence and mortality due to BC is a common occurrence in LMICs in various parts of the world, be it Latin America, Asia or Africa^[19]. In most of these countries, the rising incidence is most probably due to changing lifestyle patterns, change in reproductive risk factors and increasing obesity due to improving affluence^[23-26]. The contribution of exogenous hormonal influences cannot be ruled out^[27]. It is also quite apparent that most primary risk factors of BC and the ways in which they are changing are not easily modifiable since most of them influence the long term hormonal milieu in a woman's body^[23]. Thus, beneficial impact on BC mortality can only be created *via* implementation of population level screening/early detection and continued improvements in BC treatment^[23].

Among all forms of BC prevention, screening and early detection are the most important since they can have the maximum beneficial impact in lowering the morbidity and mortality due to BC. Consequently, a number of studies have highlighted the need for BC screening/early detection in LMICs to prevent early deaths of women presenting with late stage at diagnosis^[17,28-30]. However, many complex issues crop up in the context of planning and implementation of BC screening in LMICs. One of the important issues is the occurrence of estrogen receptor negative (ER-) BC at earlier ages in LMICs^[31]. It has been suggested that the younger age of BC in LMICs is due to the age distribution of the population^[31], although there is a possibility that the aggressive ER- BC seen in younger ages in LMICs might be a different disease subtype, as has been suggested in Asia and Africa^[32,33].

Status of mammography in LMICs

Thus far, mammography has remained the main modality of BC screening throughout the world. Adequate evidence exists from some randomized controlled trials (RCTs) that mammography screening is associated with significant reductions in BC mortality^[34-36]. Also, to be most beneficial, mammographic screening programs must be of high quality, with appropriate targeting and of sufficient frequency^[37]. Mammography itself does not lead to any excess deaths^[38], although that is currently being debated, with certain researchers suggesting that for every 10000 women invited for screening, 3-4 deaths were avoided, while 1-3 deaths were from other causes for every BC death avoided^[39]. In addition, there is data emerging from HICs that apparently denotes that implementation of screening mammography at the population level has led to probable overdiagnosis while only marginally reducing the rate at which women presented with advanced cancer, consequently having only a small effect on rate of death due to BC^[40]. Similar evidence has been accumulated from multiple other studies^[41,42] and adds to the ongoing discourse regarding the usefulness of population level BC screening using mammography^[43].

Adding to the above scenario is the fact that in LMICs, BC incidence is lower and occurs more in younger age groups when breast tissue is dense. Also, there is a lack of resources for implementing any population level screening programs using mammography. Given the above, implementation of a mammographic screening program becomes quite close to impossible since the costs are too high while the benefits are negligible. There have been very few studies that have focused on cost-effectiveness of BC screening in LMICs^[44,45]. Treating Stage 1 disease and having an extensive BC screening program were found to be most cost-effective by Groot *et al*^[44], while Okonkwo *et al*^[45] suggested CBE to be as cost-effective as mammograms for India. However, maximum cost-benefit can only occur if screening is done in an age group which has a sufficiently high incidence of BC and sufficient high longevity^[46], criteria which are very difficult to fulfil in the case of LMICs. If we look at the list

of countries with any form of population level screening program involving mammography, there are hardly any LMICs, with the exception of China where such a program was begun only in 2009^[47].

Status of CBE and BSE in LMICs

In the absence of mammography as a screening option, the other options for BC screening have been CBE and BSE. Of the two, CBE is more effective than BSE with the ability to detect much smaller tumors. CBE and BSE are more important for LMICs since the screening priorities differ between LMICs and HICs. Mostly, screening programs in HICs focus on finding asymptomatic tumors, while for most LMICs the primary issue is early detection of palpable tumors^[48]. Thus, the choice of an ideal screening program for any given LMIC needs to be based on evidence generated for each of those country settings which, however, is limited due to the paucity of data being generated regarding disease burden and cost-effectiveness of screening modalities. As has been noted by Anderson *et al*^[49], it is necessary to look very closely in any given country to best direct that particular country's screening program. As a consequence, a number of attempts have been made in various LMICs for determining a BC screening solution. Numerous evaluations of such pilot studies or national programs exist in countries such as India^[50], Egypt^[51], Colombia^[52], Lebanon^[53], Palestine^[54], Philippines^[55], Taiwan^[56], Mexico^[57], Brazil^[58], Pakistan^[59] and Nepal^[60]. The results of the above pilots have been varied due to the different combinations of screening modalities and varying compliance rates, with the most effective results being observed in studies where some degree of community penetration was possible, such as in Egypt with home visits by social workers^[61] or *via* mobile units in Brazil^[62]. Results from Taiwan and Egypt were most promising with the use of a two-phase screening, starting with CBE and continuing with mammography^[56,63]. In fact some of the earliest evidence regarding combining various screening modalities come from the breast screening trial set within the Health Insurance Plan of New York where mammography was combined with CBE and almost 70% effect was estimated to be due to CBE^[64]. The Canadian Breast Screening Study among women aged 50-59 (CNBSS 2) found no benefit from adding mammography to CBE and BSE^[65], with around 20% mortality reduction achieved due to CBE and BSE^[66].

The evidence regarding the usefulness of BSE is more indirect, with evidence observed in CNBSS 2^[66]. Furthermore, a nested case-control study discovered depicted benefits from BSE among women aged 40-49 and 50-59 in CNBSS^[67]. Similar benefits were observed in Finland^[68] with randomized trials not showing any benefits of BSE^[69,70], although these trials had limitations and BSE probably would not have led to additional benefits^[61]. In fact, our studies in LMICs have clearly shown that BSE can have a significant impact on stage reduction^[71]. It has been estimated that in India, up to 55%

reduction in mortality from BC can be attained over a 5 year period by detecting tumors of 3 cm in size in the community^[72] which is possible *via* raising awareness regarding BC and BSE. In addition, studies have indicated that women can detect 95% of BCs and 65% of early minimal BCs by themselves^[73]. Along with awareness of risk factors, the health belief model (HBM) suggests that if a woman knows about BC risks, then she is more likely to practice BSE^[74]. Evidence predicts that BSE can reduce mortality up to 18%^[75] and this figure might be higher with regular BSE practice^[76]. Thus, overall it can be said that, in the absence of mammography, CBE is a good tool to begin with at the population level for early detection. In addition, knowledge of BSE among women can also be a major factor for downstaging BC tumors.

Role of knowledge and awareness in BC prevention in LMICs

The importance of awareness when it comes to tackling cancer emerges in the quote of the title of an article in one of the recent bulletins of the World Health Organization (WHO)-“Awareness is the first step in battle against BC”^[77]. This is indeed truer for developing countries where awareness continues to be low among lay women as well as physicians and nurses. In LMICs, women present with late stage tumors which could have been detected at the primary level by physicians or nurses, but primary care physicians and nurses have not been trained to be vigilant about signs and symptoms of cancer and therefore they do not look for them^[77]. In addition, stigma and discrimination become major barriers for women who might detect a lump in their breast but hesitate in seeking medical help in time due to the fear of being abandoned by their partners or losing their jobs^[77]. It is especially important for women with cancer to have a champion or role model who has survived BC^[77]. With respect to cancer awareness interventions, there are broadly two types: one is individual-level interventions and the other community-level interventions. Evidence suggests that both individual-level and community-level interventions may increase cancer awareness. In addition, community-level interventions might increase early presentation although the evidence is limited for that^[78]. In our studies in other LMICs, we definitely found that increased individual awareness of BSE had a significant impact on early presentation^[71]. Increasing awareness also has long term impact on early presentation, as has been suggested by studies in the United Kingdom^[79].

In fact, low levels of cancer awareness have been found to be a very important risk factor for delay in presentation by the patient^[80,81]. However, studies depict that most of the research on cancer in LMICs is related to treatment with miniscule amounts of research devoted to BC prevention, awareness, early detection and palliation^[16]. Such lack of evidence on the interventions to promote cancer awareness and improve early presentation has been dampening the development of policy and action^[78], especially in LMICs.

Policy implications of BC screening in LMICs

Implementation of BC screening programs in LMICs involves complex policy implications. One of the most fundamental problems related to formulating policy making regarding BC screening in LMICs is the lack of good surveillance and monitoring systems that can provide accurate data regarding the magnitude of burden of cancer apart from cancer risk factors^[82]. Added to that is the lack of various system level factors, such as lack of trained personnel and cancer services to support screening services, which further complicates creation of effective policy^[82].

Other aspects affecting policy making includes low incidence of BC in LMICs, which means that a much larger number of women (than in HICs) need to be screened in order to find true cases of BC. Thus, implementation of screening for cancers are considered too expensive^[83]. Also, communicable diseases (CDs) are still prevalent in LMICs due to which there is reluctance to divert resources from CDs to non-communicable diseases (NCDs) like cancer, especially when there are well developed vertical programs in place in the health systems for CDs^[83]. Experts also put forth the opinion that it is unethical to screen people for cancers since treatment is unaffordable or inaccessible after screening. Thus, the final effect is that cancer screening remains accessible to a small affluent section of the population who also generally have health insurance^[83]. This situation is further aggravated by the fact that the economic evidence for implementation of BC screening strategies remains limited and of poor quality^[84]. Thus, although BC screening strategies may be economically attractive in LMICs, the evidence to create specific recommendations regarding choices such as mammography and/or CBE, frequency of screening, target population etc. remains inadequate in both quantity and quality^[84].

In general, one of the most important forces that has tried to influence policy regarding BC overall in LMICs has been the Breast Health Global Initiative (BHGI). Begun in 2002, BHGI has tried to form a global alliance for creating evidence based guidelines to improve BC outcomes according to levels of resources (basic, limited, enhanced and maximal)^[85]. Their recommendations regarding BC screening/early detection according to different resource strata are provided in Table 1^[46]. However, these recommendation need to be adapted for each country according to the various factors affecting policy in a particular country, as has been discussed before.

POSSIBLE IMPROVEMENTS IN BREAST CANCER PREVENTION IN LMICs UNDER PRESENT CIRCUMSTANCES-THE ALMOST REAL

Best possible screening strategy for BC in LMICs

Based on the evidence so far, it is becoming apparent that every country needs to customize and create its own BC

Table 1 Recommendations for breast cancer screening/early detection and public education/awareness according to the resource level of a country^[46]

Level of resources	Public education and awareness	Detection methods	Evaluation goal
Basic	Developing culturally sensitive and linguistically appropriate local education programs for target populations to convey value of early detection, BC risk factors and breast health awareness (education and self examination)	Clinical history and CBEs	Breast health awareness regarding value of early detection in improving BC outcome
Limited	Culturally and linguistically appropriate targeted outreach/education encouraging CBE for age groups at higher risk administered at district level using healthcare provider in the field	Diagnostic breast USG and/or diagnostic mammography if CBE + Mammographic screening of high risk target groups	Downsizing of symptomatic disease
Enhanced	Regional awareness programs regarding breast health related to general health and women's health programs	Mammographic screening every 2 yr in women aged 50 or older Consider mammographic screening (or USG) every 12-18 mo in women aged 40-49	Downsizing and/or downstaging of asymptomatic disease in highest yield target groups
Maximal	National awareness campaigns regarding breast health using mass media	Annual mammographic screening in women aged 40 or more	Downsizing and/or downstaging of asymptomatic disease in women in all risk groups

CBE: Clinical breast examination; USG: Ultrasonography; BC: Breast cancer.

screening and/or early detection strategy. When it comes to LMICs, most of them fall in the strata of having basic or limited resources, with only a few having enhanced resources. Considering BHGI guidelines (Table 1)^[46], it can be seen that the best that LMICs can wish for with regards to BC is downstaging of the disease. Based on the how much the health system and health expenditures of a country can allow, a LMIC can aim for CBE, CBE in combination with mammography or mammography at a population level. However, the most important factor to be considered before advocating any form of population based BC screening and/or early detection is a way to identify high risk groups of women based on their life and family history. This will ensure that any population based BC control program will be cost-effective and have maximum impact. Moreover, BC screening and/or early detection must be offered at the primary care level and primary care level workers must be properly trained in conducting CBEs. Also, proper referral and diagnostic services must be available as a part of BC screening/detection programs. A BC control program targeting prevention can be viewed as a “best buy” investment opportunity for reducing health expenditures^[86]. The above also needs to be coupled with strategies for increasing the knowledge and awareness of women regarding BC and BC screening/early detection, as emphasized below.

Education and awareness enhancement for improving BC outcomes

The majority of studies from LMICs clearly indicate that the knowledge and attitude of women regarding breast screening does not correlate well with the actual screening behavior^[87-90], with regular screening behavior such as conducting BSE ranging from 10% to 80% based on ed-

ucation, occupational and socioeconomic status^[91-93]. On average, less than 50% of women aware of BSE actually practised it, with the majority of women practising incorrect techniques. Contrary to the expected view, healthcare providers were not knowledgeable about screening techniques, neither did they encourage women to implement screening behavior^[87,94]. Healthcare providers were also not at the top of the list in terms of their importance as source of information. Instead, electronic media and television (TV) was noted to be the most important source of information on BC^[95]. For less educated women, it was relatives and friends who were the most important source of information^[96]. Also, it is important to note that, despite the low levels of awareness of women in LMICs regarding BC, very few studies exist regarding evaluating methods for increasing awareness^[97-101].

However, given the importance placed by the WHO^[77] and BHGI^[49] on the importance of awareness in controlling and preventing BC, it is of utmost importance that organized methods be applied in LMICs regarding spreading awareness of BC, especially *via* the use of electronic media and TV. There must be increased public awareness regarding disease risk factors, symptoms and screening behaviors leading on to the detection of BC at earlier stages. The impact of practising breast screening behaviors on downstaging of BC has been clearly observed in our studies in LMICs^[71].

Need for more research regarding BC prevention

As has been stressed before in this work, research on BC prevention remains highly inadequate and significant improvement is required in both quantity and quality in order to reduce morbidity and mortality due to BC. A recent review of the literature suggests a very significant

role of research in adapting the findings and experience of HICs in LMICs^[102]. A most important need in LMICs is to study “structural violence” as defined by Paul Farmer: the diffuse and indirect oppressive societal forces that routinely limit the choices that individuals have to make in LMICs^[103]. Another important area of research is to clearly define the varying etiology of BC in various LMICs, as has been delineated in some of our studies with regards to hormone receptor status^[31,104]. In fact, significant differences have been observed with respect to hormone receptor status in populations of various LMICs, such as Bangladesh, Taiwan, Philippines, Vietnam and India, relative to HIC populations^[105-107]. Similarly, differences between LMICs and HICs exist when it comes to host metabolism of systemic treatment agents, one of the cases in point being tamoxifen^[108,109]. Other differences lie in mediating effects of social and cultural factors on impact of BC interventions in LMICs, including personal representations^[110,111]. Another important area of research in LMICs is health systems which can be highly complex and thus, interventions and strategies developed for HICs may be inappropriate for LMICs with competing interests such as communicable diseases^[112] and affordability gaps^[113,114]. Overall, more research is required in various LMICs to provide the evidence base required to develop customized BC prevention strategy for each LMIC, as has also been reiterated by BHGI^[49].

Policy changes required for implementing BC screening in LMICs

It is quite apparent by now that cancer screening/early detection is not a high priority in terms of policy for LMICs. In fact, most LMICs are focused on maternal health policies based on Millennium Development Goals (MDGs). This is in spite of the fact that 200000 more women die each year due to breast and cervical cancer than from complications due to pregnancy and childbirth^[115-117]. The rising incidence of cancer in women in LMICs is quite in line with “cancer transition” described by Bray *et al*^[118]. According to these projections of cancer trends until 2030, it is plausible that cancers caused due to infections (*e.g.*, cervical cancer) will be offset due to a rise in cancers associated with NCD risks (*e.g.*, breast cancer)^[118]. Thus, overall the burden of women’s cancer will continue unabated unless the right policies are made to counteract such trends by building capacity for basic cancer services, especially screening/early detection.

Premature death and disability from cancer has maximum economic impact compared to other causes of death worldwide. Despite this, only 5% of global resources are being spent in LMICs on cancer, while 80% of the cancer burden is being borne by LMICs^[119]. With such low spending in LMICs and even without direct medical expenditures, cancer still costs approximately 895 million USD, or 1.5% of global GDP, which is 20% higher than that for cardiovascular disease^[120]. According to Knaul *et al*^[121], much of this spending can be reduced. Especially for breast and cervical cancer, the cost savings by the

“prevention/early detection and treatment approach” are much greater than by the “treatment only” approach followed currently by LMICs. The scenario is changing gradually, although with few and far between examples of implementation of cancer screening/early detection in LMICs. For example, the state of Tamil Nadu in India is the first state to launch cervical cancer screening using the VIA/VILI method^[122]. However, what is still lacking in India and most other LMICs is an overarching nationwide policy that implements screening, early detection and prevention of cancer. Thus, the need of the hour for most LMICs is the formation of a national cancer control policy which also has inbuilt strategies for increasing knowledge and awareness of people regarding cancer.

FUTURE REQUIREMENTS AND POSSIBILITIES FOR BREAST CANCER PREVENTION IN LMICS-THE SURREAL

New inventions of the future for BC screening

Although the main modalities of BC screening/early detection are still a trio of mammography, CBE and BSE, new modalities are emerging for BC prevention that may become the cornerstone of BC screening/early detection in future with greater benefits and cost-effectiveness for LMICs. One such method for BC screening/early detection has been the use of ultrasonography (USG) and there have been limited trials in LMICs^[123,124] that have proven their utility, especially when dealing with small or dense breasts, as is common with BC occurring in younger premenopausal women in LMICs^[124]. Similar trials of USG as a screening modality are required in other LMICs to evaluate its benefits as a more cost-effective and easily available way of conducting BC screening/early detection. A number of other modalities are also gradually becoming available apart from mammography and USG, such as low-dose mammography, contract-enhanced mammography, tomosynthesis, molecular imaging and magnetic resonance imaging (MRI)^[125]. MRIs are more sensitive than mammograms in picking up tumors in asymptomatic women^[46]. However, once again more trials are necessary in LMIC specific contexts to decide the best suitable technologies according to age, risk and breast density^[125]. The main issue when it comes to widespread use of better imaging technologies such as MRI is to bring down the costs to levels that can be afforded by health systems of LMICs^[126].

Use of mobile health (mHealth) for better spread of education and awareness

It will also be ideal in future if the full potential of mHealth is utilized for enhancing knowledge and changing the attitudes of women in LMICs regarding cancer and screening behaviors to limit the impact of “structural violence”^[103]. Technological interventions have gained much popularity ever since the phenomenal growth observed in Asia, Africa and Latin America in the use of

new information and communication technologies (ICTs), especially the cell phone and internet^[127]. If we look at the most recent figures, 41% of the world's households were using the internet, ranging from 7% in Africa to 77% in Europe^[127,128]. More astounding is the number of global mobile phone users which has grown to 6.8 billion, or 96% of the world's population, with the greatest growth occurring in Asia, Middle East and Africa^[127,129]. All this has become the bedrock for the growth of ICTs in health, eHealth systems or mHealth.

Mobile phone based ICTs have been used in multiple ways globally in the context of LMICs. Also termed mHealth, individuals around the world are increasingly integrating mobile technologies to access health care services and information while health professionals are integrating mobile technologies into public health and clinical activities^[130]. The main advantages of a mobile or cell phone platform has been its capability of transferring information quickly for both literate and illiterate populations. With relatively low start-up cost and flexible payment plans, mobile technology is accessible by most strata of the population^[131]. Various uses of mobile phones in health have included using SMS or even voice-recorded messages for things like reminders to take medication or dates for appointments. With further development of health-related software in mobile phones, such platforms can provide real-time feedback, pre-programmed automated message services and support an increasingly decentralized health system^[132]. Several studies from LMICs, such as Bangladesh, Laos and Egypt, have shown that introduction of mobile phones led to a more direct link between clients and health care workers, causing an increase in demand for health services and health-related information^[131,133]. Such mHealth strategies can be made a part of the cancer control programs in various LMICs for easy dissemination of information, for reminding women about appropriate screening behavior, and for scheduling appointments for screenings.

CONCLUSION

In conclusion, it can be pointed out that in spite of more women dying from cancer than from pregnancy or childbirth related complications, most LMICs are focused mainly on maternal health in terms of resources, while cancer overall and BC takes a backseat. That apart, policy maker awareness remains low in LMICs regarding BC screening/early detection being cost-effective and the "best buy" opportunity to reduce health costs. This coupled with a lack of research regarding cost-effective screening/early detection methods and little community awareness about BC being a treatable disease results in most LMICs losing a large number of women at an early age, a situation that is unfair from a human rights perspective while also creating "cancer orphans"^[86]. While creating new options for pathological diagnosis and treatment, the main focus of LMICs must be on developing national level programs that emphasize screening/early

detection of BC along with effective use of ICT for changing knowledge, attitudes and practices of women. In addition, greater encouragement for research in various aspects of public health ranging from use of newer screening methods and improving health systems cannot be emphasized enough. For the moment, the best option ahead for LMICs is to begin with ways of opportunistic screening after assessing a woman's risk using a combination of CBE followed by mammography at select centers. As a next step, such methods must be rolled out for the larger population with guidelines developed regarding frequency of screening based on the BC epidemiology in a particular LMIC. Further research regarding use of USG and development of screening guidelines regarding use of USG as a screening modality in LMICs is eagerly awaited.

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WJCO 5th Anniversary Special Issues (2): Breast cancer

Green tea compounds in breast cancer prevention and treatment

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Abstract

Breast cancer is the most common cancer among women. In recent years, many *in vitro* and *in vivo* studies indicate that green tea possesses anti-cancer effects. The epidemiological studies, however, have produced inconclusive results in humans. Likewise, results from animal models about the preventive or therapeutic effects of green tea components are inconclusive. The mechanisms by which green tea intake may influence the risk of breast cancer in humans remain elusive mechanisms by which green tea intake may influence. Here, we review recent studies of green tea polyphenols and their applications in the prevention and treatment of breast cancer. Furthermore, we discuss the effect of green tea components on breast cancer by reviewing epidemiological studies, animal model studies and clinical trials. At last, we discuss the mechanisms by which green tea

components suppress the development and recurrence of breast cancer. A better understanding of the mechanisms will improve the utilization of green tea in breast cancer prevention and therapy and pave the way to novel prevention and treatment strategies for breast cancer.

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Key words: Breast cancer; Green tea; Epigallocatechin-3-gallate; Chemoprevention; Treatment

Core tip: Green tea components, especially epigallocatechin-3-gallate, possess anti-breast cancer effects. However, their effects on breast cancer prevention and therapy are still inconclusive. The anti-tumor mechanisms of green tea remain elusive. This review focuses on epidemiological and animal studies on green tea components against tumorigenesis, as well as possible mechanisms involved.

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INTRODUCTION

Breast cancer is a malignant proliferation of epithelial cell lining the ducts or lobules of the breast. Breast cancer is still the most common cancer among women^[1]. According to the National Cancer Institute, 232340 new cases of female breast cancer and 2240 new cases of male breast cancer were reported in the United States in 2013, as well as about 39620 deaths caused by this disease. While there has been a steady decrease in breast cancer incidence and mortality since the early 90s^[1], due largely to improvements in the early detection and treatment of breast

tumors^[2], the social and economic impact of this malignancy continues to be enormous^[3]. Many risk factors can impact on a woman's likelihood of developing breast cancer^[4]. For those who are at a high risk for breast cancer, chemoprevention may be an alternative intervention to inhibit or delay carcinogenesis.

Green tea is the distinctive “liquor” produced from the evergreen plant *Camellia sinensis* leaves and is the most ancient beverage in the world. Traditional Chinese medicine has recommended drinking green tea for the prevention of disease. In recent years, many scientific and medical studies suggested that green tea possesses antiproliferative, antimutagenic, antioxidant, antibacterial, antiviral and chemopreventive effects^[5]. Green tea contains large amounts of various flavonoids. A major class of flavonoids is catechins, which include epicatechin (EC), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epigallocatechin-3-gallate (EGCG)^[6]. EGCG is the most abundant catechin and accounts for 50%-75% of the total amount of catechins. Also, EGCG appears to be the most effective constituent of green tea^[7]. Green tea polyphenols, and its major constituent EGCG, have been tested in tissue culture, animals and more recently in clinical trials^[5]. In this review, we will highlight the recent studies on tea polyphenols and their applications in the prevention and treatment of breast cancer.

GREEN TEA AND BREAST CANCER PREVENTION: EPIDEMIOLOGICAL STUDIES

Over the past three decades, green tea has attracted increasing attention for its health benefits, especially anti-cancer effects^[8]. As early as 1997^[9], there was an epidemiological study showed that increased consumption of green tea had a potentially preventive effect on breast cancer in a Japanese population, especially among females drinking more than 10 cups a day. Since then, the association between green tea consumption and breast cancer risk has been extensively investigated. To date, three meta-analyses^[10-13] have been published on the association between green tea and breast cancer risk and/or recurrence.

The most recent meta-analyses included two studies of breast cancer recurrence and seven studies of breast cancer incidence^[11,14-20]. Among the two studies of breast cancer recurrence, both found a non-significant reduction in recurrence among heavy green tea drinkers (> 3 cups a day)^[14,15]. There was no significant heterogeneity among the studies ($P = 0.65$, $I^2 = 0\%$). This analysis suggested a marginally significant reduction of 27% in recurrence among heavy green tea drinkers (> 3 cups a day) (summary RR = 0.73, 95%CI: 0.56-0.96) when compared to non-drinkers. Among the breast cancer incidence studies, two were cohort studies and five were case-control studies^[16-20]. Overall, there was a statistically significant reduction of 19% among women with high green tea

intake (summary RR = 0.81, 95%CI: 0.75-0.88). Case-control studies suggested an identical effect as the overall analysis with a 19% reduction in risk among green tea drinkers (summary RR = 0.81, 95%CI: 0.75-0.88). However, when cohort studies were analyzed separately, no association between green tea consumption and breast cancer incidence was observed (summary RR = 0.85, 95%CI: 0.65-1.22). In the second meta-analysis, seven studies were included for analyses^[16-18,21,22]. The pooled RR of developing breast cancer for the highest levels of green tea consumption in cohort studies was 0.89 (95%CI: 0.71-1.1, $P = 0.28$, $I^2 = 0\%$), and in case control studies, the odds ratio was 0.44 (95%CI: 0.14-1.31, $P = 0.14$, $I^2 = 47\%$). In summary, these meta-analyses did not find a significant effect of green tea on breast cancer prevention. For the 2 studies that assessed risk of breast cancer recurrence in relation to green tea consumption, both were cohort studies ($n = 1632$)^[15,23]. The pooled RR for breast cancer recurrence in all stages was 0.75 (95%CI: 0.47-1.19, $P = 0.22$, $I^2 = 37\%$). A subgroup analysis of recurrence in stage I and II disease showed a pooled RR of 0.56 (95%CI: 0.38-0.83, $P = 0.004$, $I^2 = 0\%$). These data indicate that high intake of green tea may be associated with a relative risk reduction in stage I and II breast cancer recurrence.

The epidemiological studies on the association between green tea and breast cancer remain inconclusive^[24,25]. Remarkably, green tea may also interact with other bioactive dietary components, such as those in soy and mushroom, to affect breast cancer risk. A study in Asian-American women demonstrated a statistically significant inverse association between green tea and breast cancer risk among women with low soy intake, but not among women with high soy intake^[26]. A case-control study indicated that higher dietary intake of mushrooms decreased breast cancer risk in pre- and postmenopausal Chinese women, and an additional decreased risk of breast cancer from joint effect of mushrooms and green tea was observed. These data suggested that combined green tea composition with other bioactive dietary components may be an appropriate way to improve its effects in cancer prevention. However, additional studies are required to elucidate the potential mechanisms of action.

GREEN TEA COMPONENTS AND BREAST CANCER: *IN VIVO* EXPERIMENTAL STUDIES

Green tea components and breast cancer prevention in animal models or clinical trials

“Cancer chemoprevention” was first introduced by M. Sporn, who defined it as the prevention of the occurrence of cancer by the oral administration of one or multiple compounds^[27]. In 1987, the chemopreventive effect of EGCG was first reported when the inhibitory effects of EGCG on teleocidin-induced tumor promotion in mouse skin was observed^[28]. There is an increasing

amount of evidence that has been presented, indicating that green tea may be chemopreventive^[29]. Here, we focus on several recent studies about the effects of green tea components on breast carcinogenesis in animal models or clinical trials (Table 1).

Kavanagh *et al.*^[30] showed that a green tea extract significantly increases mammary tumor latency and decreases tumor weight and metastases in dimethyl-benzanthracene (DMBA) treated rats. Sakata and co-workers showed that green tea, alone or in combination with other anticancer components, may have significant chemopreventive effects on carcinogen-induced mammary tumorigenesis^[31]. In the DMBA-induced mammary cancer rat models, the number of tumors per rat and the time latency to tumor development were estimated. However, animals exposed throughout life to EGCG in the drinking water showed no significant difference compared with the control group with respect to second and third tumor latency, although there was a decrease in the latency to first tumor development. Furthermore, the number of tumors per rat in EGCG-exposed rats was not significantly different from that in the controls. The authors suggested that the lack of effect of EGCG was because of the low bioavailability of pure EGCG. In 2012, Crew *et al.*^[32] reported results from a phase Ib clinical trial using EGCG over a 6-mo period, which was conducted to determine the maximum tolerated dose (MTD)^[32,33]. During the treatment period no changes in breast tissue proliferation were observed. Overall, the agent was well-tolerated, with toxicity data establishing a 600 mg twice daily MTD for polyphenon E (Poly E). A phase II trial testing the cancer preventive effects of 1 year of EGCG in postmenopausal women with high mammographic is currently ongoing, and the results are expected.

Green tea components and breast cancer therapy in animal models

So far, numerous studies have investigated the therapeutic effects of green tea on breast cancer using different rodent models and a variety of green tea products including green tea mixtures as well as specific catechins^[38]. The recent studies of green tea catechins for breast cancer treatment in animal models are summarized in Table 2.

One recent study showed that treatment with EGCG at 50 to 100 mg/kg per day in drinking water significantly inhibited the progression of breast cancer in female mice. A further study suggested that the effect of EGCG on tumour size was mediated by the inhibition of hypoxia-inducible factor 1 α (HIF-1 α) and nuclear factor κ B (NF- κ B) activation as well as vascular endothelial growth factor (VEGF) expression^[39]. Another study demonstrated that EGCG significantly reduced tumor volume in a xenograft mouse model developed using stem-like SUM-149 breast cancer cells^[40].

Remarkably, one study showed that high-dose green tea extracts strongly activated HIF-1 in T47D human breast carcinoma cells, and increased the expression of HIF-1 target genes including glucose transporter

(GLUT)-1, VEGF, and p21/CDKN1A^[41]. These results suggest that intended cancer chemoprevention with high-dose green tea extracts may be compromised by the ability of tea catechins to promote tumor cell survival pathways associated with HIF-1 activation. Therefore, the possibility of antagonistic interactions must be taken into account in the development of new cancer therapy strategies based on drug-EGCG co-treatments.

In breast cancer, EGCG has been shown to interfere with estrogen receptor function, inhibit estrogen-induced breast cancer cell proliferation, and sensitize hormone responsive tumors to drugs that target steroid receptors (*e.g.*, tamoxifen)^[44-46]. The combination of EGCG and curcumin was efficacious in both *in vitro* and *in vivo* models of ER α - breast cancer. Also, our study showed that EGCG overcame paclitaxel-induced 78 kDa glucose-regulated protein (GRP78) expression and potentiated paclitaxel-induced Jun N-terminal kinase (JNK) phosphorylation in 4T1 cells both *in vitro* and *in vivo*^[47]. When tumor-bearing mice were treated with paclitaxel in combination with EGCG, tumor growth was significantly inhibited, whereas the single-agent activity of paclitaxel or EGCG was poor. In addition, a clinical trial conducted recently in breast cancer patients undergoing radiotherapy showed that EGCG could potentiate the effect of ionizing radiation^[48]. After two to eight weeks of EGCG plus radiotherapy administration, serum levels of angiogenic factors VEGF, hepatocyte growth factor, and active matrix metalloproteinase (MMP)-2 and MMP-9 were lower compared to those in patients receiving only radiotherapy. In addition, the antioxidant and anti-inflammatory activities of green tea catechins have been suggested to contribute to the potential protective role of EGCG against chemotherapy and radiotherapy side effects^[49]. The use of green tea components, especially EGCG, could enhance the effect of conventional cancer therapies through additive or synergistic effects as well as through amelioration of deleterious side effects. Further research, especially at the clinical level, is needed to ascertain the potential role of EGCG as an adjuvant in breast cancer therapy.

MECHANISMS OF ACTION OF GREEN TEA COMPONENTS IN BREAST CANCER

To better understand the preventive and therapeutic activities of green tea components on breast cancer found in animal studies, substantial research has been conducted to uncover the mechanisms at cellular and molecular levels. Experimental studies collectively show that green tea components lead to wide range of responses in animal models or breast cancer cells.

Anti-angiogenesis

Induction of new blood vessel growth, known as angiogenesis, is required for tumor growth and metastasis^[50]. Angiogenesis permits rapid tumor growth by providing an exchange of nutrients, oxygen, and paracrine stimuli to the tumor. A recent study showed that EGCG treat-

Table 1 Studies of green tea catechins on mammary tumorigenesis in animal models or clinical trials

Ref.	Model	Intervention	Main results
Kavanagh <i>et al</i> ^[30] , 2001	4-wk-old female Sprague-Dawley rats	Treated with green tea catechins after exposure to DMBA	Green tea extract given after initiation significantly increases mammary tumor latency and decreases tumor weight and metastases in DMBA-treated rats
Hirose <i>et al</i> ^[34] , 2002	6-wk-old female F344 rats	PhIP alone or PhIP plus 1% green tea catechins for 52 wk	1% green tea catechins were associated only with reduced mean size of mammary tumors without affecting the total number of mammary tumors
Whitsett <i>et al</i> ^[35] , 2006	Female Sprague-Dawley CD rats	Treated with DMBA to induce breast cancer after previous exposure to green tea catechins or control diet throughout life	Animals exposed throughout life to EGCG in the drinking water showed a decrease in the latency to first tumor development, although there was no significant difference as compared with the control group with respect to second and third tumor latency. Furthermore, the number of tumors per rat in EGCG-exposed rats was not significantly different from the controls
Kaur <i>et al</i> ^[36] , 2007	C3(1)SV40T, t antigen transgenic multiple mammary adenocarcinoma mice	Mice received green tea catechins in drinking water at 0.01% (w/v) for 25 wk, with water as control	Green tea catechins delayed carcinogenesis as evidenced by a significant decrease in the volume and size of tumors in the mice exposed to green tea extract
Lubet <i>et al</i> ^[37] , 2007	50-d-old female Sprague-Dawley rats	Intravenous injection of methyl-nitrosourea (75 mg/kg bw) <i>via</i> the jugular vein. 5 d after treatment with the carcinogen, Poly E was given by gavage at 1000 and 333 mg/kg bw/d.	There was no effect of Poly E on the latency period of the mammary tumors. The high and low doses of Poly E decreased the number of mammary tumors by 14% and reduced the weight of the tumors by 30% and 21%, respectively
Sakata <i>et al</i> ^[31] , 2011	C3H/OuJ mice carrying preneoplastic lesions	Treated with EGCG and tamoxifen alone or in combination	The tumor incidences were decreased in the green tea extract, tamoxifen, and green tea extract and tamoxifen groups. Importantly, in the group treated with green tea extract and tamoxifen, no tumors developed
Crew <i>et al</i> ^[32] , 2012	Women with a history of histologically confirmed resected stage I-III, estrogen and progesterone receptor negative breast carcinoma.	Participants received either Poly E delivering 400, 600, or 800 mg of EGCG (2-4 capsules) twice daily with food or matching placebo for 6 mo	(1) The MTD for Poly E should be 600 mg twice daily; (2) There was about a 70% reduction in serum estradiol levels ($P = 0.05$) and a significant decrease in SHBG ($P = 0.03$) at 6 mo compared with baseline in the Poly E group. However, these changes did not differ significantly compared with the placebo group due to smaller numbers; and (3) No changes in breast tissue proliferation were observed.

DMBA: Dimethyl-benzanthracene; EGCG: Epigallocatechin-3-gallate.

ment reduced plasma VEGF levels over the control mice and the EGCG-treated tumor had lesser micro-vessels than the control tumor. The down-regulation of VEGF expression by EGCG was associated with the inhibition of HIF-1 α and NF- κ B activation^[39]. Consistently, administration of polyphenon E, a standardized green tea extract, at concentrations of 20 ng/ μ L or greater significantly decreased the formation of vascular structures. *In vivo*, quantification of micro-vessel density also indicated that polyphenon E drastically reduced angiogenesis in a dose-dependent manner^[51]. Another *in vitro* study showed that green tea extracts and EGCG decreased the RNA levels of VEGF in MDA-MB231 cells^[52]. Taken together, inhibition of VEGF transcription appeared to be one of the molecular mechanisms involved in the antiangiogenic effects of green tea, which may contribute to its potential use for breast cancer treatment and/or prevention.

Interaction with target proteins

The eight phenolic groups of EGCG can serve as hydrogen bond donors to many biomolecules. EGCG has been recently shown to bind with high affinity to several target proteins, including phosphoinositide 3 kinase (PI3K)^[53], 67-kDa laminin receptor^[54], Ras-GTPase activating protein (GAP) SH3 domain-binding protein

1 (G3BP1)^[55], Bcl-xL and Bcl-2^[56], vimentin^[57], Fyn^[58], GRP78^[59], 70 kDa zeta-associated protein (Zap-70)^[60], insulin like growth factor 1 receptor (IGF-1R)^[61] and so on. All these proteins have been demonstrated to be important for the inhibitory activity of EGCG in breast cancer cell lines or animal models.

Inhibition of cell signaling pathways

VEGF is the most significant regulator in the development of the vascular system and is commonly overexpressed in breast cancer. Green tea catechins, especially EGCG, inhibit tumor growth, proliferation, migration, and angiogenesis of breast cancer^[39,52]. Overexpression of Her-2/neu, the second member of epidermal growth factor receptor (EGFR) family, has been seen in about 30% of breast cancers and was associated with poor overall survival. EGCG treatment reduces basal phosphorylation and constitutive activation of the Her-2/neureceptor^[62,63]. Other investigators have demonstrated that EGCG blocks Wnt signaling through the HBP1 transcriptional repressor that was previously shown to inhibit Wnt signaling^[64]. In addition, Bigelow and Cardelli have investigated the effect of EGCG on inhibition of the hepatocyte growth factor signaling pathway. The results showed that EGCG (0.3 mmol/L) could completely

Table 2 Studies of green tea catechins on breast cancer treatment in animal models

Ref.	Model	Intervention	Effect on tumor size	Main mechanisms
Gu <i>et al</i> ^[39] , 2013	8-wk-old female C57BL/6 mice were inoculated with 10 ⁶ E0771 cells into the left fourth mammary gland fat pad	After cells were inoculated, mice received EGCG (around 50-100 mg/kg per day) in drinking water for 4 wk and 8 control mice received water only	(1) Tumor cross section area reduced 65% ($P < 0.01$); (2) tumour weight reduced 68% ($P < 0.01$); and (3) no difference in body weight, heart weight, kidney weight, or urinary protein	Inhibition of vascular endothelial growth factor (VEGF) expression and tumor angiogenesis <i>via</i> inhibiting hypoxia-inducible factor 1 α and nuclear factor κ B activation
Mineva <i>et al</i> ^[40] , 2013	6-wk-old female nonobese diabetic/severe combined immunodeficiency mice were implanted with 5 \times 10 ³ SUM-149 cells in the fourth inguinal mammary fat pad	After 25 d, mice were intraperitoneally injected with 16.5 mg/kg EGCG or control PBS five times a week for the first five weeks and daily for the last week	(1) Tumor volume decreased 37.7% \pm 4.4%; (2) tumor weight decreased 28.6% \pm 6.5%; and (3) the lymphatic vessel density at the periphery of tumors decreased in EGCG-treated mice	EGCG decreased levels of VEGF-D RNA and VEGF-D protein
Jang <i>et al</i> ^[42] , 2013	4T1 cells (10 ⁵) were injected subcutaneously into either side of the posterior flank of BALB/c mice	On the 7 th , 9 th , 11 th days after cell injection, mice were intraperitoneally injected with either EGCG (10 mg/kg) or PBS control	On day 30 after cell injection, a significant decrease of tumor volume and weight was observed in the EGCG-treated group <i>vs</i> the control group ($P < 0.0005$)	EGCG inhibited expression of CSF-1, CCL-2, IL-6 and transforming growth factor- β , and induced tumor necrosis factor- α expression
Thangapazham <i>et al</i> ^[43] , 2007	5-wk-old female athymic nude mice (NCR-nu/nu) were implanted with 5 \times 10 ⁶ MDA-MB-231 cells in the mammary fat pad	After cell inoculation, one group of animals received 1% polyphenols from green tea (GTP) as a sole source of drinking water and the other group received a dose of 1 mg/animal of EGCG or water as control	At the end of 10 wk, the tumor volume was reduced by 45% and 61% in the EGCG and GTP treated groups, respectively ($P < 0.05$). All animals appeared healthy with no loss of body weight	EGCG and GTP fed animals showed increased apoptosis and decreased proliferation

DMBA: Dimethyl-benzanthracene; EGCG: Epigallocatechin-3-gallate; VEGF: Vascular endothelial growth factor.

blocked phosphorylation of Met (HGF Receptor) and its downstream extracellular signal-regulated kinases 1 and 2 (ERK1/2), and Akt/protein kinase B (PKB)^[65].

Inhibition of enzyme activities

Numerous *in vivo* and *in vitro* studies have been published on the anti-tumour and anti-proliferative properties of green tea. EGCG has been reported to inhibit a number of enzymes. For example, Liang *et al*^[60] showed that cyclin-dependent kinase (CDK) 2 and CDK4 were inhibited by 30 μ mol/L EGCG in MCF-7 breast cancer lines, and this was associated with cell cycle arrest in G₀ and G₁. Also, EGCG increased the expression of the CDK inhibitor p21 in human breast carcinoma cells. Another study found that EGCG inhibited p38-regulated/activated protein kinase (PRAK; IC₅₀ = 1 μ mol/L) and dual-specificity tyrosine-phosphorylated and regulated kinase 1A (DYRK1A; IC₅₀ = 0.33 μ mol/L), but did not inhibit CDK2^[67]. A recent study shows that EGCG is an ATP-competitive inhibitor of both PI3K and mammalian target of rapamycin with *K_i* values of 380 and 320 nmol/L, respectively^[53].

Induction of cell cycle arrest and apoptosis

Dysregulated cellular proliferation and apoptosis are a hallmark of cancer. Green tea extracts and EGCG are capable of inhibiting cell growth and inducing apoptosis *via* a variety of mechanisms. Recent studies showed that EGCG suppressed proliferation and growth of triple negative breast cancer Hs578T cells^[68], estrogen and pro-

gesterone receptor positive human breast cancer cells^[69], MMTV-Her-2/neu mammary gland tumor NF639 cells^[63] and others^[70]. Also, EGCG induced apoptosis in estrogen receptor negative MDA-MB-468^[71] and MDA-MB-231 cells^[72]. Therefore, it is likely that EGCG induces cell cycle arrest and apoptosis in most, if not all, breast cancer cell lines. EGCG increases protein expression of p21 and p27^[73]. Green tea inhibited expression of Ki-67 in both benign and malignant cells^[74]. EGCG alters the activity of EGFR and its downstream targets^[75]. In addition, research showed that catechin hydrate increased the expression of pro-apoptotic genes caspase-3, -8, and -9 and TP53^[70,76]. In addition, EGCG can mediate the retinoblastoma (pRb)-E2F/DP pathway, an important regulator of cell cycle arrest and apoptosis^[77].

Effects on microRNAs

MicroRNAs (miRNAs) are small (about 22 bases), single stranded, endogenous, noncoding RNAs that negatively regulate the translation and/or stability of mRNAs. It could be affected by EGCG to cause subtle changes in multiple molecular targets and pathways. In 2010, the first global miRNA expression profile showed that there were 16 down-regulated and 7 up-regulated miRNAs in MCF-7 breast cancer cells treated with Polyphenon-60 green tea extract^[78]. Remarkably, among the miRNAs down-regulated by Polyphenon 60 treatment, MiR-27a was the most dramatic^[78]. MiR-27a directly targets FOXO1, a putative tumor suppressor, and regulates endogenous protein expression in MCF-7 breast cancer

cells^[79]. In addition, Jang *et al.*^[42] found that EGCG up-regulates MiR-16 in tumor cells, which down-regulates I κ B kinase α and subsequently induces I κ B accumulation in tumor associated macrophages, and inhibits M2 polarization. These studies suggest that the ability of green tea components to regulate miRNA expression may be one of potential mechanisms for green tea in breast cancer prevention and treatment.

Other potential mechanisms

In addition to mechanisms discussed above, there were other mechanisms involved in anticancer effects of green tea components including DNA methylation, metabolism, endoplasmic reticulum stress response and so on. Treatment of breast cancer cells with EGCG results in promoter demethylation of human telomerase reverse transcriptase, retinoic acid receptor β 2 and target of methylation-induced silencing 1^[80,81]. These studies demonstrated that EGCG has the potential to reverse epigenetic changes. A pilot study in overweight breast cancer survivors showed that intake of decaffeinated green tea for 6 mo was associated with a slight reduction in body weight and improved high-density lipoprotein and glucose homeostasis^[82]. Also, EGCG treatment inhibited the expression of fatty acid synthase in MCF-7 and AU565 human breast cancer cell lines by blocking heregulin^[83]. And our studies showed that EGCG potentiates quercetin-, taxol- and vinblastine-induced activation of pro-apoptosis arms of the endoplasmic reticulum stress response, such as JNK phosphorylation, caspase-7 and poly (ADP-ribose) polymerase (PARP) cleavage^[47,84,85]. In addition to these mechanisms discussed in breast cancer, there are other multiple mechanisms presented in colon, lung, prostate, ovarian and other cancers. It can be expected that further in-depth research on each of these specific mechanisms will uncover more details of the action of green tea in breast cancer prevention and therapy.

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Adjuvant chemotherapy in breast cancer: To use or not to use, the anthracyclines

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Abstract

Breast cancer continues to be one of the leading causes of cancer mortality in the world. The treatment generally involves multiple modalities including surgery, radiation and/or chemotherapy. Anthracyclines, one of the first chemotherapeutic agents introduced in the 1960s, has been the backbone for the last 30 years and has been used extensively so far. However, the cardiac toxicity and the concern for secondary hematological malignancy has always been a challenge. A better understanding of the tumor biology, role of Her2 expression and the discovery of trastuzumab and other anti-Her 2 agents along with other effective novel therapeutic options, have revolutionized the treatment for breast cancer. The role of anthracyclines has come under close scrutiny, especially in the adjuvant setting for patients with early stage breast cancer and those with low or intermediate risk of disease recurrence. Recent studies have highlighted such a shift in the use of anthracyclines in both the academic and community clinical practice. However, in patients with a high risk of relapse, anthracyclines still hold promise. Ongoing clinical trials are underway to further define the role of anthracyclines in such a patient population. This review highlights the development, clinical utility, limitations and potential future use of anthracyclines

in the adjuvant setting for patients with breast cancer. We consulted PubMed, Scopus, MEDLINE, ASCO annual symposium abstracts, and <http://clinicaltrials.gov/> for the purpose of this review.

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Key words: Breast cancer; Adjuvant; Neoadjuvant; Chemotherapy; Anthracyclines

Core tip: A better understanding of the tumor biology along with other effective novel therapeutic options, have revolutionized the treatment for breast cancer. The role of anthracyclines has come under close scrutiny, especially in the adjuvant setting for patients with early stage breast cancer and those with low or intermediate risk of disease recurrence, as per the recent studies. However, in patients with a high risk of relapse, anthracyclines still hold promise. Ongoing clinical trials are underway to further define the role of anthracyclines in such a patient population.

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INTRODUCTION

Approximately 230000 new cases of female breast cancer are diagnosed annually in the United States^[1]. The probability of developing invasive breast cancer in one's lifetime is one in eight^[2]. Breast cancer is a heterogeneous disease with common aspects to treatment including surgery, chemotherapy and radiation therapy. Systemic (neo)adjuvant chemotherapy, cytotoxic treatment before

or following primary surgery, is responsible in part for the reduction in cause-specific mortality from breast cancer^[3]. In the 1960s and early 1970s the anthracyclines emerged as a novel therapeutic agent against metastatic breast cancer and by the late 1970s the very first adjuvant trials with these agents were reported^[4-8]. By the 1980s, doxorubicin-based combination regimens established themselves as a primary class of chemotherapy regimens used in the treatment of early and advanced stage breast cancer^[5]. The introduction of new and effective therapeutic agents in combination with some of the irreversible and/or long term adverse events of the anthracycline group of drugs has now questioned their use in the (neo)adjuvant setting^[9].

PATIENT SELECTION/INDICATIONS FOR TREATMENT

Multiple components determine the necessity for patients requiring adjuvant chemotherapy. These include but are not limited to the tumor size, molecular subtype, histology and its grade. The axillary and regional lymph node status and the tumor hormone receptor expression are also important considerations. Finally, the patient's age, concomitant co-morbidities and their performance status play a significant role in determining the benefit of (neo)adjuvant chemotherapy. Other histologies require more information regarding size and nodal status to delineate the role of chemotherapy. Tumor size in the setting of regional disease is an independent prognostic factor with five-year overall survival (OS) for tumors ≤ 2 cm, 2.1 to 5 cm and ≥ 5 cm being 95, 82 and 63 percent, respectively^[10]. Nodal status also plays a role with any nodal involvement lowering the survival rate at five years^[11].

ESTIMATING THE BENEFIT/RISK RATIO

Despite all of the components above, selection of patients for adjuvant chemotherapy requires an individualized approach that has been enhanced by the use of benefit *vs* risk calculators. One of the most widely studied and validated tool is Adjuvant! Online^[12]. Adjuvant! Online is a web based program that aims to help health care professionals discuss the risk and benefits of getting additional therapy including chemotherapy, hormone therapy, or both after surgery for early stage cancer^[13]. The calculator uses resources such as Surveillance, Epidemiology, and End Results (SEER) database and data on adjuvant therapy from the Early Breast Cancer Trialists' Collaborative Group (EBCTCG), as well as data from several pivotal adjuvant clinical trials. It helps make approximations of the risk of a negative outcome (cancer related mortality or relapse) without systemic adjuvant therapy and calculates the estimates of the reduction of these risks afforded by therapy. These estimates are based on information entered about individual patients and their tumors (including patient age,

tumor size, nodal involvement, histologic grade, *etc.*)^[13]. In addition to the above risk calculator, the last decade has also witnessed the emergence of genomic profiling of the primary tumor with tests like Oncotype DX[®], Mammprint[®] and PAM50, which allows for better risk prognostication and in some of them a predictive benefit of adjuvant therapies^[14-16]. The review of these tools are beyond the scope of this article but needless to say, they have allowed to better define the population of patients that should consider adjuvant chemotherapy. This substantially helps both providers and patients to better assess the worthiness of the potential benefits of chemotherapy as compared to their known probable short and long term side effects.

DATA SUPPORTING ADJUVANT THERAPY WITH ANTHRACYCLINES

The EBCTCG meets every five years to review data from global breast cancer trials. The 2011 EBCTCG meta-analysis included an analysis of the utility of adjuvant chemotherapy. One analysis compared no treatment to the combination of cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) compared to an anthracycline containing regimen. Compared to no treatment, the use of CMF in 5253 women resulted in a significant improvement in the risk of recurrence at 10 years [risk ratio (RR), 0.70, 95%CI: 0.63-0.77], which translated into an absolute gain of 10.2 percent. There was also a significant reduction in breast cancer mortality (RR, 0.76, 95%CI: 0.68-0.84), validating a complete benefit of 6.2 percent. Lastly, there was a significant reduction in overall mortality (RR, 0.84, 95%CI: 0.76-0.93), thereby demonstrating an absolute gain of 4.7 percent^[17]. Comparatively the use of an anthracycline containing regimen compared to no treatment in 8575 women established a significant improvement in the risk of recurrence at 10 years (RR, 0.73, 95%CI: 0.68-0.79), which resulted in a total gain of 8.0 percent. There was also a significant reduction in breast cancer mortality (RR, 0.79, 95%CI: 0.72-0.85), ensuing an absolute improvement of 6.5 percent and a significant reduction in overall mortality (RR, 0.84, 95%CI: 0.78-0.91), confirming an absolute benefit of 5.0 percent^[17].

The 2011 EBCTCG meta-analysis also compared the dosing regimens of anthracyclines *vs* CMF. The standard dose of anthracyclines was defined as a cumulative dose of doxorubicin of 240 mg/m² *vs* the high dose defined as doxorubicin > 240 mg/m² or epirubicin > 360 mg/m²^[17]. The 10 year results of this analysis in 5122 women showed that standard dosing of anthracyclines was equivalent to CMF with no improvement in the risk of recurrence, breast cancer mortality or overall mortality^[17]. An analysis of 9527 women receiving either higher cumulative doses of anthracyclines or CMF were compared at 10 years with a reduction in the risk of recurrence (RR, 0.89, 95%CI: 0.82-0.96), which translated into an absolute gain of 2.6 percent. There was also a reduction in breast cancer mortality (RR, 0.80, 95%CI: 0.72-0.88) with

an absolute gain of 4.1 percent and a reduction in overall mortality (RR, 0.84, 95%CI: 0.76-0.92) with an absolute gain of 3.9 percent^[17]. These results suggest standard dosing anthracycline regimens are equal to CMF but slightly inferior to regimens including higher cumulative doses of anthracyclines, *i.e.*, 6 cycles of an anthracycline-based regimen being better than 4 cycles. No single regimen has been defined as the absolute gold standard treatment but based on well conducted prospective trials and meta-analyses conducted by the EBCTCG, anthracycline-based regimens have been recommended for more than 2 decades^[17-19].

The 2011 EBCTCG meta-analysis also included taxanes such as docetaxel and paclitaxel in its analysis of adjuvant therapy. Incorporation of taxanes into an anthracycline containing regimen resulted at 8 years in the reduction of the risk of recurrence, risk of breast cancer mortality, and overall mortality. This benefit was present independent of age, nodal status, tumor size, tumor grade or estrogen receptor (ER) status^[17].

Studies have also examined the dose intensity of the adjuvant regimens. A meta-analysis of dose dense therapy *vs* standard therapy in 10 trials of over 11000 women reported an improvement in disease free survival (DFS) with dose dense therapy in women with estrogen receptor (ER)-negative disease (HR, 0.71, 95%CI: 0.56-0.98), but not in women with ER-positive disease (HR, 0.92, 95%CI: 0.75-1.12)^[18]. Another analysis of three randomized trials involving 6644 women with node positive breast cancer and variable hormone receptor status demonstrated that women with ER-negative breast cancer had a larger reduction in the risk of recurrence compared to women with ER-positive breast cancer at 5 years, (55% *vs* 26%, respectively). There was also a higher absolute improvement in DFS (23% *vs* 7%) and higher absolute improvement in OS (17% *vs* 4 %)^[19].

PROBLEMS WITH THE ANTHRACYCLINES

Cardiotoxicity and secondary MDS/AML are two significant long-term toxicities of anthracycline use. Anthracycline cardiotoxicity is believed to be derived from damage to the myocardium from free reactive oxygen radicals, direct DNA damage, interference with DNA repair, and induction of immune reactions leading to cardiomyocyte apoptosis^[20-22]. This damage leads to a decrease in the left ventricular ejection function, and although could be reversed with good medical management, in many cases could be an irreversible long term problem. The risk of MDS/AML remains rare at 0.5%-1% but it carries a high mortality rate due to its association with poor cytogenetics and refractory nature to standard treatment^[23,24].

ANTHRACYCLINE TOXICITY

Cardiotoxicity

Anthracyclines are associated with cardiovascular tox-

icities including abnormal electrocardiogram (sinus tachycardia and transient arrhythmias), cardiomyopathy, acute and late-onset congestive heart failure (CHF), myocarditis, pericarditis and myocardial infarction^[25,26]. The incidence of cardiomyopathy and heart failure secondary to anthracyclines has been shown to be dose dependent and generally occurs at higher doses than the dosages administered in the adjuvant setting. However, some of the other acute cardiac events are often not dose related and could occur as soon as after the first dose^[27,28]. The risk of chronic cardiomyopathy and CHF increases substantially at cumulative doses of doxorubicin greater than 400-500 mg/m²^[28] and epirubicin greater than 800-1000 mg/m²^[28]. It is estimated that the overall risk of cardiac toxicity using standard dose anthracyclines in the general population is approximately 1%-3%, however, such risk varies greatly depending on the population of women studied. Older studies that included patients who received higher cumulative doses of anthracyclines report a higher incidence rate whereas newer clinical trials have an insufficient follow up time to accurately assess the long-term incidence of CHF^[29]. An older but long-term prospective trial of 120 patients with advanced breast cancer showed that those patients receiving high cumulative doses of epirubicin (850-1000 mg/m²) had the highest risk of CHF with 11% at 1 year, 14% at 2 years and 20% at 5 years^[30]. Also of note, those patients receiving long term treatment with an angiotensin converting enzyme inhibitor had a significant and long term recovery in cardiac function^[30].

Certain populations including older women are at increased risk with a retrospective study of 12500 women with invasive breast cancer showing a 5-year cumulative incidence of CHF of 6 percent among women aged 65 to 74 and 11 percent among women aged ≥ 75 years^[31]. This was in stark contrast to younger women with a cumulative incidence of 1-2 percent. With the addition of biologic agents including trastuzumab there is a concern for additive cardiotoxicity. In women treated with an anthracycline plus trastuzumab, there was a cumulative CHF incidence of 20 percent which represented an increased risk compared to patients who did not receive an anthracycline or trastuzumab [hazard ratio (HR), 7.19, 95%CI: 5-10.4]^[31]. The risk was also increased among patients treated with trastuzumab without an anthracycline (HR, 4.12, 95%CI: 1.11-1.76)^[31]. Of note in this study only 11.2 percent of women over the age of 65 received an anthracycline based therapy^[31]. Another study supporting the increased risk in older women reviewed 43338 patients with breast cancer treated with chemotherapy through the SEER database^[32]. The authors concluded women aged 66 to 70 years who received adjuvant anthracyclines had significantly higher rates of CHF, 38.4% of the anthracycline-treated group compared with 32.5% of the patients who received non-anthracycline chemotherapy and 29% in the no-chemotherapy group^[32]. The difference in rates of CHF continued to increase through more than 10 years of follow-up^[32].

There has also been data that have not supported long term cardiac adverse effect of adjuvant anthracycline therapy. Patients treated on the Southwest Oncology Group (SWOG) protocol S8897 were randomly assigned to adjuvant chemotherapy with or without the anthracycline doxorubicin. A retrospective study evaluated the left ventricular ejection fraction (LVEF) at 5 to 8 years and 10 to 13 years after treatment randomization^[33]. A total of 93 breast cancer survivors from a potential sample of 1176 patients completed the longitudinal assessment of LVEF^[33]. In the longitudinal analysis, there was no significant deterioration in LVEF concluding that the exposure to doxorubicin did not increase the likelihood of adverse cardiac effects^[33]. However, as noted the studied population was very small.

Risk of MDS and AML

Another well-known serious and concerning adverse event from the use of anthracycline is the development of myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML). Multiple cytotoxic agents have been implicated including those more commonly used in breast cancer including cyclophosphamide, doxorubicin, daunorubicin and epirubicin. The incidence among breast cancer patients varies among different retrospective studies. In a review of 6 adjuvant National Surgical Adjuvant Breast and Bowel Project (NSABP) breast cancer trials (B-15, B-16, B-18, B-22, B-23, and B-25) the incidence of AML/MDS was sharply elevated in patients receiving standard dose doxorubicin ($60 \text{ mg/m}^2 \times 4$) plus higher doses of the alkylating agent cyclophosphamide. In this study, those regimens with two or four cycles of cyclophosphamide at 2400 mg/m^2 (with granulocyte colony-stimulating factor (G-CSF) support) had a cumulative incidence of AML/MDS at 5 years of 1.01% (95%CI: 0.63% to 1.62%), compared with 0.21% (95%CI: 0.11% to 0.41%), for patients receiving 4 cycles of standard doxorubicin/cyclophosphamide (AC) chemotherapy^[34]. Also patients who additionally received breast radiotherapy experienced more secondary AML/MDS than those who did not (RR, 2.38, $P = 0.006$)^[34]. This study also suggested that the use of G-CSF may be an independent factor associated with increased risk of MDS/AML but this may be secondary to the use of higher doses of leukemogenic chemotherapy agents.

Therapy related myeloid neoplasms (t-MN) have on average a latency period of five to seven years^[35]. Praga and colleagues reviewed the incidence of AML/MDS in 19 randomized trials involving 7110 patients who had received adjuvant epirubicin and cyclophosphamide^[24]. Patients with administered cumulative doses of both epirubicin and cyclophosphamide in standard regimens ($\leq 720 \text{ mg/m}^2$ and $\leq 6300 \text{ mg/m}^2$, respectively) had an 8-year cumulative probability of developing AML/MDS of 0.37% (95%CI: 0.13% to 0.61%) compared with 4.97% (95%CI: 2.06% to 7.87%) for patients administered higher cumulative doses of both epirubicin and cyclophosphamide^[24]. Patients most commonly present

with persistent hematologic abnormalities such as macrocytic anemia or pancytopenia in peripheral blood and bone marrow evaluation reveals changes consistent with MDS or AML. Cytogenetics plays an important role in the prognosis of AML. In patients with t-MN there is a higher incidence of unfavorable cytogenetics compared to de novo AML (46.2% *vs* 20.4%)^[36]. Unfavorable genetics affect OS even more in treatment related AML compared to de novo AML (10 mo *vs* 15 mo, $P < 0.001$)^[36].

ANTHRACYCLINE VS NON-

ANTHRACYCLINE ADJUVANT REGIMENS

The MA.5 trial directly compared an anthracycline to a non anthracycline regimen^[37]. The trial enrolled 710 pre and peri-menopausal women with node positive breast cancer. Patients were randomly assigned to receive cyclophosphamide, epirubicin, and fluorouracil (CEF) or cyclophosphamide, methotrexate, and fluorouracil (CMF). The 10-year relapse free survival was 52% for patients who received CEF compared with 45% for CMF patients (HR for CMF *vs* CEF = 1.31; stratified log-rank, $P = 0.007$). The 10-year OS for patients who received CEF and CMF are 62% and 58%, respectively (HR for CMF *vs* CEF = 1.18; stratified log-rank, $P = 0.085$). These results support the previous 5 year follow up data with CEF being superior to CMF^[38]. This trial was not powered for comparison of treatment regimens in subgroups based on nodes or hormone receptor status, however, the HRs favored CEF in patients with one to three nodes and four or more nodes^[37].

The National Surgical Adjuvant Breast and Bowel Project (NSABP) 23 trial was another direct comparison of an anthracycline to a non-anthracycline containing regimen^[39]. 2008 patients were randomly assigned to CMF or AC with or without tamoxifen. In contrasting results a comparison between all CMF and all AC treated patients demonstrated no significant differences in relapse free survival (RFS) (87% at 5 years in both groups, $P = 0.9$), event free survival (EFS) (83% and 82%, $P = 0.6$), or OS (89% and 90%, $P = 0.4$)^[39].

As noted above, the CMF regimen has been a well-established non-anthracycline-containing regimen but its lack of superiority and longer duration of therapy led to its relative abandonment by the oncologic community in the 1990s. However, it still has an important role for patients who are not candidates for anthracycline and/or taxane-based regimens. Capecitabine, an oral prodrug that is converted to 5-fluorouracil, and approved for the treatment of metastatic breast cancer, has been evaluated in the adjuvant setting as a possibly more convenient and less toxic chemotherapy for older women. The Cancer and Leukemia Group B study CALGB 49907 was a randomly assigned trial comparing standard chemotherapy (AC or CMF per patient/provider's choice) *vs* oral chemotherapy with capecitabine in patients age 65 years or older with early-stage breast cancer^[40]. Unfortunately the

study demonstrated that capecitabine therapy was highly likely to be inferior to standard chemotherapy and patients who were randomly assigned to capecitabine were twice as likely to have a relapse and almost twice as likely to die as compared to patients who were randomly assigned to standard chemotherapy ($P = 0.02$).

Docetaxel plus cyclophosphamide has become one of the most popular adjuvant regimens of the last decade. This is based on the long term results of the US Oncology adjuvant trial 9735, which enrolled 1016 patients, age 18 to 75 years, with stage I–III breast cancer (irrespective of nodal, hormonal or HER2 status) and reported a statistically significant superiority of docetaxel-cyclophosphamide (TC) over doxorubicin-cyclophosphamide (AC)^[41]. At a median of 7 years follow-up, there was a statistical improvement in disease-free survival between TC and AC (81% TC *vs* 75% AC; $P = 0.033$; HR, 0.74; 95%CI: 0.56–0.98) as well as an OS (87% TC *vs* 82% AC; $P = 0.032$; HR, 0.69; 95%CI: 0.50–0.97). Benefit was observed irrespective of hormone-receptor status or HER-2 status. TC was noted to be superior in all age groups with the caveat that more febrile neutropenia (FN) was observed in the older population defined by a cutoff of 65 years (for TC, the rate of FN was 8% for the older population and 4% for younger patients compared with 4% in older and 2% in younger patients who received AC). Notably, the use of prophylactic granulocyte colony-stimulating factor to stimulate neutrophil production was not utilized in this study. Of concern is that 4 late deaths were observed in patients without relapse and all occurred in the AC group: a young woman died of cardiomyopathy and CHF, two older women died of complications related to myelodysplasia and myelofibrosis respectively, and 1 other patient died of acute leukemia 10 years after AC. The US Oncology group is now involved in a new study (USO 06090 phase III trial), that will further evaluate the need for anthracyclines by comparing six cycles of TC (plus/minus bevacizumab) against six cycles of TAC (docetaxel/doxorubicin/cyclophosphamide) for 3900 patients with HER2-negative resected breast cancer. We are eagerly awaiting the results of this trial.

The CALGB 40101 trial enrolled 3171 women with early stage breast cancer who were randomized in a 2 × 2 factorial design to AC once every 3 wk for four (12 wk) or six (18 wk) cycles *vs* paclitaxel (T) weekly for 12 or 18 wk (3 wk of T was considered one cycle)^[42]. So far, what has been reported is the comparison of 4 *vs* 6 cycles of therapy. The 4-year RFS was 90.9% for patients randomly assigned to six cycles of therapy and 91.8% for patients randomly assigned to four cycles. The 4-year OS for patients randomly assigned to six cycles of therapy was 95.3% as compared to 96.3% for patients randomly assigned to four cycles of therapy. The conclusion of the study established that six cycles of therapy was not superior to four cycles for either RFS or OS after adjusting for the effects of tumor size, number of positive nodes, hormone receptor status, and menopausal status. There

were a total of 28 patients in the AC arms that developed grade 3 or 4 (G3–G4) left ventricular systolic dysfunction (LVSD) and 1 G5. There was also 1 patient in the AC × 4 cycles that died of acute myocardial infarction. This is in stark contrast with no cases of LVSD in the T × 4 cycles arm and only 3 G3–4 LVSD in the T × 6 cycles arm. Six patients were diagnosed with AML/MDS between 11 and 28 mo after initiation of treatment; 5 in the AC × 6 arm and 1 in the AC × 4 arm. Patients were between 44 and 62 years of age at the time of study enrollment. No cases of AML/MDS occurred in patients treated with T. The study's data safety monitoring board has not yet released data for the efficacy comparison of AC *vs* T, but we presume that it is likely that no major differences will be noted as this trial enrolled all of these patients several years ago, between May 2002 and February 2008.

EARLY STAGE HER2 POSITIVE TUMORS

It has been hypothesized that a specific population of patients who may benefit from the use of anthracycline is that of patients with HER2 positive tumors^[43]. The National Surgical - Breast and Bowel Project 31 (NSABP B-31) trial included women with HER2 positive, node positive breast cancer. Patients were assigned to treatment with doxorubicin and cyclophosphamide (AC) followed by paclitaxel (T) with or without trastuzumab (H) therapy. In conjunction with this trial was the North Central Cancer Treatment Group (NCCTG) intergroup trial N9831 which enrolled women with HER2 positive node positive or high-risk node negative breast cancer. The women were treated with AC and T followed by no treatment, AC and T followed by sequential H or AC followed by concurrent T and H. From these two trials at a median follow up of 3.9 years, chemotherapy plus adjuvant trastuzumab compared to treatment without trastuzumab resulted in significantly superior DFS (86% *vs* 74%, HR 0.52) and OS (93% *vs* 86%, HR 0.61)^[44].

Anthracycline *vs* non-anthracycline based therapy was compared during the Breast Cancer International Research Group 006 (BCIRG-006) trial of 3222 women with HER2-positive, node-positive or high-risk node negative disease. Patients were randomly assigned to adjuvant treatment with AC-T (doxorubicin and cyclophosphamide followed by docetaxel), ACTH (AC followed by T plus trastuzumab) or TCH (docetaxel, carboplatin and trastuzumab)^[45]. With a median follow up of 65 mo patients treated with an anthracycline (ACTH) compared to treatment without an anthracycline (TCH) demonstrated a trend towards an improvement in DFS, rates at 5 years were 84% and 81%, respectively. Estimated rates of OS were 92%, and 91%, respectively. These rates for DFS and OS did not reach statistical significance but the study was actually not powered or designed to compare equivalence between the two trastuzumab-containing groups (ACTH *vs* TCH)^[45]. Conversely, patients receiving ACTH had significantly higher rates of adverse events including CHF, neuropathy and severe neutropenia^[45].

The incidence of symptomatic congestive heart failure in the two trastuzumab-containing regimens was higher in the group receiving ACTH than in the TCH group (2.0% *vs* 0.4%, $P < 0.001$)^[45]. In addition, a significant difference in sustained, subclinical loss of mean LVEF (defined as $> 10\%$ relative loss) was observed in the group receiving ACTH, as compared with the TCH group (18.6% *vs* 9.4%, $P < 0.001$)^[45]. Neuropathy was significantly worse in patients receiving ACTH (49.7% *vs* 36%, $P < 0.001$) as was neutropenia (71.5% *vs* 65.9%, $P = 0.01$)^[45].

The evaluation of whether HER-2 positivity is associated to anthracycline sensitivity has been attempted in a number of adjuvant trials. A pooled analysis of eight of such trials revealed that, for those women randomized to anthracycline *vs* non-anthracycline regimens, the DFS and OS HRs for women with HER-2 positive tumors were markedly superior, at 0.71 (95%CI: 0.62-0.85) and 0.73 (95%CI: 0.62-0.85), respectively^[46]. However, such differential benefit seems to be lost when trastuzumab is added to the adjuvant regimen^[45]. Additionally, a higher incidence of cardiotoxicity has been noted when trastuzumab is used with regimens containing an anthracycline^[47].

In evaluating HER2 positivity and anthracycline sensitivity, topoisomerase 2- α (TOP2A) has been evaluated as well to see whether TOP2A gene alterations could predict incremental responsiveness to anthracyclines in some breast cancers. Some studies have supported the concept that TOP2A co-amplification is the clinically useful target of the anthracyclines and its co-amplification in tumors could be used as a predictive marker of responsiveness to anthracyclines^[48]. A 2011 meta-analysis evaluated the relationship of HER2 and TOP2A status in patients who received either a non-anthracycline based regimen (CMF) *vs* anthracycline based regimens in the adjuvant setting of early stage breast cancer. Tumors from 3452 patients were analyzed for HER2 status (amplified *vs* non-amplified), and from 3102 patients for TOP2A (normal, amplified, or deleted) by fluorescent *in-situ* hybridization (FISH). Although there was a significant improvement in event-free survival (but not OS), for patients with HER2 overexpression treated with anthracyclines compared to CMF and for both outcomes in patients with TOP2A alterations (*vs* TOP2A normal), treated with anthracyclines, the authors concluded that there was not enough evidence to restrict the use of anthracyclines only in patients with HER2-amplified or TOP2A-altered tumors. The main reasons for this conclusion were that women with non-HER2 amplified and non-TOP2A altered tumors also derived benefits when treated with anthracyclines, and because problems exist with the reproducibility of TOP2A gene status assessment by FISH^[49]. Jones and colleagues completed a recent phase 2, single group study of adjuvant therapy with docetaxel, cyclophosphamide, and trastuzumab in HER2-amplified patients with early breast cancer and a low risk of recurrence (node negative or 1-3 positive nodes)^[50]. They enrolled 493 patients with HER2 positive tumors, that were TOP2A gene positive or negative, to receive four 21-d cycles of docetaxel (75

mg/m²) and cyclophosphamide (600 mg/m²), plus intravenous trastuzumab (4 mg/kg [loading dose] on day 1 and 2 mg/kg on days 1, 8, and 15 during chemotherapy, followed by trastuzumab 6 mg/kg every three weeks for the rest of 1 year). Follow up at 2 years revealed DFS was 97.8% (95%CI: 94.2-99.2) in the 190 TOP2A-amplified patients and 97.9% (94.9-99.1) in the 248 patients without amplified TOP2A and OS was higher than 98% in both groups. Investigators found a low occurrence of cardiotoxicity. The investigators concluded that a short course of adjuvant chemotherapy with docetaxel and cyclophosphamide plus trastuzumab might be an option in patients with lower risk HER2-amplified early breast cancer irrespective of TOP2A status as an alternative to an anthracycline containing regimen.

Targeted therapy

New agents are constantly being developed against known and novel targets. Currently such novel agents, such as VEGF (vascular endothelial growth factor), PARP (poly ADP ribose polymerase), mTOR (*mammalian target of rapamycin*) and HDAC (histone deacetylase) inhibitors, are being studied in the neo-adjuvant, adjuvant and metastatic setting, along with anthracycline and non-anthracycline containing regimens^[51]. These clinical trials will provide information on whether they can improve the outcome of patients with breast cancer and their interaction with the different standard chemotherapy agents. The targeted agent most studied has been bevacizumab, but a discussion of its potential benefits or of any of the other agents are beyond the scope of this review.

DISCUSSION

In accordance to all the data reviewed in this manuscript, the indiscriminate use of anthracyclines in the adjuvant setting has become very controversial. Humans are creatures of habit and change is often uncomfortable. Physicians are no exception and it is well known that changes in patterns of practice can take a long time. The use of (neo)adjuvant chemotherapy has certainly contributed to the decline in disease relapse and improvement in survival as noted in the last 30 years in patients with all types of early stage breast cancer. The anthracyclines have been the backbone of most adjuvant chemotherapy regimens since the 1980s and it certainly has served us well. However, emerging evidence demonstrates that anthracyclines may not be critical to the adjuvant treatment of breast cancer and such a change is being observed in practice overall. Additionally, the current evidence also suggests that the specific benefits of anthracyclines are very difficult to substantiate for HER2 positive tumors in the era of the great equalizer trastuzumab and other targeted anti-HER2 agents.

Investigators at the University of California, San Francisco Breast Cancer Center examined the charts of 1116 patients treated for breast cancer between 2000 and 2010^[52]. They examined the use of anthracycline contain-

ing chemotherapy regimens against the non-anthracycline containing. From 2000-2005, 95% of chemotherapy regimens included an anthracycline compared to 65% from 2005-2010. Another study from Giordano *et al.*^[53], looking at claims from 4458 Medicare beneficiaries and 30422 privately-insured population (Marketscan), demonstrated that a sharp increase in the use of taxane-based chemotherapy and a decline in anthracycline-based chemotherapy had occurred during the study period (1998-2009). Such change seemed to appear in late 2005 and the crossover occurred in late 2007. By the end of the study period, 51% of patients in the Medicare cohort received taxane-based and only 32% received anthracycline-based chemotherapy. A similar pattern of care was noted in the privately insured population. An additional important observation was seen among trastuzumab recipients, in the private market, use of docetaxel increased from 34.4% of women in 2005 to 61.7% of women in 2008, and for patients who did not receive trastuzumab, the use of docetaxel changed from 42.6% in 2005 to 59.4% in 2008. Similar findings were also noted in the Medicare cohort. In the trastuzumab recipients, the rate of docetaxel increased from 62.5% of patients in 2005 to 74.5% of patients in 2008, and in patients who did not receive trastuzumab, docetaxel increased from 44.2% in 2005, to 88.8% in 2008. These changes likely reflect the increasing popularity of the docetaxel/cyclophosphamide (TC) regimen for patients with HER2 negative disease and of the docetaxel/carboplatin/trastuzumab (TCH) regimen for patients with HER2 positive disease. Also of relevance is that, patients who underwent the 21-gene recurrence score testing Oncotype DX[®] were more likely to receive a taxane-based chemotherapy.

Based on the emerging data it seems that patients felt to be at lower or intermediate risk of relapse are being treated with non-anthracycline regimens in the adjuvant setting. But is there a population that should still receive an anthracycline based-regimen? We personally believe that the anthracyclines still have an important role in the (neo)adjuvant care of patients with early stage breast cancer. Anthracyclines plus taxanes are important components of what is called today “third generation regimens”^[13]. These include regimens such as 3 cycles of CEF followed by 3 cycles of docetaxel (CEF-D) as developed in the PACS 01 trial^[54]; 4 cycles of AC followed by paclitaxel or docetaxel as used in the CALGB 9741 and ECOG 1199 clinical trials^[55,56]; 6 cycles of doxorubicin, cyclophosphamide and docetaxel (TAC) as in the BCIRG 001 trial^[57] and the trastuzumab containing regimens from the NCCTG 9831, NSABP B-31 and BCIRG 006 studies previously discussed^[44,45]. These regimens have demonstrated in large prospective randomized trials to yield the best relative risk reduction in breast cancer relapse, especially for those patients with high recurrence risk (large primary tumors, presence of nodal metastasis, estrogen receptor negative tumors, HER2 positive tumors), and almost all of them were built with a backbone of anthracyclines. Until new trials, such as USO 06090

and/or others comparing the use of anthracycline *vs* non-anthracycline regimens, are able to show either the lack of superiority of the anthracycline regimen or the non-inferiority of the non-anthracycline regimen, there is still a role for its use in high-risk patients. The only high risk sub-group that could certainly obviate the use of an anthracycline is the population with HER2 positive disease. However, patients should be made aware of the small differences in 5-year DFS between the AC-T and trastuzumab *vs* the TCH regimen (84% *vs* 81% respectively), and that although this was not “statistically different”, as it wasn’t the difference in 5-year OS (92% *vs* 91% respectively), the study was not powered to detect equivalence between these 2 regimens. That 3% DFS difference may be considered clinically significant by the patient and her provider.

CONCLUSION

The current role of adjuvant anthracycline-based chemotherapy in early-stage breast cancer is very much in question. It is very reasonable to substitute such for well-established non-anthracycline regimens for patients considered at lower or intermediate risk of disease recurrence. However, for those at high risk we need more comparative studies to totally abandon a family of drugs that contributed to the decline in breast cancer relapse and improvement in related survival over the last 3 decades. Most importantly, the increasing prospectively conducted research using genomic profiling, will hopefully allow for better risk prognostication and predictive benefit of adjuvant therapies and lead us to spare many from the adverse events of any type of chemotherapy.

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Inflammatory breast cancer clusters: A hypothesis

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Abstract

Cancer clusters have long been a focus of interest because of the possibility of identifying etiologic agents. Only on rare occasions, however, have such cluster investigations been successful. One major difficulty in cluster investigations, particularly in the area of breast cancer, is the long latent period. There have been a number of publications providing a discouraging picture regarding cancer cluster investigations. The possibility of learning from a cluster investigation, however, is greatly increased if the cancer involved is relatively rare and if it has a short latent period. Inflammatory breast cancer (IBC) fits these criteria and is worth pursuing because of the strong evidence that environmental factors play a major role. In this report we describe our experience with several clusters and the lessons

learned which are now being utilized to improve investigation of future IBC clusters. The first IBC cluster that we evaluated was in 2000, when we were asked to investigate an apparent cluster of IBC in Castro Valley, California where three women in an office setting of 24 people were diagnosed with IBC in a ten month period from May 1999 to March 2000. Our investigation of this striking cluster did not yield a specific trigger for this cluster but it did indicate that the women involved all had at least two IBC risk factors that may well have made them susceptible to getting IBC. We are now investigating another apparent cluster in Texas and are aware of several others requiring careful consideration. We see a need for a consistent protocol for the evaluation of IBC clusters focusing on the laboratory investigation of environmental triggers, primarily infectious agents and chemical carcinogens.

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Key words: Inflammatory breast cancer; Environmental; Toxins; Infectious agents

Core tip: Cancer clusters are of interest because of the possibility of identifying etiologic agents. Such cluster investigations have been successful rarely. One major difficulty in the cluster investigations, particularly in the area of breast cancer, is the long latent period. The possibility of learning from a cluster investigation is greatly increased if the cancer is relatively rare and has a short latent period. Inflammatory breast cancer fits these criteria and is worth pursuing because of the strong evidence that environmental factors play a major role. In this report we describe our experience with several clusters and the lessons learned.

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INTRODUCTION

Cancer clusters have long been a focus of interest because of the possibility of identifying etiologic agents. Only on rare occasions, however, have such cluster investigations been successful^[1-4]. In Goodman *et al.*'s^[5] review of 428 cancer cluster investigations in a particular geographic area or community, they found a confirmed increased incidence for only 72 investigations, of which only three were linked to the exposure, and in only one of those, the pleural cancer investigation in South Carolina, was there clear evidence that there was an exposure to some chemical in the naval shipyard^[3]. In the other two investigations in which there was an association, there was statistical uncertainty. In the leukemia investigation in Woburn, Massachusetts, the association with contaminated water was not statistically significant and the association was found only in the boys and not the girls^[1,6]. In the pediatric cluster in Dover Township, New Jersey^[2], which was a superfund site, there were elevated incidences of leukemia and central nervous system cancers in children. However, a statistically significant association was found only with leukemia and for females only.

One major difficulty in cluster investigations, particularly in the area of breast cancer, is the long latent period. There have been a number of publications providing a discouraging picture regarding cancer cluster investigations^[5,7-10]. The possibility of learning from a cluster investigation, however, is greatly increased if the cancer involved is relatively rare and if it has a short latent period. Inflammatory breast cancer (IBC) fits these criteria and is worth pursuing because of the strong evidence that environmental factors play a major role^[11-13]. The evaluation of each cancer cluster is a separate experiment in which the outcome, while usually not identifying a specific etiologic factor, is a learning experience which can assist in preparing a more definitive evaluation when another cluster is identified. In this report we describe our experience with several clusters and the lessons learned which are now being utilized to improve investigation of future IBC clusters.

FOLLOW UP OF THE NILES, ILL. LEUKEMIA CLUSTER

The first cancer cluster we investigated was in 1967 when we were asked to obtain biological samples from the only survivor (JB) of a well described acute leukemia cluster in Niles, Ill.^[14]. This cluster was the first of a series investigated by the Centers for Disease Control and involved acute leukemia occurring among children primarily in one Catholic school 1957-1963. These early studies were primarily descriptive although some biospecimens, primarily blood samples, were provided to the Viral Leukemia Section of the National Cancer Institute (NCI)^[17]. The serologic tests available at that time were less specific than those in use today. In 1967 we learned that a survivor of the Niles, Ill. cluster, now 13 years old, was having

a tonsillectomy and the virologists in the National Cancer Institute who were involved in the original cluster investigation hoped to obtain biospecimens from the tonsillectomy to isolate a leukemia virus. One of us (PHL) traveled to Chicago and obtained blood, bone marrow and tonsillar tissue placed in fresh media and brought them back to NCI for the laboratory studies. While no human leukemia virus was isolated from this patient, this and similar projects involving collection of biospecimens for NCI's Virus Cancer Program (VCP) proved to be valuable for years to come, providing reagents for research investigators, such as Robert Gallo, who credited the VCP with accelerating his discovery of human lymphotropic virus type I (HTLV-I) by providing appropriate biospecimens (Gallo, personal communication).

A CLUSTER OF CHRONIC FATIGUE SYNDROME ASSOCIATED WITH NON-HODGKIN'S LYMPHOMA IN NORTHERN NEVADA

A second cancer-related cluster that provided useful experience, although not IBC, was an outbreak of chronic fatigue syndrome (CFS) in 1984-6 in Incline Village, NV^[15] which was focused on a single internal medicine practice since the two primary physicians were recognized as the regional experts on this controversial disorder. These physicians suspected an excess of cancer in their CFS patients, particularly non-Hodgkin's lymphoma and brain cancer, and asked for our epidemiologic assistance^[16]. To investigate this possibility, we worked with the Nevada Cancer Registry and compared the cancer patterns in Washoe County in northern Nevada (which contains Reno) where the cluster was focused, to the patterns in Clark County in southern Nevada (which contains Las Vegas) which was selected as a control county. NHL and brain cancer were the targeted malignancies, and the control cancers chosen were breast and lung cancer. An abrupt relative increase in NHL was noted in 1986 in Washoe County compared to Clark County and a more sustained increase in brain cancer followed^[17,18].

AN IBC CLUSTER IN CASTRO VALLEY, CALIFORNIA

The first IBC cluster that we evaluated^[19] was in 2000, when we were asked to investigate an apparent cluster of IBC in Castro Valley, California where three women in an office setting of 24 people were diagnosed with IBC in a ten month period from May 1999 to March 2000.

The office had major environmental concerns. It was located one floor above a mammography and MRI imaging center and employees were concerned about the bad air and water quality in the office as well as the potential radiation exposure from the floor below. File boxes were stacked in front of windows that did not open in an area

where the ventilation system did not operate adequately when in use and was turned off at night. In addition, the office stored billing records printed on carbonless copy paper at a temperature above 68 degree Fahrenheit in the same area where the employees worked.

Although the complaints about the air and water quality and possible exposure to radiation began in 1986, the air quality studies and a water sampling study did not begin until 1988 and the radiation leakage studies began in 1999 when new mammography equipment had replaced the old units. The water sampling studies indicated that the only organic compounds detected were present at allowable California maximum contaminant levels. The radiation leakage studies found no radiation despite the absence of concrete between the two floors. The indoor air quality studies showed that the office had poor air circulation and higher levels of CO and CO₂. All three cases had their offices located on the side of the office that had the worst air circulation according to the studies.

All employees were interviewed by telephone using a structured questionnaire that obtained demographic information and information on IBC and breast cancer risk factors, such as pregnancy history, family history of breast cancer, oral contraceptive and hormone replacement therapy use, medical history, and exposure to possible oncogenic agents and tumor promoters.

The three cases did not live in the same residential area and therefore did not share the same residential environmental exposures. While the sample was too small to do statistical analyses, there were some interesting differences between the cases and the controls. The cases had a higher BMI, higher oral contraceptive and hormone replacement therapy use, and were exposed to pesticides and herbicides for a longer period of time. The one case that had children had her first child at age 19 and did not breast feed her children. Two of the cases had a family history of breast cancer, and the third case's mother was adopted.

All three cases were seen by the same oncologist and all cases had pathologically confirmed IBC with invasion of the dermal lymphatics. Although IBC is a clinical diagnosis and pathologic confirmation is not necessary, the surgeon believed documentation was important and obtained skin biopsies. It required 15 skin biopsies before a positive skin biopsy was obtained for one of the cases. We obtained tissue samples for all three cases.

Our attention was focused on the environmental exposures because of the concerns of the office workers and at that time infectious agents were not considered.

AN IBC CLUSTER IN FRIENDSWOOD, TEXAS

In 2011, a possible IBC cluster in Harris and Galveston counties in Texas was brought to our attention by a patient advocate/IBC survivor as part of our recruitment of cases for the IBC Registry and a biospecimen repository, which we established in 2002^[20]. The registry links

epidemiologic information with laboratory studies from our biospecimens. We were referred to an IBC Google map (www.terrismap.org) that reports IBC cases in the United States and is constantly updated. The IBC map showed approximately fifteen cases diagnosed between 1998 and 2011, nine in Harris County and six in Galveston County. All women in this apparent cluster were diagnosed at major cancer centers in the Houston area, including the MD Anderson Medical Center.

We were encouraged by the patient advocate/IBC survivor to enroll these patients in our Registry which only enrolls patients who contact us first and complete Informed Consent forms as per our Institutional Review Board (IRB) approval. Based on information from concerned patients, one focus of research was a hazardous waste site designated by the Environmental Protection Agency (EPA) which allegedly had waste draining into a stream going through the community. In order to investigate possible environmental triggers for this IBC cluster, we tried to identify the potential hazardous waste sites in the region utilizing the National Priorities List (NPL) developed by the Environmental Protection Agency (EPA)^[21]. Harris County with its largest concentration of chemical manufacturing and refining facilities has 27 EPA regulated Superfund Sites out of 31 for both counties. Key concerns regarding the quality of outdoor air include elevated levels of ozone and particulate matter. Two seafood advisories have been issued in the past for the Galveston bay system, tributaries of which run through parts of both the counties. The first was based on dioxin contamination. The second advisory was based on the three toxic compounds, namely dichloromethane, trichloroethane (carcinogens) and carbon disulfide discovered in fish from Clear Creek, a principal tributary of the Galveston bay. The contaminated fish were found in the vicinity of an EPA Superfund site. A study of the map showed two cases in the Friendswood city where the Clear Creek flows and which also includes a former refining site. Four cases were also concentrated near Clear Lake in Galveston County.

As this cluster was being investigated, the possibility of an infectious trigger was raised by a report suggesting infectious agents as possible etiologic factors in a series of IBC patients in Pennsylvania^[22] and our observation of a seasonal incidence of IBC in the northeastern United States^[13]. We therefore investigated infectious agents prevalent in Harris County where this IBC cluster appeared, and noted that Saint Louis encephalitis (SLE) is endemic and occasionally epidemic in that particular region. In 2002, West Nile virus (WNV) appeared in Houston and quickly spread throughout that region. Both SLE and WNV are transmitted by a particular species of mosquitoes, called *Culex quinquefasciatus* which is the prevalent species in Harris County. It is also a dominant vector of lymphatic filariasis, a disease caused by thread like parasitic worms. This raises the possibility of a parasite or a virus transmitted by mosquitoes. *Culex quinquefasciatus* larvae breed and thrive abundantly in stagnant dirty

water. The elaborate drainage system of Harris County creates ideal biotic and abiotic conditions conducive for mosquito larval development, particularly during relatively dry periods when stagnant water remains in the storm sewer system. Approximately 99% of the mosquitoes that develop in these storm sewers are *Culex quinquefasciatus*. These mosquitoes use the storm drains as daytime resting sites as well as for larval development. Outbreaks of *Vibrio* diseases have also been reported. Out of 176 *Vibrio* infections reported statewide between May of 1981 and September of 1991, 68 were reported in counties surrounding Galveston Bay which also includes Harris County. Contact recreation and consumption of molluscan shellfish, primarily oysters, were roughly equal sources of *Vibrio* infections, which accounted for 75% of the reported cases. The consumption of oysters, especially raw, can pose a significant health risk since oysters can concentrate pathogens in their gut as they feed. Of particular concern is *Vibrio Vulnificus*, which can cause severe infection with exposure of open wounds to sea water, especially in those with underlying chronic diseases or who are immunocompromised. This particular bacterium showed a greater genetic diversity in Galveston Bay water and oysters. An interesting feature of this organism is its response to low temperature stress where it goes into a viable but non-culturable state during winter months (December to February). These observations are intended to show the feasibility of infectious agents as a trigger to the Texas cluster but no evidence for such a link currently exists.

An attempt has been made to confirm the reported IBC cases and to get updated information from the Texas Cancer Registry, comparing the incidence of IBC in the Houston area with other metropolitan areas, such as Dallas and Austin. These studies are in progress.

DISCUSSION

Cancer clusters are notoriously difficult to evaluate, and we have tried to take advantage of our experience and those of others to develop a hypothesis consistent with the information currently available on IBC. One hypothesis that we believe is tenable is based on our studies of CFS and NHL where a cluster of CFS showed an ecologic association to NHL^[16,17,23] and the link was subsequently documented by a linkage of population-based registries of CFS patients in Medicare and cancer patients in SEER^[24], where NHL was the only malignancy with a statistically significant association with CFS. The virus most closely linked to CFS is EBV, which is consistently reactivated in those patients with acute onset CFS presenting with an infectious illness^[25], and, not infrequently, is a long term sequel of infectious mononucleosis which is the most common clinical manifestation of primary EBV infection. EBV is a ubiquitous virus, however, and more than 90% of the population has been infected early in life and is therefore immune to new infection. Therefore EBV is unlikely to trigger a cluster of CFS. Since CFS can be trig-

gered by a variety of infectious agents^[26], we hypothesize that a new agent struck the community and in certain susceptible individuals, perhaps those with a predisposing susceptibility to an autoimmune disorder, CFS developed. Clusters of CFS (initially described as epidemic neuromyasthenia)^[27,28] have been well documented^[29] and occasionally the precipitating agent has been identified, including EBV, herpes simplex-1, herpes zoster and giardia^[26,30]. We therefore postulate that for IBC, it is feasible that a precipitating agent, either chemical or infectious, could strike a community and those individuals with a predisposition (possibly a combination of factors such as obesity, family history, and/or infection with the proposed human tumor virus with sequences similar to mouse mammary tumor virus^[31-34], subsequently develop IBC. While we were successful utilizing the Nevada Cancer Registry to show the clustering of NHL after CFS, similar efforts investigating IBC is very problematic. NHL is a defined disease histologically and should be captured by a cancer registry in virtually 100% of cases. IBC, however, is a far more difficult problem for two reasons. First, the diagnosis has been evolving, and, although the situation has improved since we first documented the conflict between two major institutions, the American Joint Committee on Cancer and NCI's the Surveillance, Epidemiology, and End Results program (SEER), who used different case definitions^[20], the situation is far from satisfactory. Our clinical experience in Tunisia and with the IBC Registry indicates even the current criterion of clinical involvement of one third of the breast is too restrictive and the diagnosis should be made in any patient with acute onset of erythema, induration, and/or peau d'orange regardless of the size. This suggestion is supported by research efforts documenting the similar outcome and laboratory findings in IBC regardless of the extent of erythema^[35,36]. Second, there are many reasons why IBC may not be recorded in the cancer registry even though the physician has the diagnosis in his office records because it may not be noted by the abstracter reviewing the final hospital diagnoses. Attempts are being made to investigate the degree of "slippage" between the number of cases identified by practicing physicians and the number of cases recorded in the central registry^[37]. In addition to the concern that many IBC cases are not appropriately coded in cancer registries, there are constraints that hinder the collection of data on IBC in small geographic areas, even counties, since registries often will not release data where less than 50 patients are included because of concerns about confidentiality being maintained.

Although the extensive experience of the Centers for Disease control^[7] and others have often been disappointing, investigation of clusters of rare aggressive cancers such as IBC should not be completely neglected. For optimal pursuit of such clusters, several items should be considered to bring optimal results.

The importance of a coordinating physician(s)

Coordinating physicians were extremely important in the

success of cluster evaluations in Niles, Ill (the patient's internist), Incline Village, NV (the two internists in the practice) and Castro Valley, California (the three patients' medical oncologist). These physicians facilitated the signing of the informed consent forms, documentation of diagnosis, collection of good historical data, and collection of biospecimens. In contrast, thus far the absence of a coordinating physician in Texas has made interviews of the patients and collection of biospecimens in the Texas cluster extremely difficult.

Preservation of biospecimens

Obtaining biospecimens is critical. Techniques for utilizing a variety of biospecimens, including formalin fixed paraffin embedded tissues, in molecular studies continue to improve. We have been able to use the biospecimens in our repository for a number of projects^[36,38-40] and several hypotheses can be proven with these samples, such as the detection of organochlorines should chemical toxicity be responsible for the Friendswood clusters. In future studies, blood samples on cases and controls should be obtained to investigate the possibility of infectious agents as triggers of clusters. Other hypotheses might require other biospecimens, such as saliva or urine.

The importance of moving quickly

Early investigation is critical because of the frequently poor outcome in IBC. For example, our prompt investigation of the Castro Valley cluster allowed us to obtain a critical positive family history not recognized by clinicians from a patient who died shortly after our interview. Ideally one should obtain the biospecimen before treatment as neoadjuvant chemotherapy may affect the tumor biology.

Identification of appropriate laboratory support

The thorough evaluation of biological specimens requires experienced laboratory support that is primarily found in large research institutions. As noted below, it will be useful to have consistent protocol for the evaluation of IBC clusters to allow an exchange of relevant information among various investigators. We are currently attempting to develop a protocol focusing on infectious agents and environmental toxins with potential carcinogenic properties but we expect this protocol to change as laboratory techniques improve and additional information is obtained from other cluster investigations. Our latest studies are taking advantage of available molecular techniques allowing identification of bacterial pathogens in paraffin blocks of tumors but we expect eventually to explore viral pathogens and collect blood samples for antibody studies. The identification of potential toxins will be difficult because they are rapidly metabolized and/or excreted but we hope to identify some persistent chemical byproducts, such as organochlorines, which may be useful.

The issue of statistical significance

Statistical analysis of cancer clusters has been addressed

in several publications^[41,42] and various analytic methods have been used, but it is important to recognize that biological significance can be independent from statistical significance. Statistical significance is particularly problematic because of the small number of IBC cases compounded by changes in the case definition and Registry coding. Occasional misdiagnosis or unusual presentations have been encountered^[43-47], but these are uncommon and are unlikely to deter epidemiologists from focusing laboratory studies on the typical cases. We suggest that the identification of a cluster of IBC cases in a short period of time, such as the three women in a small office within ten months, deserves investigation regardless of whether or not it is statistically significant.

CONCLUSION

In summary, the apparent importance of environmental factors on the etiology of IBC and the identification of clusters of this relatively rare aggressive malignancy indicate the need to investigate these clusters for possible etiologic triggers. A variety of biospecimens should be collected for immediate testing but also for storage as future hypotheses and technology may provide clues not initially apparent. Tissue samples could be examined for toxic chemicals or infectious agents, blood samples could be used to identify adducts signifying exposure to carcinogens or antibodies to infectious agents, and DNA (saliva or white blood cells) could be used to identify genetic markers of susceptibility. Detailed histories could not only reveal potential important exposures but systemically applied questionnaires such as those used by the IBC Registry could identify predisposing factors that could add to our understanding of risk factors for IBC.

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Pleomorphic lobular carcinoma *in situ* of the breast: Can the evidence guide practice?

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Abstract

The clinical significance of pleomorphic lobular carcinoma *in situ* (PLCIS) is a subject of controversy. As a consequence, there is a risk of providing inconsistent management to patients presenting with PLCIS. This review aims to establish whether the current guidelines for the management of PLCIS are consistent with current evidence. A systematic electronic search was performed to identify all English language articles regarding PLCIS management. The data was analysed, specifically looking at: incidence of concurrent disease, recurrence rates, long-term prognosis and PLCIS management. A search was also performed for PLCIS management guidelines for the United Kingdom, United States, Canada, Australia, Germany and pan-European. The results of the evidence analyses were compared to the guidelines in order to establish whether the recommended management is consistent with the published evidence. Nine studies (level 3-4 evidence), involving a total of 176 patients and five management guidelines

(from United Kingdom, United States, Australia and pan-European) were included in the review. From the evidence, 46 of 93 (49%) patients were found to have PLCIS with concurrent invasive disease on excision specimen analysis. Regarding recurrence rates, 11 of 117 (9.4%) patients developed a recurrence of PLCIS. There were no instances of invasive disease or ductal carcinoma *in situ* (DCIS) on recurrence histology. There were no studies assessing long-term outcomes in PLCIS cases. With regards to the management guidelines, the Association of Breast Surgery (United Kingdom) and the National Breast and Ovarian Cancer Care (Australia) do not mention PLCIS. The National Comprehensive Cancer Network (United States) suggest considering excision of PLCIS with negative margins. The NHS Breast Screening Programme (United Kingdom) and the European Society of Medical Oncology (pan-European) recommend PLCIS should be treated as with DCIS. We conclude that high quality evidence to inform guidance is lacking, thus recommendations are relatively vague. However, based on the available evidence, it would seem prudent to treat PLCIS in a similar manner to DCIS.

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Key words: Pleomorphic lobular carcinoma *in situ*; Breast cancer; Breast carcinoma; Carcinoma *in situ*; Guideline; Excision margin; Recurrence rate

Core tip: Pleomorphic lobular carcinoma *in situ* (PLCIS) is a breast lesion, the clinical significance of which is a subject of controversy. To date, this systematic review is the largest pooled series of clinical data regarding PLCIS. We aimed to establish whether current guidelines for management are consistent with the evidence. The results demonstrate a lack of high quality data and guidelines for management are variable. Analysis revealed a high incidence of concurrent invasive disease with PLCIS (49%) and following excision, a recurrence rate of 9.4%. We conclude that it would seem prudent

to manage PLCIS as with ductal carcinoma *in situ*, although there is a dire need for long-term outcome studies.

Pieri A, Harvey J, Bundred N. Pleomorphic lobular carcinoma *in situ* of the breast: Can the evidence guide practice? *World J Clin Oncol* 2014; 5(3): 546-553 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i3/546.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i3.546>

INTRODUCTION

The pleomorphic subtype of lobular carcinoma *in situ* (PLCIS) was first described by Frost *et al*^[1] in 1996. Frost described the lesion as being composed of one or multiple distended lobules with enlarged and dyscohesive cells, irregularly shaped nuclei and abundant eosinophilic cytoplasm^[1]. PLCIS is also regularly associated with comedo necrosis and calcification and hence is often mammographically detectable, in contrast to classical LCIS (CLCIS)^[1-5]. The clinical significance of PLCIS is a subject of controversy. It is generally accepted to be a risk factor for invasive disease, as with CLCIS. However, there is a suspicion that PLCIS may carry a higher risk of progression to invasive disease due to its more aggressive molecular and histopathological features, which are more consistent with those of ductal carcinoma *in situ* (DCIS) than CLCIS^[1,2]. There is an acceptance that PLCIS should be considered a non-obligate precursor to invasive disease and managed in a similar way to DCIS, though this is largely based on the histopathological similarities rather than studies providing evidence on clinical outcomes^[1,6].

It has historically been difficult to differentiate PLCIS from DCIS due to the similarities in their histomorphology. Due to recent advances in immunohistochemical staining differentiating PLCIS from DCIS has become much more achievable. Use of E-cadherin immunohistochemistry can differentiate between DCIS and LCIS^[7,8]; E-cadherin is a cell adhesion molecule, expression of which is lost in lobular neoplasia but retained in ductal. As a consequence, there has been an increase in the number of PLCIS cases reported^[9].

There are multiple reviews relating to PLCIS in the literature, but they are predominantly narrative in nature^[6,9-14]. Hussain *et al.* published the only systematic review to date that includes clinical data regarding PLCIS^[12]. However, this review included only 22 PLCIS cases. Compared to CLCIS and atypical lobular hyperplasia (ALH), they demonstrated that PLCIS is the most likely to have concurrent malignancy on excision specimen (41% of PLCIS being associated with malignancy compared with 19% in ALH ($P = 0.003$). Since the Hussain *et al*^[12] systematic review there has been a large increase in the published literature for PLCIS. Guidance on the management of PLCIS may be historical, based on a small number of reported cases. The aim of this systematic re-

view is to compare the current evidence base for PLCIS with international guidance on its management.

SEARCH STRATEGY: EVIDENCE

This systematic review was conducted in line with the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) Guidelines. Electronic literature search of Medline, Embase, the Cochrane database, and the WHO International Clinical Trials Registry Platform were performed by a single author (AP). Search terms were “pleomorphic lobular carcinoma *in situ*”, “pleomorphic lobular carcinoma *in-situ*” and “PLCIS”. Articles were then selected based on title and abstract and then on the full text manuscript. A manual search of the references from key articles was also performed to identify any articles potentially missed by the systematic search.

The search was limited to human studies published in English, from 1996 onwards. The search was conducted in October 2013. We excluded editorials, case reports, reviews and letters or comments, and case series with less than five patients. Small studies (less than five patients) were excluded to focus the review on studies that would more likely influence practice.

SEARCH STRATEGY: GUIDELINES

In order to obtain a global representation of management guidelines for PLCIS, searches were performed for the United States, Canada, United Kingdom, Germany, pan-European and Australia. Electronic literature search of Medline and Embase were performed by a single author (AP). Search terms were “guideline” AND “breast cancer” AND the respective country (“United States”, “Great Britain”, “Canada”, “Australia” and “Europe”). National oncology, pathology and surgical societies, colleges, associations and governing bodies were also manually searched for each country. Independent organisations’ websites were searched for guidelines for breast cancer management. For the United Kingdom, the National Institute for Health and Care Excellence^[15], the Association of Breast Surgery^[16] and the National Health Service Breast Screening Program^[17] sites were searched. For the United States, the American Society of Clinical Oncology^[18], the College of American Pathologists^[19], the National Comprehensive Cancer Network^[20], the American College of Surgeons^[21] and the Society of Surgical Oncology^[22] websites were searched. For Canada, the Royal College of Physicians and Surgeons of Canada^[23], the Breast Cancer Society of Canada^[24], the Canadian Cancer Society^[25] and the Cancer Care Ontario (CCO)^[26] websites were searched. For Australia, the Cancer Australia and National Breast and Ovarian Cancer Care^[27] site was searched. For Germany, the German Cancer Society^[28] website was searched. Pan-European guidelines were searched for on the European Society of Medical Oncology^[29] website. Google search engine was used and Google Translate was used for non-English websites.

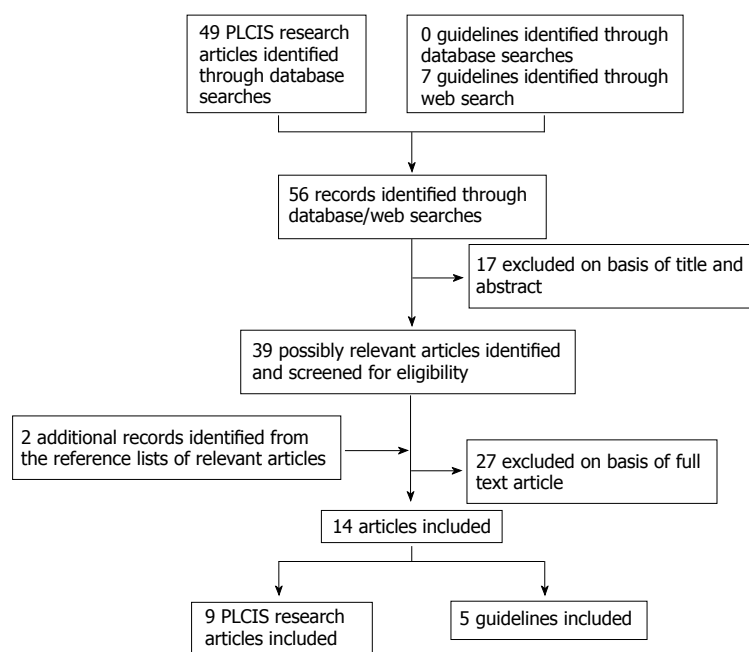


Figure 1 Study selection. PLCIS: Pleomorphic lobular carcinoma *in situ*.

Guidelines that did not have a version available in English were excluded. Guidelines that were specific to invasive disease (*i.e.*, did not give recommendations regarding *in situ* disease) were excluded.

DATA ANALYSIS

AP extracted data and JH and AP assessed its quality. Disagreements were resolved by discussion and study review. The following topics were specifically considered: incidence of concurrent invasive disease, recurrence rate of disease, long-term prognosis and the management of PLCIS alone.

DESCRIPTION OF STUDIES AND GUIDELINES

There were nine research articles and five guidelines that met the inclusion criteria (Figure 1). The nine studies involve a total of 176 patients. There were no meta-analyses, randomised control trials or cohort studies. All studies were case series, with data collected retrospectively. The dates of publication ranged from 2000 to 2013. Seven of the studies (93 patients) aimed to determine the risk of concurrent invasive carcinoma or DCIS on excision specimen, following a core needle biopsy diagnosis of PLCIS. Three studies (117 patients) documented recurrence rates in PLCIS. There are no studies published to date that investigate comparative treatment options, or oncological outcomes such as local and systemic recurrence disease in PLCIS patients.

Guidelines for “*in-situ*” breast disease management were found for the United Kingdom (Association of Breast Surgery and NHS Breast Screening Programme), United States (National Comprehensive Cancer Network), Canada (Cancer Care Ontario), Australia (National

Breast and Ovarian Cancer Care), Germany (German Cancer Society) and pan-European (European Society of Medical Oncology). An English version of the German guidelines could not be found and thus was excluded. The Canadian guidelines were pertaining to invasive disease only and thus were excluded.

INCIDENCE OF CONCURRENT INVASIVE DISEASE

The risk of concurrent invasive disease was based on the pooled data from seven studies, involving 93 patients^[3,4,30-34]. All patients were women, with a mean age of 58.4 years at PLCIS diagnosis (age range 35-84 years). Of those with a documented mode of presentation, 40 (91%) patients presented after a screen-detected abnormality and 4 (9%) had a symptomatic lump. Initial core biopsy results showed PLCIS as the most significant lesion in 58 (62%) cases and PLCIS with DCIS or invasive carcinoma in 35 (38%) cases. The breakdown of number of patients diagnosed with concurrent PLCIS with DCIS, and PLCIS and invasive carcinoma on core biopsy are not specified. After core biopsy, all patients went on to have surgery: 2 had a diagnostic biopsy, 67 had a wide local excision, 8 had a segmental mastectomy, 15 had a mastectomy and 1 patient had a bilateral mastectomy (reason not stated). On surgical specimen histology, 34 (37%) patients had PLCIS alone and 59 (63%) patients were found to have concurrent DCIS or invasive carcinoma (Table 1). Of these patients with DCIS or invasive disease; 13 (22%) were DCIS, 7 (12%) were invasive ductal carcinoma, 26 (44%) were invasive lobular carcinoma and 13 (22%) were invasive carcinoma with type not specified. The risk of concurrent invasive disease was 49% (46 of 93 patients). Looking specifically at the risk of upstaging from a core biopsy result of PLCIS only,

Table 1 Risk that ductal carcinoma *in situ* or invasive carcinoma is found on subsequent surgical excision pathology following a core biopsy diagnosis of pleomorphic lobular carcinoma *in situ*

Ref.	No. of PLCIS cases	Diagnosis on core biopsy	Surgical procedure	PLCIS alone on surgical specimen	Concurrent DCIS or invasive carcinoma on surgical specimen	Concurrent DCIS (%)	Concurrent invasive cancer (%)
Carder <i>et al</i> ^[4]	10	10 PLCIS	2 DB, 8 WLE	7	3 ILC	0	30
Chivukula <i>et al</i> ^[3]	12	12 PLCIS	1 DB, 1 WLE, 8 SMx, 1Mx, 1BMx	9	3 ILC	0	25
Fasola <i>et al</i> ^[30]	34	13 PLCIS 21 PLCIS + DCIS or IC	PLCIS: 11 WLE, 2 Mx PLCIS with DCIS or IC: 9 WLE, 12 Mx	4	9 DCIS 15 ILC 6 IDC	26	62
Morris <i>et al</i> ^[31]	17	3 PLCIS 7 PLCIS + DCIS 7 PLCIS + IC	17 WLE	3	3 DCIS 11 IC	18	65
Niell <i>et al</i> ^[32]	5	5 PLCIS	5 WLE	1	1 DCIS 2 ILC 1 IDC	20	60
Georgian-Smith <i>et al</i> ^[33]	5	5 PLCIS	5 WLE	3	2 IC	0	40
Lavoue <i>et al</i> ^[34]	10	10 PLCIS	10 WLE	7	3 ILC	0	30
Total	93	58 PLCIS 7 PLCIS + DCIS 7 PLCIS + IC 21 PLCIS + DCIS or IC		34	13 DCIS 26 ILC 7 IDC 13 IC	14	49

At initial assessment PLCIS is diagnosed on a core biopsy specimen, this table details the subsequent histology from a surgical resection of the area, demonstrating how many of the core biopsies are upgraded to invasive carcinoma or DCIS. PLCIS: Pleomorphic lobular carcinoma *in situ*; DCIS: Ductal carcinoma *in situ*; ILC: Invasive lobular carcinoma; IDC: Invasive ductal carcinoma; IC: Invasive carcinoma (with type not specified); DB: Diagnostic biopsy; WLE: Wide local excision; Mx: Mastectomy; SMx: Segmental mastectomy; BMx: Bilateral mastectomy.

to invasive disease on surgical specimen, data from five of the studies were used (in two studies, the correlation between core biopsy specimen and respective surgical specimen is unclear^[30,31]). Of 42 patients with PLCIS only on core biopsy, 14 were upstaged to invasive carcinoma on surgical specimen histology (33%).

RECURRENCE RATE OF DISEASE

There are three articles reporting recurrence rates following excision of PLCIS^[5,30,35]. Downs-Kelly *et al*^[5] report a retrospective series of 26 patients with PLCIS on surgical excision. They included both patients with PLCIS alone (20 cases) and patients with PLCIS and concurrent invasive disease where excision margin for the invasive component was more than 10 mm (6 cases). Patients were offered adjuvant chemoprevention and/or radiotherapy. The article does not state which chemoprevention agents were used. Of the six PLCIS cases with concurrent invasive disease, one had adjuvant radiotherapy and five had both chemoprevention and radiotherapy. Of the 20 cases of PLCIS alone, three patients received radiotherapy, six had chemoprevention and one received both chemoprevention and radiotherapy. The authors state that three of the cases of PLCIS alone had been misdiagnosed as DCIS at the time of treatment and thus received adjuvant therapy. The rationale for the remaining seven cases of PLCIS alone whom received adjuvant treatment is unclear. Downs-Kelly *et al*^[5] report one episode of recurrent PLCIS at 19 mo (3.8% recurrence rate). The recurrence was biopsy-diagnosed following a mammogram with new suspicious calcification at the surgical site (patient had

previous normal mammograms at 7 and 12 mo). Of note at initial excision, PLCIS was present at the margin. The patient had received adjuvant chemotherapy after the initial excision but no radiotherapy (Table 2).

Khoury *et al*^[35] report a series of 57 PLCIS cases and compare their recurrence rate to that of 615 cases of DCIS, who had presented over the same 12-year period. Their data shows 7 episodes of recurrent PLCIS with no invasive disease (12.3% recurrence rate). This series reports that the recurrence rate was higher in younger women and in cases where the margin remained positive for CLCIS ($P = 0.02$ and 0.01 respectively). PLCIS had a higher rate of recurrence than low and intermediate grade DCIS cases observed in the study ($P = 0.06$ and 0.04 respectively). It is not stated as to whether margin clearance was the same for DCIS and PLCIS cases. The margin status for PLCIS recurrences and the time to recurrences are not reported. Fasola *et al*^[30] consider the role of radiotherapy in PLCIS, comparing 13 patients with a diagnosis of PLCIS alone and 21 patients with PLCIS in the setting of invasive carcinoma. Patients with PLCIS alone were more frequently treated with lumpectomy as opposed to mastectomy (85% *vs* 43%, $P = 0.03$) and none of the PLCIS only patients received adjuvant radiotherapy or chemotherapy compared to 16 (76%) of the patients with PLCIS and invasive disease receiving radiotherapy and/or chemotherapy. They report that local recurrence rate in the PLCIS only group is 15% compared with 5% in the PLCIS and carcinoma group ($P = 0.8$). The comparative adequacy of margin clearance is not stated for the two groups. However, they conclude that this increased rate of recurrence may be a result of the

Table 2 Rate of recurrent disease after surgical excision of pleomorphic lobular carcinoma *in situ*

Ref.	No. of PLCIS cases	PLCIS at margin	Histology of recurrence	Time to recurrence	Local recurrence rate
Downs-Kelly <i>et al</i> ^[5]	26	13 cases ≤ 1 mm 4 cases 1.1-2 mm 9 cases > 2 mm	1 PLCIS	19 mo	3.8%
Khoury <i>et al</i> ^[35]	57	Not stated	7 PLCIS	Not stated	12.3%
Fasola <i>et al</i> ^[30]	34	Not stated	3 PLCIS	≤ 5 yr	8.85

This details the number of cases of histologically diagnosed recurrence following a previous excision of PLCIS. PLCIS: Pleomorphic lobular carcinoma *in situ*.

less aggressive adjuvant therapy and there may be a role for radiotherapy in PLCIS. They report an overall 5-year recurrence rate of 8.8% (3 of 34 patients) for PLCIS.

Combining the data from the three studies gives a PLCIS recurrence rate of 9.4% (11 of 117 cases) regardless of margin (not reported in all cases). There were no cases reported where invasive disease was found on recurrence specimens in any of the three studies.

REVIEW OF NATIONAL GUIDELINES FOR MANAGEMENT

A total of 5 national guidelines (United Kingdom, United States, Australian and pan-European) were found that met the inclusion criteria and are summarised in Table 3. Associated guidelines for management of CLCIS and DCIS by the same organisations are included in the Table for comparison.

There is no mention of PLCIS in the Association of Breast Surgery and National Breast and Ovarian Cancer Care guidance. The National Comprehensive Cancer Network suggest considering resection of PLCIS with negative margins. The European Society of Medical Oncology recommend that PLCIS should be managed as with DCIS and similarly, the NHS Breast Screening Programme recommend grading PLCIS on core biopsy as B5a, *i.e.*, non-invasive carcinoma (in contrast to CLCIS which is graded as B3 indeterminate) and excising with negative margins.

EVIDENCE QUALITY

There are significant limitations to this review, currently there is a lack of quality evidence supporting the management of PLCIS, the majority being level 3-4 evidence^[36]. There are no management comparison studies measuring long-term survival and very limited data on recurrence rates. Nonetheless, there are significant findings within the data available. This is the only systematic review to focus specifically on PLCIS management and with 173 cases, this is the largest pooled series of clinical data regarding PLCIS.

ASSOCIATION OF PLCIS WITH INVASIVE DISEASE

PLCIS is associated with invasive lobular carcinoma on

excision in at least 28% of cases (26 of the 93 cases, but in 13 more cases of associated invasive carcinoma, the type is not reported). When DCIS is diagnosed on core biopsy there is a 10%-20% risk of associated invasive carcinoma on subsequent excision^[37-40]. By comparison with DCIS, this study demonstrates PLCIS to have a higher association with its invasive counterpart. Reis-Filho *et al*^[41] identified that invasive pleomorphic carcinoma and its *in situ* counterpart share the same genetic changes, supporting the argument that PLCIS is a precursor lesion (rather than simply a risk factor) of invasive disease. The high rate of upstaging of PLCIS to invasive disease seen in this review further supports the “non-obligate precursor” hypothesis. These findings suggest that it would not only be advisable to perform a surgical biopsy for PLCIS to look for associated malignancy, but to ensure excision with margins negative for PLCIS. Thus, a grading of pre-invasive (B5a) similar to DCIS would seem appropriate.

SENTINEL NODE BIOPSY AND PLCIS

For pure DCIS on core biopsy, the Association of Breast Surgery recommend that sentinel lymph node biopsy should be considered in cases where there is a high risk of finding invasive disease on the subsequent excision specimen^[42]. They state that this would include patients undergoing surgery for: an extensive area of microcalcification; a palpable mass; or high grade disease. In this review, the overall rate of concurrent invasive disease associated with PLCIS was found to be 49%. In 67% of these cases, the invasive component was missed on core biopsy and thus wasn't diagnosed until analysis of the surgical specimen. Given the high risk of upgrading PLCIS to invasive disease after definitive surgery, combined with the fact that histologically, PLCIS is considered to have a similar appearance and characteristics to high grade DCIS, sentinel node biopsy should be considered in PLCIS.

ADJUVANT THERAPY FOR PLCIS

There are no studies that consider the role of adjuvant chemoprevention in PLCIS. Fasola *et al*^[30] assessed whether there is a potential role for radiation therapy in PLCIS by looking at the comparative recurrence rates^[30]. However, they demonstrated no significant difference between the radiation therapy and non-radiation therapy groups.

Table 3 Summary of guidelines for the management of *in situ* breast disease

Guideline source	Recommendation-PLCIS	Recommendation-CLCIS	Recommendation-DCIS
ABS, 2009 ^[16] (United Kingdom)	PLCIS not mentioned	Should consider diagnostic biopsy Clear margins not required Post-op surveillance is appropriate (No adjuvant treatment mentioned) (No lymph node surgery required)	Resection with clear margins (> 1 mm) required (WLE or Mx) Intra-op radiography should be used for all DCIS as majority impalpable Lymph node surgery not usually required but may be considered in high risk cases
NCCN, 2013 ^[20] (United States)	"Consider excision with negative margins"	Diagnostic biopsy Risk reducing treatment discussion with patient (options: risk reducing surgery, hormone therapy, no further treatment) Surveillance indicated	Consider MRI WLE or Mx Margin controversial but certainly > 1 mm SLNB usually not required but may be considered in high risk cases Consider RTx
ESMO, 2013 ^[29] (pan-European)	"May behave similarly to DCIS and should be treated accordingly"	Risk factor for future development of invasive cancer and does not require active treatment	Resection with clear margin (≥ 2 mm) required (WLE or Mx) SLNB usually not required but may be considered in high risk cases
NBOCC, 2003 ^[27] (Australia)	PLCIS not mentioned	Consider surgical biopsy Surveillance ≥ 15 yr No role for clear margin excision established	Clear margin excision Usually adjuvant RTx Consider hormone therapy
NHSBSP "In situ lobular neoplasia: overview and recommendations" [pending publication] ^[17] (United Kingdom)	Should be classified B5a (as with DCIS) and excised with negative margins	Merits MDT discussion and usually diagnostic biopsy	

ABS: Association of Breast Surgery; NCCN: National Comprehensive Cancer Network; ESMO: European Society of Medical Oncology; NBOCC: National Breast and Ovarian Cancer Care; NHSBSP: National Health Service Breast Screening Programme. PLCIS: Pleomorphic lobular carcinoma *in situ*; DCIS: Ductal carcinoma *in situ*.

ARGUMENT FOR MRI IN PLCIS CASES

Invasive lobular carcinoma is associated with over a quarter of the PLCIS cases, it is more frequently multifocal and bilateral compared with ductal carcinoma^[43,44]. Consequently, MRI is often performed pre-operatively in known lobular carcinomas in order to better assess the extent of disease and thus reduce re-excision rates. MRI may have a future role in imaging of patients with PLCIS due to the high rate of upstaging from PLCIS to invasive lobular carcinoma.

RECURRENCE RATES AFTER PLCIS EXCISION

Safety of PLCIS management ultimately can be determined by recurrence rates. No conclusions could be drawn from a small sample size of 173 cases, especially given the heterogeneous mix of margin management and adjuvant therapies. Information on recurrence is also not stated in all series. Using DCIS again as a comparator, Boyages *et al* reported that after excision, 43% of local recurrences were invasive, not *in situ*^[45]. In this review, there were no episodes of invasive disease on recurrence - only PLCIS.

PLCIS MANAGEMENT GUIDELINES

Looking at the guidelines for management, PLCIS is not mentioned in the Association of Breast Surgery or the

National Breast and Ovarian Cancer Care publications. These guidelines were last updated in 2009 and 2003 respectively and thus pre-date the majority of the evidence regarding PLCIS. Thus, it is no surprise that PLCIS does not feature. The National Comprehensive Cancer Network publication (2013) states that PLCIS may have a similar behaviour to DCIS, and proposes that negative margin excision should be considered. Qualification of this statement is made by explaining that the outcome data regarding the efficacy of surgical excision to negative margins is lacking. This guidance is certainly consistent with the data reviewed. The NHS Breast Screening Programme (pending publication) and the European Society of Medical Oncology (2013) make similar statements that PLCIS may behave similarly to DCIS and should be excised with negative margins. The European Society of Medical Oncology also states, with regards to *in situ* lobular neoplasia, "radiotherapy is not warranted, perhaps with the exception of the pleomorphic subtype". This statement is presumably made, based on the histomorphological similarities between DCIS and PLCIS, thus using DCIS-based data as surrogate evidence, but there is no data regarding the clinical efficacy of radiotherapy for PLCIS directly. The lack of guidance from many National and International organisations on the management of PLCIS reflects the lack of data on which to support treatment guidance. The few guidelines that do pertain to PLCIS generally recommend excision with no clear definition of margin width.

CURRENT VARIATIONS IN PRACTICE

Blair *et al.*^[46] recently published a survey completed by surgeons in the United States, regarding the management of positive margins in PLCIS cases. They report considerable heterogeneity in the management. Only 24% felt they would always wish to re-excite PLCIS at the margin. The survey did not address the reasons for the varied responses, but they postulate that it may be due to a lack of familiarity with this unusual variant of LCIS or an active decision to await better evidence to inform further intervention. Either way, such diversity in responses suggests a requirement for clearer evidence and guidance.

PLCIS has historically been a rarely diagnosed phenomenon. In the past, some cases will undoubtedly have been diagnosed as DCIS due to its histological similarities. However, due to the relatively recent universal use of E-cadherin immunostaining, combined with the ever-growing numbers in breast screening programs, PLCIS is likely to become a more frequent diagnosis. Its clinical characteristics remain largely unknown, but are not entirely consistent with either DCIS or CLCIS. Thus independent, clear guidelines for the management of PLCIS are required, although there remains a need for quality evidence on a national and international scale, to inform practice.

CONCLUSION

There is a lack of quality evidence to inform guidance on the management of PLCIS. The limited data demonstrates a high rate of concurrent invasive disease associated with PLCIS. Based on the available evidence, it would seem safe to surgically excise PLCIS in a similar manner to DCIS. There is no evidence on the efficacy of adjuvant treatments. As with high grade DCIS, a sentinel lymph node biopsy may be considered at the time of excision.

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Is exercise ignored in palliative cancer patients?

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Abstract

Exercise and rehabilitation approaches in palliative care programs for cancer patients affect patients' symptoms, physical functioning, muscle strength, emotional well-being, psychological symptoms, functional capacities, quality of life, mortality and morbidity positively. Based on scientific data, palliative cancer patients should be recommended to participate in exercise programs. There is no standard approach to recipe an exercise regimen for a palliative cancer survivor. Studies for demonstrating the positive effects of exercising in palliative care patients are increasing in number day by day. At this point, increasing awareness about exercising in the entire team monitoring the patient and our efforts in this matter seems to be very important.

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Key words: Palliative care; Cancer; Exercise; Rehabilitation; Muscle

Core tip: Although cancer patients are in great need of physiotherapy and rehabilitation, this is often ignored by health professionals. For this reason, the role of physiotherapy and rehabilitation during palliative care in cancer patients is limited in both clinical practice and literature. Exercise in palliative care should definitely be considered since it is easily administered and safe and is beneficial for the patient.

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INTRODUCTION

Cancer and intensive medical therapies administered due to cancer affect the physical, functional, emotional, psychological and social statuses of patients^[1]. There are many factors that cause disability in cancer patients. These factors include fatigue, treatment complications, malnutrition, neurological and musculoskeletal problems, pain, intestinal and bladder dysfunctions, thromboembolic disease, depression, and presence of comorbid diseases^[2-9]. The resulting physical disability leads to depression, decreased quality of life, and increased need for care and health expenditures^[2,10-12]. Right at this point, we, rehabilitation physicians assume great responsibility. The first study that documented the need for cancer rehabilitation was published by Lehman *et al*^[5]. In that study, 805 cancer patients were examined and 438 patients were found in need of rehabilitation; this need was reported to be as high as 70% in patients with breast, lung, and head-neck tumors. There was functional loss associated with physical weakness in 35% of the assessed patients, a need for an assistive device to be able to perform daily living activities in 32% of them, difficulty in ambulation in 23% and inadequacy in transfers in 7%^[5]. Despite the crucial need for physical activity and exercise in this patient population, patients' demand for physical treatment varied from 2% to 81%^[13]. It has been stressed that terminal cancer patients also have significant functional incapacity^[4,6]. Although emphasizing its importance date back to many years ago in the literature, utilization of these study results in clinical practice has not unfortunately reached the desired level.

Physical strength, time spent in bed during the day, ability to do something intended and being dependent on others are important factors for cancer patients and their

loved ones in terms of their quality of life. It is stressed in the literature that progressive disability and the feeling of being dependent on others are among the reasons that deplete the desire to live in cancer patients^[14,15]. In another study made in a nursing home with terminal-stage cancer patients, it was found that the desire to be physically active came first even during the last three days of life. In that study, 88% of the patients wished to have increased mobility and 50% of them complained about the problems in their daily living activities^[16].

Rehabilitation in cancer patients is very important in that it ensures maintenance of their functional capacity and particularly their mobility^[17,18]. Although cancer patients are in great need of physiotherapy and rehabilitation, this is often ignored by health professionals^[19,20]. For this reason, the role of physiotherapy and rehabilitation during palliative care in cancer patients is limited in both clinical practice and literature^[21]. Especially in terminal-stage cancer patients, we see assumptions that rehabilitation is useless with the underlying thought that death is approaching. On the contrary, patients we see in our clinical practice seem to have a desire for exercising even if they will be lost (death) in a day. Treatment approaches for protecting and restoring physical functioning particularly in patients with diminished life expectancy have started to appear in small numbers in the literature related to palliative care^[22]. Physical functions appear to be the major factor determining the length of life and quality of life^[22,23]. Although, the additional effect of exercise in palliative care has been investigated in numerous studies throughout the last decades, there have been no clear consensus or a standard approach yet.

EFFECT OF EXERCISE IN PALLIATIVE CARE

It has been found in recent studies that exercise has a positive effect on life expectancy in cancer patients during their palliative care^[22,24-26]. Prospective-observational studies have demonstrated that physical activity after cancer diagnosis is associated with a reduced risk of cancer recurrence and improved overall mortality among multiple cancer survivor groups, including breast, colorectal, prostate and ovarian cancer^[27]. In a multi-center, randomized and controlled study made by Courneya *et al*^[28], resistance and aerobic exercises were prescribed for 17 wk to 242 patients with breast cancer who had been started adjuvant chemotherapy. Although the exercises given did not affect the individuals' quality of life associated with cancer, they improved their physical conditions, body compositions and general well-being^[28]. There is increasing evidence that exercise therapy is beneficial during palliative care of cancer patients. Studies have reported that 30% of total cancer deaths are associated with lack of exercise and malnutrition and 250000 early deaths occur every year due to inactivity^[29,30].

Exercises have positive effects on energy use, insulin

resistance, inflammation, and on many tissues and organs. The cardiovascular capacities and quality of life of the cancer patients who exercise increase, their fatigue, sleeping problems, body structures and immune responses improve and therefore a general well-being occurs^[18,19,31-34]. Exercise has been shown to have positive effects not only on physical and functional condition but also on psychological symptoms in cancer patients^[35,36]. This positive effect on both physical and psychological symptoms may explain the increase in life expectancy in these patients.

QUALITY OF LIFE

Although quality of life decreases in patients with advanced cancer, this decrease is less in those who receive exercise therapy^[37]. There are only a few studies investigating the effect of rehabilitation in terminal-stage cancer patients. In one of those studies, 301 people who have been staying in a nursing home and who have exercised for 6 years were examined. They had an average of 27% improvement in their Barthel mobility indexes after their rehabilitation. Forty-nine patients gained their independence in their mobility and daily living activities^[11,12]. In the study of Porock *et al*^[38] where they investigated the effect of exercise on palliative care patients, fatigue, anxiety and quality of life improved in patients who received an exercise therapy for 28 d^[38]. In another study, fatigue and quality of life improved in patients who received an intensive exercise therapy for 6 wk^[22]. Montagnini *et al*^[39] also evidenced that exercising resulted in an improvement in the scores of the daily living activities scale in 56% of the palliative care patients within 2 wk^[39].

FATIGUE

Fatigue is a very frequently seen symptom in palliative care patients (80%-90%). Fatigue is argued as being associated with anxiety, depression, pain, dyspnea, insomnia, loss of appetite, nausea, and dizziness. It is an important symptom in that it affects daily living activities of people and lowers their quality of life. Exercising is known to have positive effects on fatigue^[40]. It is stressed that group exercises, energy protection techniques and regular physical activities are significant in reducing cancer-related fatigue^[41]. A 20-30 min daily progressive walking exercise program was administered 4-5 times a week to patients with breast cancer who were receiving radiotherapy and a decrease was seen in fatigue^[42]. A bicycle exercises program was administered for 30 min to 50% of the patients after bone marrow transplantation and fatigue and physiologic stress levels were found to be lower in the exercising group than in those who did not exercise^[43]. In a study where fatigue and its causes as well as the efficacy of rehabilitation were investigated, 32 cancer patients were included in a rehabilitation program consisting of aerobic and muscle strengthening exercises, sports activities and psychological support. After the rehabilitation activities which lasted fifteen weeks, improvement was

seen in physical parameters and a marked decrease in fatigue^[44]. There are not adequate data as to how exercise reduces fatigue or its role in decreasing cancer risk.

It should be borne in mind that aerobic exercises must be employed when fighting against fatigue in cancer patients. In many studies, light and moderate walking and bicycle exercises have been administered a few times a week to activate large muscle groups and to increase oxidative muscle fibers. In view of the data available to us, it can be said that a less intense exercise program should be administered to patients whose cancer treatment actively continues and more intense exercises can be prescribed to those whose cancer treatments have been completed.

MUSCLE MASS AND MUSCLE STRENGTH

Cancer cachexia is a multifactorial condition that cannot be fully reversed with conventional food supplements; it leads to progressive functional impairment and progresses with loss of muscle mass^[45]. There are very few treatment options for patients with advanced cancer. Loss of muscle mass and muscle strength in cancer cachexia results in unintentional weight loss. Due to the potential effect of exercising on muscle mass and muscle strength, exercise is recommended as a treatment method that can be chosen before and during cachexia in advanced cancer cases^[46-52]. In a meta-analysis that evaluated the effect of physical exercise on muscle mass and muscle strength during an active cancer treatment and that reviewed 16 studies, it was demonstrated that aerobic and resistance exercises administered during an active cancer treatment could prevent muscle mass loss^[53]. Therefore, exercise therapy in cancer patients is effective for patients in sustaining their daily living activities^[8,26,53].

Measuring muscle mass objectively requires experienced staff and expensive equipment. There is not a clear consensus about the dose and type of exercise that has to be performed for the recovery of muscle mass^[53]. For this reason, it is difficult to comment on the changes an exercise therapy makes in muscle mass. There is a need for further studies evaluating muscle strength in advanced cancer cases.

The importance of protecting and increasing muscle mass for daily living activities has been stressed. There are also studies on the positive effects of muscle strengthening exercises^[26,48,50-53].

TREATMENT PLAN AND COMPLIANCE

A rehabilitation program should be custom made. When planning the rehabilitation, disease location, its stage, previous and present treatments received by the patient, their previous functional condition, their life expectancy, comorbidities, pain, medications used, the patient's cognitive and emotional status, their nutrition, physical capacity and potential limitations should all be considered^[54]. The patient's neurological and musculoskeletal examinations

should be performed thoroughly and their motor deficits, range of motion (ROM), walking patterns and risk of falling should be inquired. Indications for the exercise treatment in this patient population include to regain or improve physical functions, aerobic capacity, strength, flexibility, body image, body composition, quality of life, ability to withstand physically and psychologically to any current and future cancer treatments and to withstand the anxiety associated with the current or recurrent disease^[55].

For example, while the main target is the restoration of arm and shoulder mobility in a patient who was administered a curative surgery due to breast cancer, mobility and daily living activities come first for a patient who had a hip metastasis associated with breast cancer and who developed a pathologic fracture. In rehabilitation, targets should be objective and realistic; education and psychosocial consultation facilities should be provided. The patient's family and loved ones should be active participants of the rehabilitation process.

Adherence to treatment in cancer patients and our efforts in this matter also become very important. Generally speaking, compliance of patients with the treatment is quite high. The reasons patients discontinue an exercise therapy include lack of interest in the therapy, medical complications, exercising time and intensity, program being too long, disease stage, and transportation problems. The intensity and length of the exercise program should be adjusted in a way not to tire or bore the patient especially when terminal-stage cancer patients are in question^[54].

When exercising with palliative care patients, the patient groups are kept small and there is no definite standard in treatment programs. This is why it is difficult to determine what type of exercise will be given to what patient group and how. There is no agreement about the type, frequency, intensity and length of exercises to be given to cancer patients, nor is there an agreement as to at what stage of the disease it should be given and how. The exercise types usually given are exercises performed by the patient in bed with the help of a caregiver, exercises performed by the patient alone in line with the directives given, exercises involving simple support devices and weights, strengthening and aerobic exercises such as walking and swimming. Patients often prefer walking and home exercising programs^[20].

A rehabilitation or exercise program which disregards the patient's general condition and in which expectations are kept high will make patient compliance difficult and will demoralize the patient. For example, it would certainly not be appropriate to give 2 tours of walking exercise in the corridor mornings and evenings to a patient who can only walk from the bed to the patient room door due to fatigue. To give only ROM and breathing exercises to a patient who can hardly move in bed will be more relaxing for both the patient and his/her family. Patient compliance is better when the patient is able to do and comply with the programs.

Workouts involving resistance exercises are usually

less common; the majority of workouts involve aerobic exercises^[31,56]. Resistance exercises increase muscle strength and overall body mass^[57]. Although there are data that resistance exercises are more effective in increasing muscle mass and muscle strength than aerobic exercises, there are no strong evidences yet^[28,53].

According to the data pooled from cancer studies, the American Cancer Society recommends cancer survivors to engage in regular physical activity avoiding inactivity, to exercise for at least 150 min per week including strength-training for at least 2 d per week and to keep a healthy weight^[27]. Joyful exercises like Pilates, Tai Chi, Yoga, Nordic walking and dance may be chosen according to the expectation and motivation of the patient by carefully adjusting the intensity to the abilities of the individual.

CONCLUSION

Exercise and rehabilitation approaches in palliative care programs for cancer patients affect patients' symptoms, functional capacities, quality of life and length of life positively. Studies for demonstrating the positive effects of exercising in palliative care patients are increasing in number day by day. Exercise in palliative care should definitely be considered since it is easily administered and safe and is beneficial for the patient. We, rehabilitation physicians, must certainly be a major part of this team, because patients can benefit from this facility only when they are directed to us. At this point, increasing awareness about exercising in the entire team monitoring the patient and our efforts in this matter seems to be very important.

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WJCO 5th Anniversary Special Issues (1): Lung cancer**Apoptosis block as a barrier to effective therapy in non small cell lung cancer**

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the treatment of non-small cell lung cancers with platinum-based combination chemotherapy. New biomarkers of prognosis as well as new therapies focusing on molecular targets are emerging helping to identify patients who are likely to benefit from therapy. These are as yet only available to the minority of patients. Drug resistance remains the major cause for treatment failure. Apoptosis block as a mechanism for drug resistance and potential routes to overcome this will be reviewed.

Paul I, Jones JM. Apoptosis block as a barrier to effective therapy in non small cell lung cancer. *World J Clin Oncol* 2014; 5(4): 588-594 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i4/588.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i4.588>

Abstract

Lung cancer, is the most common cause of cancer death in men and second only to breast cancer in women. Currently, the first line therapy of choice is platinum-based combination chemotherapy. A therapeutic plateau has been reached with the prognosis for patients with advanced non-small cell lung cancer (NSCLC) remaining poor. New biomarkers of prognosis as well as new therapies focusing on molecular targets are emerging helping to identify patients who are likely to benefit from therapy. Despite this, drug resistance remains the major cause for treatment failure. In this article we review the role of apoptosis in mediating drug resistance in NSCLC. Better understanding of this fundamental biological process may provide a rationale for overcoming the current therapeutic plateau.

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Key words: Apoptosis; Lung cancer; Adjuvant therapy; Mitochondria; BAX; BAK; BCL2

Core tip: A therapeutic plateau has been reached with

INTRODUCTION

Lung cancer is the leading cause of cancer death in the United Kingdom accounting for 24% of all male related cancer deaths and 20% in females^[1]. Similar trends are seen in the United States with lung cancer representing the most common cause of cancer death in men and women, recently overtaking breast cancer in the latter^[2]. In the United States, more than 213000 new cases were diagnosed in 2007 with a total of 1.61 million cases worldwide in 2008^[2,3]. The majority of these cancers (75%-80%) are non-small cell lung cancers (NSCLC)^[4].

Surgery is the mainstay of treatment for early stage and localised disease (Stage I and II and selected IIIA). Multimodal therapy is the norm for regionally advanced disease in the form of adjuvant chemotherapy. Palliative chemotherapy forms the mainstay of treatment in patients with advanced metastatic disease^[5]. Five year survival rates following lung resection for NSCLC are I A-73%, I B-54%, II A-48%, II B-38%, IIIA-25%^[6]. The majority of patients have advanced disease at the time of

diagnosis and therefore are not surgical candidates. This is illustrated by the fact that in the United Kingdom only 14% of patients diagnosed go on to have surgical resection^[7]. In those patients that are surgical candidates, more than 50% will develop a recurrence. Adjuvant chemotherapy has been used with limited success to decrease the recurrence rates but this has only yielded a survival benefit of 5%-15%^[8].

For patients not suitable for surgery due to advanced disease or those who have suffered recurrence following resection, chemotherapy forms the mainstay of treatment with evidence that platinum based therapies are most effective in the first line setting^[9,10]. Despite this, in a recent trial of platinum combination therapies in advanced NSCLC, only 30% of patients showed objective disease response and a significant proportion suffered toxic side-effects such as neutropenia (27%), anaemia (10%), thrombocytopenia (13%), alopecia (21%) and nausea (4%). During the trial, deaths due to study drug toxicity were in the region of 1%^[11]. This demonstrates the major problem of drug resistance in NSCLC to standard platinum based therapies and the associated toxicities.

MOLECULAR TARGETED THERAPY

A recent major advance in the management of NSCLC has been the identification of activating mutations in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR)^[12]. These mutations, found in 10% of NSCLC specimens in the United States and Europe, are associated with increased sensitivity to EGFR tyrosine kinase inhibitors erlotinib and gefitinib^[13]. EGFR mutations are often accompanied by gene amplification^[14]. Mutation of the EGFR can lead to signal transduction independent of ligand stimulation, relaying signals to the PI3K-AKT-mTOR pathway involved in cell survival, or to the RAS-RAF-MEK-ERK pathway involved in cell proliferation^[15]. The fact that kinase inhibition leads to apoptosis in cells with mutant EGFR supports the notion that these cells are "addicted" to signalling *via* the mutant proteins^[16]. This explains the dramatic response to EGFR tyrosine kinase inhibitors in EGFR mutated NSCLC with associated improvement in survival^[17,18].

Molecular profiling in NSCLC has revealed a number of other mutations such as EML4-ALK translocation^[19]. This is associated with extreme preclinical sensitivity to respective ALK kinase inhibition in both preclinical and clinical settings^[20,21]. Additional molecular subclasses with associated somatic gene alterations have been discovered, predominantly in lung adenocarcinomas and include mutations of BRAF^[22], Her2^[23] and PIK3CA^[24]. The continual discovery of new molecular subclasses represents significant progress in the treatment of NSCLC but they should not necessarily be viewed as a panacea as resistance to these novel targeted therapies are being reported^[25]. The above molecular subclasses still only account for less than half all new cases of the disease. The majority of patients still rely on standard combination

chemotherapy of platinum based doublet regimens.

In addition to molecular tumour markers, there is increasing interest in developing biomarkers associated with NSCLC. These may be predictive for response to treatment or prognostic. An area of interest in our unit relates to the link between plasma fibrinogen and NSCLC. In early stage disease amenable to surgical resection, raised fibrinogen is associated with increased risk of incomplete resection and correlates with T stage of tumour^[26]. More recently, serum fibrinogen has been shown to be an independent predictor for both disease recurrence and overall survival in both resectable and advanced disease^[27,28]. The mechanism by which fibrinogen acts as a marker is not fully understood. Its role as an extracellular matrix, in promoting angiogenesis and metastasis has been proposed^[29-32].

APOPTOSIS AND DRUG RESISTANCE

Cisplatin (*cis*-diamminedichloroplatinum II) is the most effective platinum based therapy in the first line setting for NSCLC^[33]. It forms DNA-platinum covalent adducts resulting in inhibition of DNA replication, suppression of RNA transcription and protein translation, attempted DNA repair as well as disturbance of the cell cycle and the activation of apoptosis^[34].

Apoptosis serves as a natural barrier to cancer development^[35]. Normally the accumulation of genetic mutations required to drive uncontrolled cell cycle progression and tumorigenesis would result in triggering of apoptosis within a cell^[36]. The ability of cancer cells to evade apoptosis is, therefore, an essential hallmark of cancer^[37]. Defects in apoptosis, harboured by cancer cells, not only underpin tumorigenesis but also drug resistance^[38]. Resistance of NSCLC cells to a diverse range of cytotoxic therapies suggests a defect in apoptosis signalling^[39]. Further understanding of the mechanisms by which apoptosis resistance occurs and how this can be overcome will be important in order to administer a more rational approach to anticancer drug design and therapy.

APOPTOSIS

Apoptosis, a genetically encoded programme of cell death, was originally defined based on the morphological features observed as the cell died; nuclear condensation, nuclear and cellular fragmentation, membrane blebbing and phagocytosis of the dying cell in the absence of inflammation^[40]. The process of apoptosis is conserved in a wide range of multicellular organisms from worms to humans and plays a key role in normal development and homeostasis. The apoptosis phenotype is produced through the activity of cysteine-aspartic proteases, a family of cysteine proteases termed caspases. A hierarchical cascade of activation occurs which results in apoptosis. Two groups of caspases have been classified; the initiator caspases and the effector caspases.

The initiator caspases, caspase-8, -9 and -10, are ac-

tivated early in apoptosis signalling and have restricted cleavage targets, limited to self cleavage, the effector caspases and BID (caspase-8). In contrast, the effector caspases, caspase-3, -6 and -7, have hundreds of cleavage sites broadly distributed throughout the cell^[41]. The effector caspases are held in the cytosol as inactive dimers. The activating event, catalyzed by the initiator caspases, involves conversion to catalytically active enzymes by cleavage in the linker region between the large and small active subunits. This allows intramolecular rearrangements to form an enzymatically active dimer^[42]. Caspase-3 and -7 display highly similar substrate specificity and carry out redundant but essential functions in apoptotic cell death as mouse embryonic fibroblasts lacking both enzymes are resistant to intrinsic and extrinsic apoptotic stimuli^[43].

Caspase-8 and -10 are involved in the death receptor signalling pathway whereas caspase-9 is involved in mitochondrial apoptosis.

It is clear caspase activation is a critical step in the execution of apoptosis and is generally a terminal event for the cell. Regulation of the process occurs through the extrinsic, death receptor apoptosis pathway and the intrinsic mitochondrial apoptosis pathway.

EXTRINSIC APOPTOSIS PATHWAY

Apoptosis occurs via the extrinsic apoptosis pathway as a result of signalling through death receptors expressed on the surface of mammalian cells. In the 1970's it was identified that certain products of lymphocytes and macrophages caused the lysis of certain types of cells, especially tumour cells. This product was termed 'tumour necrosis factor (TNF)'^[44,45]. It has been established that TNF has its effect via cell surface receptor binding and activation. At least 18 TNF family ligands and 29 receptors have been identified in humans^[46]. In cancer research, interest has grown around the use of TNF-related apoptosis-inducing ligand (TRAIL) and targeting its receptors, members of the TNF superfamily, since the observation that recombinant human (rh) TRAIL induces apoptosis in various tumour cells but not in normal cells^[47].

TRAIL activates apoptosis by binding to specific transmembrane receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5)^[48,49]. It can also bind truncated, non-functional receptors TRAIL-R3 and TRAIL-R4 known as decoy receptors (DcR1 and DcR2)^[50]. Upon binding TRAIL to the death receptors (DR4, DR5), trimerization of the receptors occurs and a complex is formed termed the death-inducing signalling complex (DISC). This is the key step for subsequent initiator caspase activation to occur.

INTRINSIC APOPTOSIS PATHWAY

The other major apoptosis pathway leading to caspase activation and cell death is the Intrinsic (mitochondrial) apoptosis pathway. As DISC formation is the key step

in extrinsic (death receptor) signalling so mitochondrial outer membrane permeabilization (MOMP) is the key requirement for caspase activation and apoptosis via the mitochondrial apoptosis pathway. As the name suggests, this apoptosis pathway is engaged primarily as a result of internal cellular stresses such as DNA damage, ER stress^[41].

MOMP during apoptosis is primarily controlled by the BCL2 family of proteins^[51]. The pro-apoptotic members, BAX^[52] and BAK^[53], contain BH1-3 domains. BAX and BAK are often referred to as effector proteins as there is an absolute requirement for their activation at the outer mitochondrial membrane for MOMP to occur^[54].

A diverse range of death signals caused by eg DNA damage, growth factor deprivation leads to a shift in the balance of anti- and pro-apoptotic BCL2 family members with signals produced to engage MOMP. Activation of a "multidomain" proapoptotic member, BAX or BAK, is an essential gateway to mitochondrial dysfunction required for cell death in response to these diverse stimuli^[54].

Upon receipt of a death signal, BAX translocates to the mitochondrial surface where BAK already resides^[55,56]. A detailed sequence of events from BAX translocation to the OMM, subsequent activation and MOMP has been described recently^[57]. The requirement for activated BAX and/or BAK for MOMP to occur is clear, the mechanism by which they bring about MOMP is not. The key feature of MOMP in bringing about caspase activation and apoptosis is cytochrome C release. This is clear from evidence that cells lacking cytochrome C fail to activate caspases in response to UV irradiation, serum withdrawal, or staurosporine^[58]. The primary function of cytochrome C is in oxidative phosphorylation as it is a key component of the electron transport chain and it is found loosely associated with the mitochondrial inner membrane. The other important mitochondrial intermembrane space protein released during MOMP is second mitochondria-derived activator of caspase (SMAC) also termed DIABLO (direct IAP binding protein with low pI). It binds XIAP's, antagonising their ability to inhibit caspases^[59,60].

APOPTOSIS BLOCK IN CANCER

Having outlined how apoptosis proceeds, the mechanisms by which cancer cells evade this process will be explored. Block in mitochondrial apoptosis has been broadly divided into three groups^[61]: (1) Class A block occurs when normal generation of proapoptotic activators by aberrant behavior is inhibited. The mechanisms by which aberrant behaviour, such as genomic instability and oncogene activation, generate death signals via BH3-only proteins is as yet poorly understood. This is an area of ongoing study; (2) Class B block occurs when there is a significant loss of the BCL2 family effectors, BAX and BAK; and (3) Class C block occurs when increased expression of an anti-apoptotic BCL2 family protein is present, thereby inhibiting or sequestering pro-apoptotic BH3-only proteins. In this scenario, the cell has generated

an appropriate BH3-only death signal but it is inhibited by opposing anti-apoptotic expression. Cells in this state are referred to as “primed for death” and are “addicted” to the overexpression of the anti-apoptotic protein^[62].

Apoptosis blocks such as described above may determine the sensitivity of a cancer cell to a wide range of diverse chemotherapy agents and explain frequently observed phenomenon of multidrug resistance^[51]. The fact that being primed for death (class C block) is apparently more common in tumours than in normal cells may help explain why chemotherapy is often more toxic to cancers^[51].

Having classified apoptosis block in tumours, a new technique for determining what type of block a given cell employs has been developed termed BH3 profiling^[61,62]. The basic method involves incubation of mitochondria isolated from tumour cells with a panel of BH3 peptides. By assessing the pattern of response, the type of apoptosis block present can be identified. This may be used in the future to select drugs targeting anti-apoptotic proteins as a strategy for improving efficacy of drug treatments.

EXPRESSION OF BCL2 FAMILY MEMBERS IN NSCLC

Much focus has been placed on the role of BCL2 as a prognostic predictor in NSCLC. Many studies over the last 15-20 years have assessed its expression in many differing solid tumours. Given its function as an antiapoptotic protein, it would be predicted that overexpression would result in a more aggressive and treatment resistant phenotype. The published data is mixed with regard to its role as a prognostic marker.

A meta-analysis from 2003 compiled 28 studies from 1993 to 1999 which report the expression of BCL2 in NSCLC and the prognostic value of its expression in the primary tumour^[63]. Immunohistochemistry was used to detect expression in all studies. Of the 28 studies included, 11 concluded BCL2 expression was a good prognostic marker, 14 concluded it was not prognostic for survival with only 3 linking BCL2 expression to poor prognosis. Having performed the meta-analysis, the authors concluded that BCL2 positive tumours had a significantly better survival than those with BCL2 negative tumours. Given its function in the apoptotic pathway this would appear a paradoxical conclusion. One theory suggests that loss of BCL2 expression correlates with tumour de-differentiation and therefore a more aggressive phenotype^[64]. As discussed above, increased expression of BCL2 would likely confer a class C block in apoptosis and as such these tumours would be primed for death perhaps explaining the increased survival. This should be interpreted with caution as a result of heterogenous treatment patients in these studies received. Given the highly complex interplay between BCL2 and its other family members in regulating apoptosis, it is not surprising that the study of one anti-apoptotic member yielded such a result. As knowledge about the interplay between BCL2

family members improves, other targets or combination of targets may be more relevant to study rather than a single protein in isolation.

The crucial role the proapoptotic multidomain proteins BAX and BAK play in mitochondrial outer membrane permeabilisation warrants further study as prognostic markers. Loss of both proteins would confer a class B apoptosis block. Many studies exist reporting the status of BAX and BAK expression in NSCLC but again only report in isolation and each is considered separately as prognostic markers.

Altered BAX expression is frequently reported in NSCLC^[65-68]. None of these studies conclude that altered BAX expression has significant value as a prognostic marker, although none have investigated the expression of BAK together with BAX. Fewer studies report the incidence of altered BAK expression^[69,70]. These studies report the incidence of BAK loss at 42% and 59%. They also include data on BAX expression with loss reported in 34% and 47%. Neither study reports the incidence of double loss. Both conclude that no prognostic value is attributed to BAX or BAK expression in NSCLC but again each protein is analysed in isolation. Data from the International Adjuvant Lung Cancer Trial (IALT) suggests a trend toward increasing chemosensitivity with increasing BAX level^[71]. The converse effect of BAX negativity was not reported.

STRATEGIES TO OVERCOME MITOCHONDRIAL APOPTOSIS BLOCK

Given the frequent loss of expression of each protein, it is likely a significant portion of patients with NSCLC will have BAX and BAK double loss and given the evidence that this results in a highly apoptosis resistant phenotype it would be important to assess the impact on double loss on both survival and response to standard chemotherapy and radiotherapy in NSCLC.

Given what is known about the mitochondrial apoptosis pathway, class B block in apoptosis is likely to prove resistant to a range of targeted therapies. BH3 mimetics would be predicted to be ineffective due to absence of effector proteins BAX and BAK. Alternative strategies will be required to treat these potentially multidrug resistant cancers. *In vitro* evidence exists to show that in the absence of BAX and BAK, detachment of hexokinase from mitochondrial VDAC can lead to cytochrome C release and therefore mediate cell death^[72]. This can be achieved using either a competitive peptide, or by inhibiting AKT (which in turn regulates hexokinase interaction with VDAC) and prove a rational approach to treating cancers exhibiting Akt activation or an imbalance in the expression of antiapoptotic and proapoptotic members of the BCL2 family.

CONCLUSION

NSCLC presents a major health burden worldwide. Plati-

num based chemotherapy is the mainstay of treatment in the clinic today, although denovo and acquired drug resistance has resulted in a therapeutic plateau since its introduction over 30 years ago. Novel targeted therapies are beginning to emerge that induce apoptosis in certain molecular subclasses. Apoptosis resistance underpins tumorigenesis and drug resistance. Understanding how apoptosis resistance occurs in NSCLC will allow tailoring of therapy and development of novel targets to overcome this problem.

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WJCO 5th Anniversary Special Issues (1): Lung cancer

Management of tyrosine kinase inhibitor resistance in lung cancer with EGFR mutation

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molecular mechanisms of resistance and the options for clinical management of disease progression. Promising investigational strategies for overcoming TKI resistance are highlighted.

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Abstract

The identification of driver mutations and drugs that inhibit their activity has been a major therapeutic advance for patients with advanced lung adenocarcinoma. Unfortunately, the success of these drugs is limited by the universal development of resistance. Treatment failure can result from inadequate drug exposure or selection of resistant malignant clones. Clinically distinct mechanisms of disease progression have been identified and can inform treatment decisions. Investigations into the biochemical mechanisms of tyrosine kinase inhibitor resistance may provide additional therapeutic targets by which the efficacy of targeted therapy can be improved.

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Key words: Epidermal growth factor receptor mutation; Tyrosine kinase inhibitor; Lung cancer; Adenocarcinoma; Resistance; Targeted therapy

Core tip: The causes of epidermal growth factor receptor tyrosine kinase inhibitor (TKI) treatment failure including pharmacokinetic failure, intrinsic resistance and acquired resistance are discussed. We review the

INTRODUCTION

In the treatment of lung adenocarcinoma, drugs that inhibit unique driver mutations have proven superior to conventional chemotherapy in molecularly-defined subgroups, altering treatment paradigms and research agendas. The observation that dramatic responses to erlotinib or gefitinib occurred in patients with epidermal growth factor receptor (EGFR) mutations affirmed the concept of “oncogene addiction^[1]” in non-small cell lung cancer: despite the complexity of genetic and epigenetic changes in malignant cells, interfering with the activity of a single dominant oncogene can induce tumor regression. Translating this concept into clinical benefit required identification of the driver mutations to which the cancers are “addicted” and the development of drugs capable of selectively blocking their activity. Mutually exclusive driver mutations can be detected in approximately 60% of lung adenocarcinomas through multiplexed testing techniques^[2]. Single agent anti-tumor activity has been reported with drugs that inhibit the kinase activity of EGFR, EML4-ALK, ROS1, HER-2, BRAF, RET and MET^[3-9]. Moreover, targeted therapy with afatinib, gefitinib or erlotinib in EGFR-mutated lung cancer and crizotinib in lung cancer harboring EML4-ALK translocations have dem-

Table 1 Drugs that may lower serum levels of targeted therapies

Erlotinib/gefitinib	Afatinib
Rifampin, rifabutin, rifapentine, phenytoin, phenobarbital, carbamazepine, St. John's wort, proton pump inhibitors, H2-blockers, antacids, tobacco	Rifampin, phenytoin, carbamazepine, St. John's wort, primidone, tipranavir

onstrated clinically significant improvements in response rates, progression-free survival (PFS) and quality of life when compared with standard chemotherapy^[3,4,10-12]. In the LUX-Lung 3 trial, for example, afatinib produced longer PFS (11.1 mo *vs* 6.9 mo) and higher response rates (56% *vs* 23%) compared with pemetrexed and cisplatin in the first-line treatment of patients with EGFR mutations^[3]. Based on these results, practice guidelines recommend targeted therapy as first-line treatment for lung cancers with EGFR mutations or ALK translocations^[13]. Unfortunately, this therapeutic success is invariably temporary as all patients ultimately develop resistance to currently available targeted therapies. The goals of this review are therefore to (1) examine the mechanisms of failure of tyrosine kinase inhibitors (TKIs); and (2) discuss the strategies for preventing or overcoming resistance that are currently in development. We will focus on resistance to targeted therapy in lung cancers harboring EGFR mutations where these concepts are best characterized, though some concepts may be applicable to targeted therapy in general.

DIFFERENTIATING DRUG RESISTANCE FROM PHARMACOKINETIC FAILURE

The first step in identifying the mechanism of treatment failure is to differentiate pharmacokinetic failure from true drug resistance. Pharmacokinetic failure refers to disease progression due to inadequate drug exposure. True drug resistance occurs when malignant cells survive and divide in the presence of therapeutic drug levels and can be further characterized as intrinsic or acquired. In cases of pharmacokinetic failure, interventions to achieve therapeutic drug levels may effectively halt or prevent disease progression.

Interactions

Gefitinib and erlotinib are metabolized by the cytochrome p450 system and therefore have the potential for numerous interactions (Table 1). Concurrent medications and homeopathic remedies that induce p450 enzymes may lower systemic drug levels of these targeted therapies. The clinical significance of such interactions is demonstrated in the published case of a patient with advanced EGFR-mutated lung cancer that did not respond to initial treatment with erlotinib while concurrent medications included fenofibrate, a CYP3A4 inducer^[14]. Serum trough levels of erlotinib were sub-therapeutic and disease regression was achieved after dose escalation re-

sulting in therapeutic drug concentrations. Furthermore, current smokers have increased clearance of erlotinib, likely due to induction of CYP1A2 and CYP1A1^[15] and similar interactions are possible with gefitinib based on pharmacokinetic studies^[16]. In a study to determine the maximum tolerated dose of erlotinib in patients currently smoking at least 10 cigarettes daily, the trough plasma concentration and toxicity profile at 300 mg daily was similar to the standard dose of 150 mg in non-smokers^[15]. In patients taking erlotinib who cannot achieve smoking cessation, dose escalation to 300 mg daily as tolerated is recommended^[17]. Afatinib undergoes minimal metabolism by the cytochrome P450 system but is a substrate of p-glycoprotein. P-glycoprotein inducers may therefore lower systemic drug concentrations of afatinib^[18]. As oral drugs, gastric contents and pH may also impact bioavailability. Afatinib absorption is reduced when taken with a high fat meal whereas erlotinib absorption is increased and patients are directed to take both medications on an empty stomach. Drugs that increase gastric pH can reduce absorption of erlotinib and gefitinib, and have been shown to lower drug levels^[17,19]. When patients require antacid therapy, twice-daily histamine receptor blockers are recommended over proton pump inhibitors when possible and patients are advised to take erlotinib ten hours after the last dose and two hours prior to the next dose to minimize the effect on absorption^[17].

Blood-brain barrier

Central nervous system (CNS) involvement in the form of brain or leptomeningeal metastases is common in patients with advanced non-small cell lung cancer, either at the time of diagnosis or as a site of disease progression. The blood-brain barrier restricts most large and hydrophilic substances from passing from the circulation into the CNS. The cerebrospinal fluid (CSF) to plasma concentration ratios for erlotinib and gefitinib have each been shown to be less than 0.01^[20,21]. While confirming the reduced penetration of these drugs into the CNS, these measurements likely underestimate the exposure of brain metastases to each of these drugs due to local disruption of the blood-brain barrier in abnormal tumor vasculature. Despite CSF concentrations that would predict sub-therapeutic drug exposure, radiographic responses have been observed in brain metastases treated with erlotinib, gefitinib and afatinib at standard doses^[22,23]. Moreover, dose escalation of gefitinib or high-dose weekly erlotinib can increase drug levels in the CSF and reverse CNS disease progression that occurred during standard dosing. In one case report, a patient with an exon 19 mutation exhibited progressive brain and leptomeningeal metastases despite systemic disease control on gefitinib. The gefitinib concentration in the CSF was found to be less than that required to inhibit the growth of a cell line derived from the patient's tumor. After dose escalation to 1000 mg daily, the CSF concentration of gefitinib exceeded the half maximal inhibitory concentration and the patient experienced radiographic and symptomatic improvement

with clearing of malignant cells from the CSF^[20]. Since sustained escalated doses of erlotinib are poorly tolerated, high-dose weekly administration was investigated as a strategy to improve erlotinib CNS penetration. In a retrospective series of nine patients with CNS progression while on standard dose erlotinib, the CNS response rate to 1500 mg once weekly was 67%^[24].

DEFINING MECHANISMS OF DRUG RESISTANCE

True drug resistance, the survival and proliferation of malignant cells in the presence of therapeutic drug levels, is observed to varying degrees in all EGFR-mutated lung cancers. The complete response rates for currently available agents are less than 5% suggesting the presence of intrinsic resistance in a population of tumor cells of most patients at the time of treatment initiation. Furthermore, disease progression after initial response occurs due to emergence of acquired resistance with median PFS ranging from 9 to 13 mo in clinical trials. Given that some factors present at the time of diagnosis can predict both reduced probability of response and shorter duration of response, there is clearly overlap between the mechanisms of intrinsic and acquired resistance.

The response to EGFR-targeted therapy varies according to EGFR mutation

Both the probability and the duration of response to EGFR-targeted therapy vary according to the specific EGFR mutation. Activating EGFR mutations occur within exons 18-21 of the tyrosine kinase binding domain. Exon 19 deletions and L858R point mutations in exon 21 comprise 85%-90% of EGFR mutations and most reliably predict response to EGFR-targeted therapy^[25]. For this reason, FDA approval for first-line therapy with erlotinib or afatinib is limited to cancers harboring these mutations. However, differential sensitivity within this group has been observed: patients harboring exon 19 deletions show longer progression free survival compared with patients with L858R mutations^[26,27], despite similar *in vitro* activity^[28]. The reasons for the differences in clinical activity observed are not clear.

The efficacy of EGFR TKIs in the treatment of uncommon EGFR mutations is less predictable, in part due to their relative rarity. This is a heterogeneous group that includes exon 20 mutations, exon 19 insertions, exon 21 missense mutations (other than L858R), and exon 18 point mutations^[25]. The data on TKI efficacy in these cancers is limited to subset analyses of larger trials, small series and case reports. No partial responses were observed in patients with uncommon EGFR mutations in a phase II trial of first-line gefitinib, although some patients achieved prolonged stable disease^[29]. In the LUX-lung 3 trial of first-line treatment with afatinib *vs* pemetrexed and cisplatin, the progression free survival of patients treated with afatinib was improved when less common mutations were excluded from the analysis, sug-

gesting a higher prevalence of intrinsic or acquired resistance in this group^[3]. Exon 20 mutations in particular are generally associated with clinical resistance to all currently available EGFR TKIs. However, even within the group of tumors bearing exon 20 mutations there is heterogeneity and responses to EGFR TKIs have been observed with selected mutations^[30].

EGFR mutation abundance and heterogeneity

EGFR activating mutations occur *de novo* during tumor development and heterogeneity with regard to EGFR mutation status in a particular tumor nodule has been reported^[31,32]. While conventional DNA sequencing can detect an EGFR mutation present in at least 10% of tumor cells, a more sensitive method, the Scorpion amplification refractory mutation system (ARMS; DxS, Manchester, United Kingdom) uses unimolecular fluorescent probes to detect mutations present in 1%-10% of cells^[33]. In a retrospective study of 100 randomly selected archived cases, treatment with EGFR TKIs achieved a longer progression free survival of 11.3 mo in patients whose tumor demonstrated high EGFR mutation abundance (more than 10%) than those with low EGFR mutation abundance (1%-10%, PFS 6.9 mo) and the PFS in both cases were longer than that in patients with wild type tumors (PFS 2.1 mo)^[34]. This notion that higher EGFR mutation abundance in the tumor correlates with treatment effect in prolonging tumor control requires prospective validation. The heterogeneity of EGFR mutation status is not only observed in the primary tumor, but also between the primary and the metastatic lung nodules, with a discordance rate as high as 24%^[35]. The cases with discordance appear to show mixed response to EGFR TKIs^[35]. Therefore, genetic heterogeneity could be another mechanism for apparent TKI resistance at tumor progression.

Additional pathways that modulate response to targeted therapy

Several additional pathways appear to influence the depth and duration of response to TKIs in patients with EGFR-mutated tumors and provide new targets for improving therapeutic efficacy. The pro-apoptotic protein BIM has been shown to be necessary for EGFR TKI-induced apoptosis and tumor regression in EGFR-mutated cell lines and xenograft models^[36,37]. In a small retrospective series, treatment with an EGFR TKI was associated with a higher response rate (57% *vs* 29%, $P = 0.04$) and longer PFS (13.7 mo *vs* 4.7 mo, $P = 0.007$) in patients whose tumors showed higher pre-treatment levels of BIM RNA^[38]. An intronic deletion polymorphism of BIM found in 12% of East Asian patients and associated with reduced anti-apoptotic activity correlated with inferior response to EGFR TKIs^[39]. In cell lines and xenografts with this polymorphism, restoration of BIM function with BH3-mimetic drugs or HDAC inhibition overcame TKI resistance^[39,40]. This suggests that therapeutic strategies to augment BIM function, particularly in low-BIM expressing tumors, may reduce the problem

of TKI resistance in oncogene-addicted tumors. In addition, activation of the NF- κ B pathway has been shown to confer *in vitro* resistance to erlotinib in EGFR-mutant cell lines. Patients whose tumors showed high expression of the NF- κ B inhibitor I- κ B were more likely to respond to treatment with erlotinib and had longer progression free and overall survival, suggesting that NF- κ B signaling may have a clinically significant role in EGFR TKI resistance^[41]. Therefore, combined EGFR and NF- κ B inhibition presents another potential opportunity for improving the efficacy of targeted therapy.

Acquisition of secondary EGFR mutations

An important strategy for defining mechanisms of resistance to EGFR TKIs has been to re-biopsy the tumor at the time of disease progression. In two reported series comprising 192 patients treated with erlotinib or gefitinib, a distinct histologic change or molecular mechanism of resistance could be identified in the majority of cases^[42,43]. Importantly, all TKI-resistant tumors retained the original EGFR mutation. In over half of tumors analyzed, a second EGFR point mutation, T790M in exon 20, was newly detected. T790M mutations are thought to reactivate EGFR signaling by increasing the receptor's affinity for ATP over TKIs^[44]. Though a systematic analysis of the mechanisms of resistance to afatinib has not yet been published, resistance due to emergence of T790M has been reported^[45].

EGFR-independent mechanisms of acquired resistance

Signaling through alternate oncogenic kinases can bypass EGFR inhibition to re-activate proliferation and survival pathways in EGFR-mutated cells. Amplifications of MET and HER2 were identified in a minority of resistant tumors examined in the two re-biopsy series mentioned above^[42,43,46]. In one of the studies, PIK3CA mutations were also identified in two patients^[42]. Furthermore, an analysis of a large tissue database identified BRAF mutations as a possible mechanism of resistance in 2% of specimens^[47]. In addition, histologic changes such as transformation to small cell histology and epithelial to mesenchymal transition have also been observed, though the mechanisms by which they develop and lead to resistance are incompletely understood^[42,43]. So far, mechanisms of resistance have been studied in a limited number of tumors and therefore the prevalence of each resistance mechanism is likely to change as more data accrues.

CLINICAL MANAGEMENT OF TKI-RESISTANT DISEASE

When a patient who previously responded to a TKI develops progression of disease, acquired TKI resistance occurs due to the various mechanisms described above. Although its clinical utility is debated, re-biopsy at progression in selected cases could be critical to understanding the mechanism of TKI resistance and hence guide

management decisions. While it is standard practice to discontinue chemotherapy at the time of disease progression, there is definitely a rationale for TKI continuation as discussed below. Treatment options include adding local therapy or conventional chemotherapy, or TKI continuation alone. Two hypotheses guide current strategies for treatment of TKI-resistant disease: (1) a population of TKI-sensitive clones remains at the time of disease progression; and (2) Resistant clones may be detected radiographically before widespread dissemination occurs.

As discussed above, the T790M secondary mutation is by far the most common mechanism of resistance, responsible for disease progression in more than half of patients treated with erlotinib or gefitinib. *In vitro* data has demonstrated that cells that acquire T790M second site mutations grow at a slower rate than parental cells without the mutations^[48]. Furthermore, the same study suggested that in the presence of TKIs, resistant cell populations are heterogeneous and consist of slow-growing cells harboring T790M along with quiescent cells without the secondary mutation. Clinical observations support these *in vitro* results. In two patients with acquired TKI resistance and T790M mutations, serial biopsies during treatment with and without TKIs showed that T790M becomes undetectable after a period without TKI treatment^[42]. Moreover, patients with T790M mutations identified at the time of disease progression have longer post-progression survival than those without the mutation^[49]. Presumably, continuation of the original TKI exerts selective pressure that inhibits more aggressive TKI-sensitive clones and allows only the indolent T790M-harboring cells to proliferate. Therefore, in patients with T790M-mediated resistance or asymptomatic patients with radiographic evidence of progression and limited overall increase in disease burden, immediate change of systemic therapy may not be necessary and continuation of targeted therapy may still provide some measure of disease control.

It is conceivable that TKI-resistant clones develop in a single site of disease and can be detected on imaging before widespread dissemination. Patients who initially achieve disease control with EGFR-targeted therapy may subsequently show signs of disease progression in only one or a few sites of disease while other sites remain suppressed. Several groups have described their experience with the use of local therapies such as radiation or surgery to these sites of limited disease progression and have observed prolonged disease control after local therapy without a change in systemic therapy^[50-52]. Progression-free-survival after local therapy of 6-10 mo^[50,51] has been reported and the time until change in systemic therapy in one study was 22 mo^[50]. Clearly, patient selection is key to the success of this strategy. Factors that might predict prolonged stable disease after local therapy include EGFR exon 19 deletions and longer duration of initial disease control on targeted therapy^[52]. These observational studies suggest that local therapy may be of benefit, though prospective trials are needed to determine

whether local therapy can truly alter disease course.

In patients with symptomatic or rapid radiographic progression, re-biopsy of a rapidly growing lesion should be considered. If transformation to small cell histology is discovered, small cell chemotherapy regimens are appropriate for those patients. The remaining majority of patients are generally treated with chemotherapy. In this group of patients, the question of whether the original TKI should be continued is under investigation. Although no benefit was observed with the addition of TKIs to chemotherapy in unselected non-small cell lung cancer populations^[53,54], several observations suggest benefit in the treatment of patients with EGFR mutations and acquired TKI resistance. A retrospective series reported higher response rates in patients who continued the original TKI after initiating chemotherapy, though no difference in progression-free survival was observed^[55]. Furthermore, accelerated disease progression or “disease flair,” defined by hospitalization or death attributable to disease progression, was observed in the short wash out period in some patients who stopped TKIs awaiting further chemotherapy^[56]. These results suggest that some clones remain sensitive to EGFR blockade at the time of disease progression and that maintaining EGFR suppression is beneficial. Clinical trials are underway to prospectively assess the benefit of continuing TKI when chemotherapy is initiated (NCT01544179, NCT01928160 clinicaltrials.gov). If the TKI is not continued during chemotherapy, re-responses to erlotinib can be seen after post-progression “drug holidays”^[57].

FUTURE DIRECTIONS

The identification of common recurring mechanisms of resistance to TKIs provides the opportunity for rationally designed treatment of resistant disease. One strategy is the development of new TKIs with activity against secondary resistance mutations. Though there was initial optimism based on preclinical data that afatinib, an irreversible TKI, would overcome resistance to erlotinib or gefitinib including T790M, the response rate was only 7% in a phase II b/III trial in patients with disease progression after initial disease control on erlotinib or gefitinib^[58]. CO-1686, a third-generation EGFR TKI, has shown *in vitro* and *in vivo* activity against cells and tumors harboring T790M mutations and is currently being studied in a phase 1/2 clinical trial of EGFR TKI-resistant disease^[59]. In addition, an alternate dosing regimen incorporating intermittent high-dose afatinib showed *in vitro* activity against T790M and is being studied in a phase Ib clinical trial (NCT01647711 clinicaltrials.gov). Furthermore, combination therapy with afatinib and cetuximab showed promising activity in erlotinib resistant disease including cancers harboring the T790M mutation^[60]. Other strategies to prevent or treat TKI-resistant disease include the addition of an inhibitor of one of the bypass pathways (MET, AKT, PI3K, IGF1R) and HSP-90 inhibitors, which may decrease signaling through EGFR by decreasing the

stability of the protein^[61].

The identification of driver mutations in lung adenocarcinoma and the subsequent development of drugs that inhibit their oncogenic activity has been a major therapeutic advance benefitting patients with advanced disease. An understanding of the reasons for drug failure enables the optimal use of currently available EGFR targeted TKIs and maximizes their clinical benefit. Current evidence to guide management of TKI-resistant disease is limited but suggests that new principles may apply in the era of targeted therapy. The field of targeted therapy of lung cancer is rapidly evolving and the full potential of this treatment strategy is yet to be realized.

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WJCO 5th Anniversary Special Issues (1): Lung cancer

Proton beam therapy for locally advanced lung cancer: A review

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Abstract

Protons interact with human tissue differently than do photons and these differences can be exploited in an attempt to improve the care of lung cancer patients. This review examines proton beam therapy (PBT) as a component of a combined modality program for locally advanced lung cancers. It was specifically written for the non-radiation oncologist who desires greater understanding of this newer treatment modality. This review describes and compares photon (X-ray) radiotherapy (XRT) to PBT. The physical differences of these beams are described and the clinical literature is reviewed. Protons can be used to create treatment plans delivering significantly lower doses of radiation to the adjacent organs at risk (lungs, esophagus, and bone marrow) than photons. Clinically, PBT combined with chemotherapy has resulted in low rates of toxicity compared

to XRT. Early results suggest a possible improvement in survival. The clinical results of proton therapy in lung cancer patients reveal relatively low rates of toxicity and possible survival benefits. One randomized study is being performed and another is planned to clarify the clinical differences in patient outcome for PBT compared to XRT. Along with the development of better systemic therapy, newer forms of radiotherapy such as PBT should positively impact the care of lung cancer patients. This review provides the reader with the current status of this new technology in treating locally advanced lung cancer.

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Key words: Protons; Proton beam therapy; Lung cancer; Photons; X-rays; 3-D radiotherapy; Intensity modulated photon radiotherapy; Intensity modulated radiotherapy; Intensity modulated proton therapy

Core tip: This review was written for the non-radiation oncologist who wishes to understand the use of proton beam therapy (PBT) for locally advanced lung cancer. PBT can be used to create treatment plans delivering significantly lower doses of radiation to the adjacent organs at risk (lungs, heart, esophagus, and bone marrow) than photon (X-ray) radiotherapy (XRT). PBT combined with chemotherapy has resulted in relatively low toxicity and favorable survival. One randomized study is being performed and another is planned to clarify the differences in outcome for PBT compared to XRT. Newer forms of radiotherapy such as PBT should positively impact lung cancer patients.

Schild SE, Rule WG, Ashman JB, Vora SA, Keole S, Anand A, Liu W, Bues M. Proton beam therapy for locally advanced lung cancer: A review. *World J Clin Oncol* 2014; 5(4): 568-575 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/>

INTRODUCTION

Lung cancer is the leading cancer killer in the United States and worldwide. It has been estimated that lung cancer has taken the lives of 159480 Americans in 2013^[1]. Lung cancer has been historically divided into 2 major histologic types: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC).

Thoracic radiotherapy using photon (X-ray) radiotherapy (XRT) has been standard therapy for lung cancer since the 1960's when the Veterans Administration Hospital System performed a phase III trial in patients with unresectable lung cancer (including both SCLC and NSCLC)^[2]. Patients with localized but clinically inoperable tumors were assigned either XRT or a placebo. The median survival of patients given XRT was 142 d compared to 112 d ($P = 0.05$) for those who received a placebo. This study established the role of XRT in the treatment of unresectable lung cancer. XRT improved survival modestly in spite of the fact that many of these patients likely had distant metastatic disease that wasn't recognized because this trial was performed prior to the advent of modern imaging (computed tomography, magnetic resonance imaging, or positron emission tomography).

Standard therapy for locally advanced SCLC (stages II-III) and NSCLC (stage III) includes concurrent chemotherapy plus XRT^[3]. SCLC patients who complete chemotherapy plus RT and have achieved stable disease or a better response should also receive prophylactic cranial irradiation (PCI)^[3,4].

In spite of many years of investigation, the outcome for lung cancer patients remains poor due to the cancer's propensity to persist locally and metastasize widely^[5]. Local control rates have been poor when defined using post-chemo-RT tumor persistence on biopsy rather than radiologic imaging criteria^[6]. The overall 5-year survival rates for locally advanced NSCLC and SCLC range from approximately 15% to 25%^[3,4]. Concurrent chemotherapy plus XRT results in better survival than XRT alone or sequential therapy but with higher rates of severe toxicity^[7,8]. For example, The North Central Cancer Treatment Group (NCCTG) performed a phase III trial to determine whether chemotherapy (cis-platin and etoposide) plus either twice daily (BID) XRT or daily (QD) XRT achieved a better outcome for patients with stage III NSCLC. Severe, grade ≥ 3 (3+) and grade 4+ toxicity was very common, occurring in 87% and 67% of patients, respectively^[9]. Grade 3+ and 4+ hematologic toxicity occurred in 80% and 62%. Grade 3+ and 4+ esophageal toxicity occurred in 18% and 1%. Grade 3+ and 4+ pneumonitis occurred in 12% and 2%. The 5-year survival was 17%. These results form a reasonable standard for chemotherapy plus XRT to which other treatment programs can be compared. It is clear that

more research is needed to develop therapies that result in better survival and less toxicity.

PHYSICS, RADIOBIOLOGY, AND RATIONALE FOR PBT

One potential method to improve patient outcome is to optimize the radiotherapy. Historically, we have improved patient outcomes each time newer methods of radiation delivery were invented. First, radiotherapy was delivered with radio-isotopes placed directly into the tumor. This was problematic when treating lung cancer since the act of placing potential sources within a lung tumor would require physical damage to the lung. Low energy X-ray devices were then developed which delivered kilo-voltage X-rays. Unfortunately, these beams penetrated the tissues poorly and delivered the maximum dose on the skin with only a small fraction of the dose to a deep seated tumor. Kilovoltage X-ray beams were used in the VA study noted above. The isotope cobalt 60 was then used and produced a beam composed of gamma rays with two distinct energies of 1.17 and 1.33 million volts (MV) resulting in better skin sparing and improved depth-dose penetration. Linear accelerators were then developed which produced still higher energy MV X-rays or photons. These photon beams penetrated better but still delivered the maximal dose between 1.5 and 3.5 cm beneath the surface with the dose gradually decreasing as the beam penetrated straight through without stopping until exiting the body. The dose-distribution of X-rays within the body is due to their unique characteristics of having almost no mass and no charge.

Protons, in contrast, have mass (approximately 1800x that of an electron) and hold a positive elementary charge. These characteristics create a much different distribution of dose deposition within the body. First, the accelerated protons enter the body with a high momentum which carries them to a specific depth dependent on the initial kinetic energy imparted upon them by the accelerating device (generally a cyclotron or synchrotron). As the proton beam travels to that depth, there is a relatively small amount of energy transmitted to the tissues. As the protons slow down, more and more energy is transferred to the surrounding tissues. The energy lost per unit path length is almost inversely proportional to the square of the speed of the proton. Shortly before the entire energy of the proton is lost, the energy loss rate reaches a sharp peak. Once the kinetic energy of the proton is entirely dissipated into the tissue, the proton comes to rest within the body. The energy of the proton is dissipated in collisions with the electrons of the neighboring atoms in the surrounding tissues causing ionizations which produce radiation damage. The region in the body where the maximum energy loss and final stopping of protons occurs is narrow and is located at a specific depth depending on the initial energy of the proton beam. This sharp, well defined peak of maximal dose from charged particles is referred to as the "Bragg peak". Beyond this

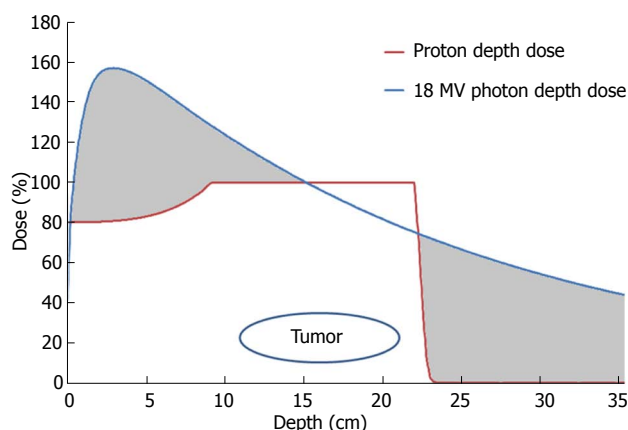


Figure 1 Depth-dose comparisons of a single photon (X-ray) beam and a proton beam.

point, there is no radiation energy imparted upon surrounding tissues as the protons have stopped. So far we have only discussed mono-energetic proton beams, *i.e.*, proton beams in which all protons have the same initial energy. The peak of a mono-energetic proton beam is so narrow that in order to generate a clinically useful proton beam one has to spread it out by giving repeated mono-energetic beams of protons with successively lower energies to cover a mass or tumor within the high dose region otherwise known as the “spread-out Bragg Peak” (Figure 1).

Figure 1 reveals depth-dose comparisons of a single photon (X-ray) beam and a proton beam. Each beam enters the body at 0 cm depth on the left side of the graph and travels into the body traveling to the right. The depth within the patient is shown on the X-axis and the percent of the prescribed dose delivered on the Y-axis. The 18 MV photon beam delivers the greatest dose at 3.5 cm depth within the patient and then decreases in dose delivered as it enters and exits the tumor at depths of 11 and 22 cm, respectively. The photon beam then continues through the body until it exits. In contrast, the proton beam administers the maximum dose in the tumor stopping just beyond the deepest portion of the tumor. There is less dose delivered as the proton beam travels to the tumor and no exit dose beyond the tumor. The grey areas correlate to the excess dose delivered by photons to normal healthy tissue that would not be irradiated by the proton beam.

Protons are also different from photon irradiation in terms of killing power. The relative biological effectiveness (RBE) for proton RT is generally estimated at 1.1 X that for photons. Thus, one gets 10% more cancer kill power for each gray (Gy) of proton RT than photon RT. To simplify discussions, photon doses are described in Gy and proton doses described in Gy equivalence (GyE) to describe 2 beams with similar killing properties. One Gy is the addition of one joule of energy per kilogram of tissue. The energy absorbed results in free radical formation. The free radicals ionize and fracture DNA

molecules within the cells’ nuclei. These DNA fractures result in cell death especially in rapidly reproducing cells which are the most sensitive to radiotherapy.

The primary benefits of PBT compared to XRT are based on the above mentioned interactions with matter when traveling into a patient to treat a tumor. The fact that the proton beam stops results in no radiation exposure beyond the tumor allows for the sparing of distally placed tissues. In contrast, photons don’t stop and travel through the entire body from the entrance to the exit point.

If one has a tumor in the anterior portion of the chest, it would be desirable to treat from the anterior perspective with PBT. After the proton beam treats the tumor, it stops. This results in substantially less dose than X-rays to deeper structures including the spine (spinal cord and marrow), esophagus, lung, and heart. Each of these normal dose-limiting organs is sensitive to radiation and can be injured during treatment. As discussed earlier, the standard therapy of concurrent chemotherapy plus XRT to 60 Gy in 30 daily fractions results in severe (grade 3+) toxicity in the vast majority of patients^[9]. Thus, maximal sparing of these critical organs is important in potentially improving patient outcomes (survival, quality of life, and toxicity).

Detailed studies comparing the XRT plans using both 3-D planning and intensity modulated photon RT (IMRT) technology to proton TRT plans have been performed. Nichols *et al*^[10] examined the dose distributions (dosimetry) from 8 consecutive stage III NSCLC patients. In all patients, 3-D XRT, IMRT, and proton therapy plans achieved the dose goals for the tumor volumes. Compared with 3-D XRT plans, proton plans offered a median 29% reduction in normal lung V20 (total lung volume receiving > 20 Gy), a median 33% reduction in mean lung dose (MLD), and a median 30% reduction in the volume of bone marrow receiving a dose of > 10 Gy. The V20 and MLD have been established to correlate well with the risk of radiation pneumonitis^[11-14]. The 10 Gy dose to the bone marrow would be sufficient to suppress myelopoiesis within the irradiated marrow. Compared with the IMRT plans, the proton plans offered a median 26% reduction in normal lung V20, a median 31% reduction in MLD, and a median 27% reduction in the volume of bone marrow receiving a dose of > 10 Gy. They concluded that by reducing the volumes of normal structures irradiated, protons can potentially improve the therapeutic index for stage III NSCLC compared to with either 3-D XRT or IMRT. Similar results were found by Chang *et al*^[15] when they compared the RT plans that delivered high dose RT with either protons or photons. PRT reduced the dose to normal tissues significantly, even with dose escalation, compared with standard-dose photon therapy, either 3-D XRT or IMRT.

The dosimetric studies highlight significant differences in the dose distributions when comparing protons to photons in the treatment of lung cancer. Because the proton beam can stop just beyond the far end of the target, normal tissues beyond the tumor can be spared from

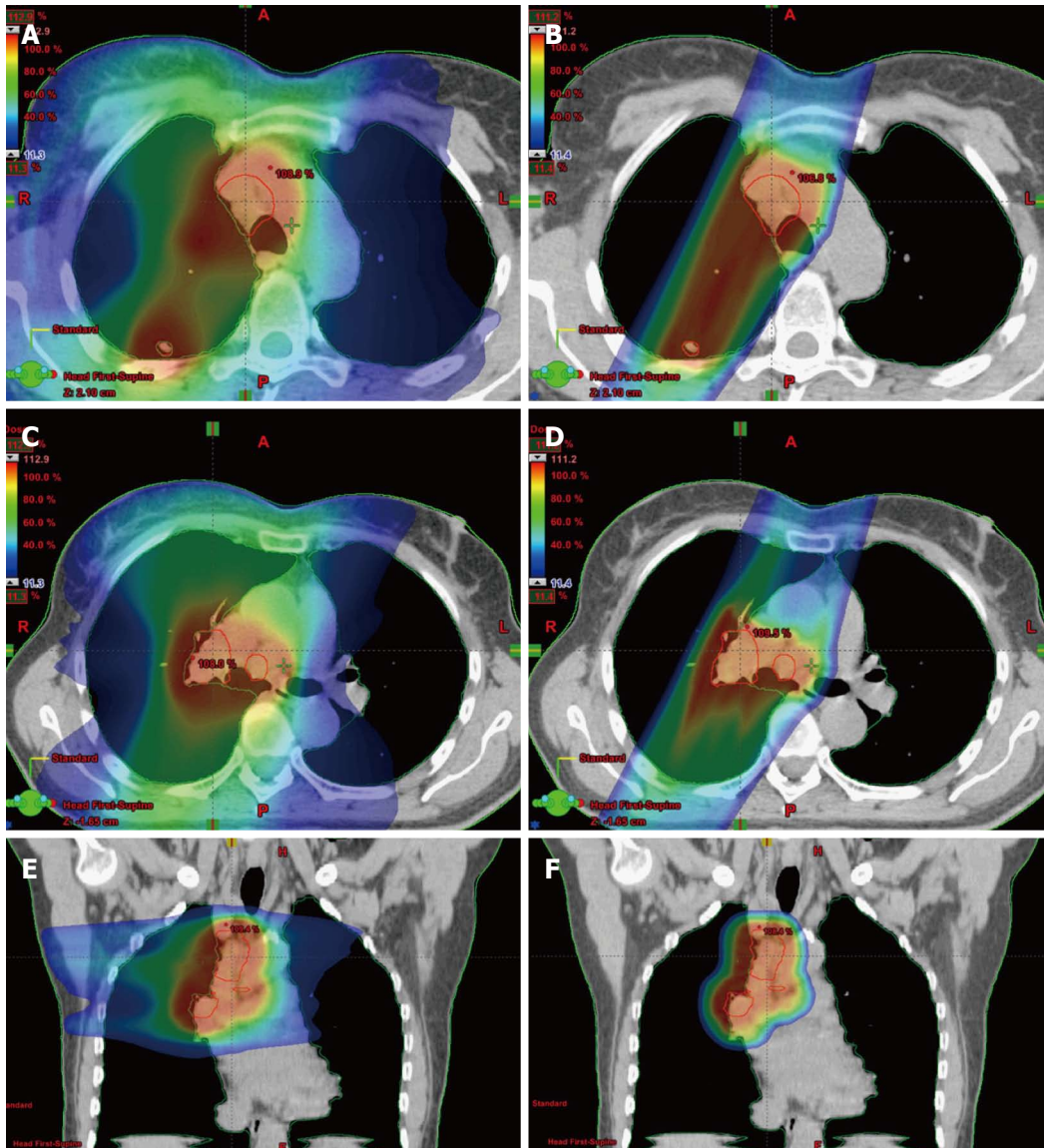


Figure 2 Dosimetric comparisons between photon [left (A, C, E) and proton right (B, D, F)] plans for a stage III lung cancer patient. Radiation doses shown as color washes warmer colors (red) represent higher doses compared to cooler colors (blue). The first pair of axial slices (A and B) are at a level superior to axial cuts C and D; It is apparent that more normal lung is irradiated on the left than the right; E and F are coronal cuts with dosimetry.

receiving radiation when compared to XRT. These differences can be used to produce potentially safer RT plans.

Clinical data is required to substantiate that the gains found dosimetrically translate into improvements in patient outcome. Newer proton facilities are employing scanned pencil beams instead of the broad passively scattered proton beams which are shaped with apertures and range compensators. Pencil beams are very narrow proton beams steered with magnetic fields which move in a raster pattern back and forth through the tumor volume. The intensity (or dose) can be modified as the beam moves allowing the delivery of intensity modulated proton beam therapy (IMPT). Zhang *et al.*^[16] was able to show that IMPT produced better dosimetric results than more conventional proton beam therapy. Figure 2 includes dosimetric comparisons between photon and proton plans for a patient with stage III lung cancer. The doses delivered shown as a color wash with lower doses denoted in blue and higher doses in red. On

the left is a rapid arc intensity modulated photon RT (IMRT) plan which included 6 MV photons which delivered IMRT while the gantry rotated around the patient. On the right are shown the same patient treated with intensity modulated proton RT. It is clear from figures that the IMPT plan delivers the same dose to the target (red outlined region) with less dose to the surrounding normal tissues.

Proton beams are more sensitive to a variety of uncertainties such as respiratory motion, changes in patient positioning, and tumor shrinkage. This creates more technical challenges in planning and delivery of radiotherapy. The term, “robust” is used to describe treatment plans which can accommodate these uncertainties.

RESEARCH

Results in patients with limited stage SCLC

Colaco *et al.*^[17] reported the first known series of limited

stage SCLC (L-SCLC) patients treated with PBT. All 6 patients also received chemotherapy and PCI. Five patients received 60–66 GyE in 30–34 daily fractions and one patient received 45 GyE in 30 BID fractions. With one year of median follow up, the one-year overall survival rates was 83%. Treatment was well tolerated; there were no cases of acute grade 3+ esophagitis or grade 2+ pneumonitis, and no other grade 3+ non-hematological toxicities were seen. Dosimetric comparison revealed better sparing of lung and esophagus with PBT than IMRT. They concluded that PBT merits further investigation as a method of reducing toxicity in L-SCLC.

Results of PBT in patients with stage III NSCLC

Clinical outcomes of phase I and II studies are available. Standard therapy for stage III NSCLC in fit patients is concurrent radiation and chemotherapy. As such, this summary focuses on the studies which delivered combined modality therapy. Chang *et al.*^[18] reported the early findings of a phase II trial of high-dose PBT that included 44 patients with stage III NSCLC treated with 74 GyE in 37 fractions with weekly carboplatin and paclitaxel. Protons were delivered as passively scattered beams and adaptive re-planning. The median overall survival time was 29.4 mo and no patients suffered grade 4 or 5 proton-related toxicity. The most common non-hematologic grade 3 toxicities were dermatitis ($n = 5.11\%$), esophagitis ($n = 5.11\%$), and pneumonitis ($n = 1.2\%$). Nine (20.5%) patients experienced local disease recurrence, four (9.1%) patients had regional lymph node recurrence, and 19 (43.2%) patients developed distant metastasis. Of the 44 patients, 9 (20%) had their original plans adapted to changes in their tumor volume^[19]. Changes in tumor volume due to response can sufficiently alter the proton stopping power of the tissues to require changing the plan in order to administer the prescription dose to the tumor and protect normal tissues. The authors concluded that concurrent high-dose PBT and chemotherapy were well tolerated and that the median survival was encouraging.

Hoppe *et al.*^[20] reported on 19 NSCLC patients ($n = 18$:stage III, $n = 1$:stage II b) treated with carboplatin, paclitaxel, and PBT to 74 Gy/37 fractions. There were only 1 (5%) acute grade ≥ 3 non-hematologic toxicity and 2 (11%) chronic non-hematologic toxicity with a median follow up of 16 mo. Oshiro compiled a series of 57 patients with stage III NSCLC treated with a median dose of 74 Gy with PBT and no chemotherapy^[21]. The median survival was 21.3 mo which was similar to many combined modality series. Toxicity was very modest with grade 3+ lung toxicity in 11% and no grade 3+ esophageal toxicity.

Comparative results of protons vs photons

Clinical comparisons of patients treated with either photon or proton therapy for lung cancer are rare. This is in part due to the relatively few clinical experiences with proton therapy for lung cancer. This is in part due to the

difficulty in getting lung cancer covered by insurance for PBT. Seijpal *et al.*^[22] reported a retrospective comparative analysis of the MD Anderson experience in patients with stage 3 NSCLC. Their rationale for the use of proton beam therapy was the recognition that concurrent chemotherapy plus XRT, the standard of care for stage 3 NSCLC, causes severe toxicity in most patients. Photon based TRT cannot be given at doses associated with a high chance of cure without excessive toxicity. They hypothesized that PBT could permit higher tumor doses with less normal-tissue toxicity than XRT delivered as 3-D XRT or IMRT. They compared the outcome of PBT + chemotherapy ($n = 62$), 3D-XRT + chemotherapy ($n = 74$) or IMRT + chemotherapy ($n = 66$). RT was delivered to the gross tumor volume with weekly paclitaxel and carboplatin. The median total radiation dose was 74 GyE for the proton group and 63 Gy for the other groups. Severe (grade 3+) pneumonitis and esophagitis in the proton group (2% and 5%) were lower despite the higher radiation dose (3-D XRT, 30% and 18%; IMRT, 9% and 44%; $P < 0.001$ for all). The median survival times were 17.7 mo for the 3-D XRT group, 17.6 mo for the IMRT group, and 24.4 mo for the proton therapy group ($P = 0.1$). They found that higher doses of PBT could be delivered to lung tumors with lower rates of esophagitis and pneumonitis.

The above findings were provocative enough to justify a randomized trial of IMRT *vs* PBT. This trial is entitled, “A bayesian randomized trial of image-guided adaptive conformal photon *vs* proton therapy, with concurrent chemotherapy, for locally advanced NSCLC.” The primary objective is to compare the incidence of grade 3+ treatment related pneumonitis (TRP) or local failure. In addition, the Radiation Therapy and Oncology Group (RTOG) is planning a phase III trial (RTOG 1308) comparing chemotherapy plus either XRT or PBT in doses of 60 to 70 Gy. The primary endpoint is overall survival. Other trials exist and can be found at: <http://clinicaltrials.gov> and are summarized in Table 1.

PBT offers stage III lung cancer patients potentially safer radiotherapy plans with significantly lower doses delivered to the lungs, esophagus, and bone marrow. This has resulted in less toxicity^[22] in retrospective comparisons. Safe dose escalation with photon therapy was not possible in stage III NSCLC patients treated on RTOG 0617 which compared chemotherapy plus either 60 Gy or 74 Gy of photon irradiation. The higher dose arm resulted in poorer survival which appears to be secondary to increases in normal tissue (pulmonary and/or cardiac) doses^[23]. PBT can better spare the normal surrounding organs at risk and, thus, offers investigators an opportunity to safely deliver higher tumor doses which may result in higher local control rates and improved survival.

One randomized study is being performed and another is being planned to further elucidate the potential clinical benefits of PBT compared to traditional XRT. Technology continues to improve and pencil beam PBT and IMPT may produce better clinical results than scat-

Table 1 Proton beam therapy trials for stage 3 lung cancer patients**Non-small cell lung cancer**

- 1 ClinicalTrials.gov identifier NCT00881712 (LU02: University of Florida): A phase II trial of 3-D proton radiotherapy with concomitant chemotherapy for patients with initially unresectable stage III non-small cell lung cancer:
 - (1) Arm 1: Concurrent chemotherapy and PBT (74 GyE/37 fractions) for unresectable stage 3 patients with nodes > 15 mm in diameter.
 - (2) Arm 2: Concurrent chemotherapy and PBT (60 GyE/30 fractions) for unresectable stage 3 patients with nodes < 15 mm in diameter.
 - (3) Arm 3: Resectable stage 3 diseases: preoperative PBT (50 GyE/25 fractions) and surgery.
- 2 ClinicalTrials.gov identifier NCT01993810: (RTOG1308) phase III randomized trial comparing overall survival after photon vs proton chemoradiotherapy for inoperable stage II-III B NSCLC:
 - (1) Arm I : XRT (70 Gy/35 fractions) and either paclitaxel and carboplatin or etoposide and cisplatin.
 - (2) Arm II: PBT (70 GyE/35 fractions) and either paclitaxel and carboplatin or etoposide and cisplatin.
- 3 ClinicalTrials.gov identifier NCT01770418 (Proton Collaborative Group): A Phase I / II study of hypofractionated proton therapy for stage II-III non-small cell lung cancer:
 - (1) Proton radiotherapy
 - Dose Level 1: 60 Gy (RBE) at 2.5 Gy (RBE) per fraction × 24 fractions
 - Dose Level 2: 60 Gy (RBE) at 3 Gy (RBE) per fraction × 20 fractions
 - Dose Level 3: 60.01 Gy (RBE) at 3.53 Gy (RBE) per fraction × 17 fractions
 - Dose Level 4: 60 Gy (RBE) at 4 Gy (RBE) per fraction × 15 fractions
 - (2) Paclitaxel and carboplatin or cisplatin and etoposide
- 4 ClinicalTrials.gov identifier NCT01565772 (MGH): A phase I trial of hypofractionated PBT with cisplatin and etoposide followed by surgery in stage III non-small cell lung cancer:

Radiation (PBR): 45-55 Gy total, 1.8-2.2 Gy × 25 fractions with cisplatin and etoposide followed by resection
- 5 ClinicalTrials.gov identifier NCT01165658 (MD Anderson) phase I study of Hypofractionated Proton Radiation Therapy in Thoracic Malignancies: The radiation prescription dose ranges from 45 Gy in 3 Gy fractions to 60 GyE in 4 Gy fractions. This is for patients ineligible for chemotherapy.
- 6 ClinicalTrials.gov identifier NCT00614484 (Loma Linda University): phase I / II study of combined chemotherapy and high dose, accelerated proton radiation for the treatment of locally advanced non-small cell lung carcinoma. Carboplatin and taxol and 5 wk of daily proton therapy.
- 7 ClinicalTrials.gov identifier NCT01629498 (MD Anderson): phase I / II trial of Image-guided, intensity-modulated photon or scanning beam proton therapy. Both with SIB dose escalation to the GTV with concurrent chemotherapy for stage II / III NSCLC
 - (1) Experimental. IMPT + SIB + chemotherapy phase I starting IMPT dose: 60 Gy (RBE) in 30 fractions; phase I starting SIB dose: (72-84) Gy (RBE). All patients receive standard concurrent chemotherapy.
 - (2) Experimental. IMRT + SIB + chemotherapy: phase I Starting IMRT dose: 60 Gy (RBE) in 30 fractions; phase I starting SIB Dose: (72-84) Gy (RBE). All patients receive standard concurrent chemotherapy
- 8 ClinicalTrials.gov identifier NCT00915005 (MD Anderson): A bayesian randomized trial of image-guided adaptive conformal photon vs proton therapy, with concurrent chemotherapy, for locally advanced NSCLC (treatment related pneumonitis and locoregional recurrence)
 - (1) Experimental group 1 (photon therapy): 74 Gy/37 fractions + carboplatin and paclitaxel
 - (2) Experimental group 2 (proton therapy): 74 Gy/37 fractions + carboplatin and paclitaxel
 - (3) Experimental group 3 (proton therapy): 66 Gy/33 fractions + carboplatin and paclitaxel
- 9 ClinicalTrials.gov identifier NCT01076231 (University of Pennsylvania) feasibility and phase I / II trial of preoperative PBR with concurrent chemotherapy for resectable stage IIIA or superior sulcus NSCLC: pbr over 5.5-7.5 wk plus concurrent chemotherapy comprising cisplatin and etoposide. Then, patients may undergo surgical resection or additional chemoradiotherapy
- 10 ClinicalTrials.gov identifier NCT01108666 (University of Pennsylvania): phase I dose escalation trial of PBR with concurrent chemotherapy and nelfinavir for inoperable stage III NSCLC. Determine MTD of nelfinavir and MTD of PBR when given with chemotherapy for stage III NSCLC.
- 11 ClinicalTrials.gov identifier NCT01808677 (MD Anderson): registry study of thoracic reirradiation for NSCLC utilizing PBT or intensity modulated radiation therapy. Primary objective to assess the prevalence of high-grade toxicity in patients being treated with thoracic re-irradiation with PBT or IMRT for NSCLC, with or without chemotherapy.
- 12 ClinicalTrials.gov identifier NCT01386697 (University of Pennsylvania): A prospective radiation oncology planning study for lung, gastrointestinal and lymphomatous malignancies using proton radiotherapy as compared to 3D conformal and intensity-modulated X-ray therapy for dosimetric evaluation of tumor coverage and dose to organs-at-risk. The overall objective is to estimate the actual or potential benefit of DIBH treatment in the context of proton radiotherapy as compared to 3DCRT and IMXT.

PBT: Proton beam therapy; RBE: Relative biological effectiveness; IMRT: Intensity modulated radiation therapy; NSCLC: Non-small cell lung cancer; SIB: Simultaneous integrated boost; GTV: Gross tumor volume; DIBH: Deep inspiration breath holding; IMXT: Intensity-modulated X-ray therapy; 3DCRT: Three dimensional conformal radiation therapy.

tered PBT. Along with the development of better systemic therapy, improvements in radiotherapy technology should positively impact on the care of unresectable lung cancer. Protons may allow the safe escalation of dose to levels considered to be tumoricidal while sparing the critical normal tissues^[24]. This was not possible using photons either with 3-D treatment planning or IMRT when tested in RTOG 0617^[25].

More research is needed to optimize proton administration especially for the newer pencil beam systems. This requires greater physics understanding in order to create plans which are robust in the face of uncertainty. Comparative studies will elucidate the potential value of this newer radiotherapy modality in terms of both clinical outcomes (survival, toxicity, and QOL) and cost effectiveness.

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WJCO 5th Anniversary Special Issues (1): Lung cancer

Review of the current targeted therapies for non-small-cell lung cancer

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Core tip: The development of oncogene-directed targeted therapies has significantly changed the treatment of non-small-cell lung cancer. We review the data demonstrating efficacy of small-molecule tyrosine kinase inhibitors against epidermal growth factor receptor, anaplastic lymphoma kinase, ROS1, and other oncogenes. We also discuss the challenge of acquired resistance to these therapies and review promising agents which may overcome resistance.

Abstract

The last decade has witnessed the development of oncogene-directed targeted therapies that have significantly changed the treatment of non-small-cell lung cancer (NSCLC). In this paper we review the data demonstrating efficacy of gefitinib, erlotinib, and afatinib, which target the epidermal growth factor receptor (EGFR), and crizotinib which targets anaplastic lymphoma kinase (ALK). We discuss the challenge of acquired resistance to these small-molecular tyrosine kinase inhibitors and review promising agents which may overcome resistance, including the EGFR T790M-targeted agents CO-1686 and AZD9291, and the ALK-targeted agents ceritinib (LDK378), AP26113, alectinib (CH/RO5424802), and others. Emerging therapies directed against other driver oncogenes in NSCLC including *ROS1*, *HER2*, and *BRAF* are covered as well. The identification of specific molecular targets in a significant fraction of NSCLC has led to the personalized deployment of many effective targeted therapies, with more to come.

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INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths around the world with approximately 1.3 million deaths per year and a poor prognosis for those with advanced stage disease treated with traditional chemotherapy agents^[1,2]. However, the last decade has witnessed the discovery of molecular changes that drive lung cancer in a substantial minority of patients and development of many targeted therapies that have significantly changed treatment in this setting. In this paper we review the data leading to approval of targeted therapies against the epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK), discussing the challenges of overcoming acquired resistance to these small-molecular tyrosine kinase

inhibitors (TKIs). We also review several other promising targeted therapies currently in development.

FIRST-GENERATION EGFR TKIS

The first available targeted therapies for advanced NSCLC were gefitinib and erlotinib, both of which are small-molecule TKIs against EGFR, also known as HER1 or ErbB-1. The dimerization of EGFR activates its tyrosine kinase, which in turn activates intracellular signal transduction pathways involved in many cellular processes. Early work on EGFR in lung cancer has shown that EGFR overexpression is commonly seen in NSCLC^[3,4], motivating the development of EGFR TKIs.

Phase II studies of gefitinib and erlotinib in the second- or third-line setting for advanced NSCLC in unselected patients were promising, showing a partial radiographic response rate of about 12% with symptomatic improvements^[5-7]. The first clinical trial to show an improved overall survival (OS) was the Canadian phase III BR.21 trial, which randomized patients with stage III B or IV NSCLC, neither clinically nor molecularly selected for EGFR mutations, who had received one or two chemotherapy regimens, to either erlotinib or placebo. Patients receiving erlotinib had a median OS of 6.7 mo, compared with 4.7 mo for those on placebo^[8]. Interestingly, a similar phase III study, the Iressa Survival Evaluation in Lung Cancer (ISEL) trial, comparing gefitinib to placebo in the second- or third-line setting failed to demonstrate an improved OS. However, subgroups of never smokers and Asians did have statistically significant survival advantage on gefitinib compared to placebo^[9]. That erlotinib apparently had greater efficacy than erlotinib might be due to the fact that erlotinib was dosed at its maximum tolerated dose (MTD)^[8] while gefitinib was dosed at one-third of its MTD^[9].

However, data from these clinical trials and others suggested that EGFR immunohistochemical staining intensity was not predictive of therapeutic benefit^[5]. Subsequently, somatic activating EGFR mutations, most commonly including exon 19 deletions and exon 21 L858R missense mutations, were discovered to be a dominant predictor of responsiveness to EGFR TKIs^[10-15]. It is estimated that these activating EGFR mutations are present in tumors from about 50% of Asian patients with NSCLC and 15% of Western patients^[16-19]. The cause for this difference in the prevalence rates of EGFR mutations among various ethnic groups remains unknown, yet EGFR mutations are also observed most frequently in women, patients with no or minimal history of smoking, and tumors of adenocarcinoma histology^[16,17,20].

More recent first line studies in advanced NSCLC attempted to enrich patients with activating EGFR mutations to compare EGFR TKI therapy with conventional chemotherapy. The pivotal Iressa Pan-Asia Study (IPASS) randomized over 1200 untreated patients who were never smokers or former light smokers to either gefitinib or the combination of carboplatin and paclitaxel. The progres-

sion-free survival (PFS) at 12 mo was 25% for gefitinib and 7% for chemotherapy. For patients with activating EGFR mutations, gefitinib was associated with a hazard ratio for progression of 0.48 ($P < 0.001$) compared to chemotherapy, while for patients who were negative for EGFR mutations, gefitinib was associated with shorter PFS with a hazard ratio for progression of 2.985 ($P < 0.001$). OS was similar between the two groups, presumably due to crossover^[18,19]. Similar results have been observed in other trials involving gefitinib conducted in Asia. The First-SIGNAL trial from the South Korea comparing gefitinib to cisplatin and gemcitabine in the first-line setting for advanced pulmonary adenocarcinoma in never smokers demonstrated a PFS benefit for gefitinib but also no OS difference. This study also had significant crossover. For the subgroup of patients with EGFR-mutant adenocarcinoma, gefitinib was associated with a higher overall response rate (ORR) (84.6% *vs* 37.5%; $P = 0.002$) and a trend toward longer PFS (HR = 0.544; 95%CI: 0.269-1.100; $P = 0.086$) compared to chemotherapy. For those patients with tumors harboring wild-type EGFR, the reverse was found: chemotherapy showed a trend toward higher ORR and longer PFS^[21]. Together, the IPASS and First-SIGNAL studies demonstrated that activating EGFR mutations are predictors of benefit with gefitinib and that wild-type EGFR patients do poorly with first-line gefitinib compared to platinum-based chemotherapy.

Instead of selecting patients by smoking status, subsequent studies included only patients with activating EGFR mutations. In randomized controlled trials, Japanese researchers confirmed the PFS superiority of gefitinib to chemotherapy as first-line treatment for patients with advanced EGFR-mutant NSCLC. In the West Japan Thoracic Oncology Group trial 3405, patients on the gefitinib arm had a median PFS of 9.6 mo, compared to 6.6 mo for those on cisplatin plus docetaxel^[22,23]. In a North-East Japan Study Group trial, gefitinib was associated with a PFS of 10.8 mo *vs* 5.4 mo for carboplatin-paclitaxel^[24]. In both Japanese trials, the differences in OS were not statistically significant^[23,24].

Similar to gefitinib, erlotinib has also demonstrated PFS advantages compared to chemotherapy in patients with EGFR-mutant NSCLC in the first-line setting. The Chinese OPTIMAL trial showed a PFS of 13.1 mo for erlotinib *vs* 4.6 mo for carboplatin and gemcitabine^[25]. The EURTAC trial demonstrated that EGFR TKIs were also effective for European patients with EGFR-mutant NSCLC in the first-line setting. In this study, patients receiving erlotinib had a PFS of 9.7 mo, compared to 5.2 mo for those receiving a platinum-based chemotherapy regimen^[26]. OS was not statistically different between the erlotinib and chemotherapy arms in either the OPTIMAL or EURTAC trial^[26,27].

More recent efforts have focused on newer-generation EGFR TKIs. Afatinib is an irreversible ErbB family inhibitor that, in preclinical models, has been shown to have activity against activating EGFR mutations as well

as the *EGFR* T790M mutation that confers resistance to erlotinib and gefitinib^[28]. The initial randomized studies of afatinib addressed its efficacy in the *EGFR*-TKI resistance setting. In LUX-Lung 1, patients with *EGFR*-mutant NSCLC who had received a first-generation *EGFR* TKI and chemotherapy were randomized to either afatinib or placebo. PFS was 3.3 mo for afatinib, compared to 1.1 mo for placebo ($P < 0.0001$)^[29]. The drug was then studied as a first-line treatment for *EGFR*-mutant NSCLC. The global LUX-Lung 3 phase III study randomized 345 patients to either afatinib or cisplatin-pemetrexed. The median PFS was 11.1 mo for afatinib and 6.9 mo for chemotherapy (HR = 0.58; 95%CI: 0.43-0.78; $P = 0.001$)^[30]. Similarly, the LUX-Lung 6 phase III study randomized 364 Chinese patients with *EGFR*-mutant NSCLC to either afatinib or cisplatin-gemcitabine in a 2 to 1 ratio. The median PFS of patients on the afatinib arm was 11.0 mo *vs* 5.6 mo for chemotherapy, HR = 0.28, $P < 0.0001$ ^[31]. In July 2013, nine years after the initial approval of erlotinib for treatment of advanced NSCLC (second or third line, regardless of *EGFR* mutation status) and only two months after the approval of erlotinib for first-line treatment of advanced *EGFR*-mutant NSCLC, the United States Food and Drug Administration (FDA) approved afatinib, for the first-line treatment of advanced NSCLC with activating exon 19 deletions and L858R *EGFR* mutations. A pooled subgroup analysis from trials of afatinib in TKI-naïve patients demonstrated good activity with a PFS of 10.7 mo in patients with other *EGFR* mutations that are classically sensitive to erlotinib, like L861Q and G719X^[32]. However, tumors initially harboring historically TKI-resistant alterations including T790M and exon 20 insertions appear markedly less sensitive, with PFS of under 3 mo in both groups. The ongoing clinical trial LUX-Lung 7 is comparing afatinib against gefitinib in the first-line setting for *EGFR*-mutant NSCLC to help determine relative efficacy of the two TKIs (ClinicalTrials.gov identifier: NCT01466660).

OVERCOMING RESISTANCE TO EGFR TYROSINE KINASE INHIBITORS

The discovery of *EGFR* TKIs has thus revolutionized treatment of NSCLC with activating *EGFR* mutations, with erlotinib, gefitinib, and afatinib approved for use in various countries. While a small minority of patients have disease control for years on these drugs, on average these TKIs have a median response duration seldom exceeding one year due to acquired resistance. The mechanisms of resistance vary, with the *EGFR* T790M point mutation in exon 20 being the most common cause of acquired resistance, accounting for about 50% of cases. The T790M “gatekeeper” mutation was initially thought to simply exclude binding of *EGFR*-TKI drugs by steric hindrance, but more importantly it appears to restore the *EGFR* affinity for ATP, thus decreasing the binding of the ATP-competitive TKIs^[33-35]. There is increasing evidence that a low level of the T790M mutation exists before treatment in many patients with *EGFR*-mutant NSCLC and pre-

dicts a worse PFS on erlotinib compared to those without pre-treatment T790M^[36]. Another clearly described cause of acquired resistance to TKI is the amplification of the mesenchymal epithelial transition (MET) proto-oncogene, which activates an AKT-mediated signaling pathway, bypassing *EGFR*^[37,38]. Several other *EGFR* mutations have also been implicated in conferring resistance to *EGFR* TKIs: D761Y^[39], T854A^[40], and L747S^[41], in addition to activating *BRAF* mutations^[42] and *HER2* amplification^[43]. Interestingly, some TKI-resistant tumors undergo histologic changes, including transformation from non-small-cell to small cell or epithelial-mesenchymal transition, leading to resistance through less direct mechanisms^[44].

Since half of acquired resistance is dependent on the T790M missense mutation, newer *EGFR* TKIs are in development to overcome resistance. The second-generation inhibitors afatinib and dacomitinib irreversibly inhibit both wild-type and mutant *EGFR* proteins, and to a lesser extent, T790M *EGFR*. In clinical trials designed to test activity in patients with acquired resistance, however, these drugs have not routinely induced reliable, robust responses. In the LUX-Lung 4 single-arm phase II study from Japan, patients were enrolled with *EGFR*-mutant NSCLC that had progressed on gefitinib/erlotinib and chemotherapy. Treatment with afatinib was associated with a modest response rate of 8.2%, and median PFS of 4.4 mo with median OS of 19.0 mo^[45]. Similarly, the larger placebo controlled trial of LUX-Lung 1 trial of afatinib after failure of chemotherapy and erlotinib or gefitinib evaluated 390 patients on afatinib and 195 patients on placebo. Compared with the afatinib group, the placebo group had an identical OS (10.8 mo *vs* 12.0 mo; HR 1.08; 95%CI: 0.86-1.35; $P = 0.74$). However, median PFS was statistically better in the afatinib group (3.3 mo *vs* 1.1 mo; HR 0.38; 95%CI: 0.31-0.48; $P < 0.0001$), yet the response rate was still unimpressive in this group (7%)^[29]. Dacomitinib (PF-00299804) is another irreversible TKI active against *EGFR*, *HER2*, and *HER4*. In a preliminary report of a phase II studying patients with NSCLC after failure of chemotherapy and erlotinib, responses were seen in 3 of 62 evaluable patients (5%)^[46]. To confirm the activity in this population, a large phase III study, the Canadian BR.26 trial, randomized 720 patients to dacomitinib or placebo for progressive disease after treatment with chemotherapy and an *EGFR* TKI (ClinicalTrials.gov identifier NCT01000025). According to a recent press release, however, this trial failed to meet its primary objective of prolonging overall survival *vs* placebo, with results to be reported in upcoming meetings^[47]. Another phase III study comparing dacomitinib *vs* gefitinib in treatment-naïve patients with *EGFR*-mutant NSCLC is still ongoing (ClinicalTrials.gov identifier NCT01774721). With other second-generation *EGFR* inhibitors including neratinib^[48] and XL647 (which also inhibits VEGFR)^[49], similarly low response rates were reported in trials of acquired resistance.

The strategy of combination therapy incorporating second-generation inhibitors has also been employed with

mixed success. Cetuximab, a monoclonal antibody against EGFR, adds little activity when added to erlotinib in the setting of acquired resistance^[50]. However, the combination of afatinib and cetuximab is surprisingly effective for acquired resistance. In a phase I b study, patients with acquired EGFR inhibitor resistance were given 40 mg daily of afatinib plus 500 mg/m² of cetuximab every other week. Of 61 reported patients at the recommended dose, 35% had confirmed response and 95% had stable disease or better, including patients with tumors with and without T790M mutations. Side effects including rash, diarrhea, and mucositis were significant^[51]. Follow-up trials of these agents for patients with *EGFR*-mutant NSCLC are currently in development. The strategy of combined EGFR and MET inhibition has also been employed in phase I / II trials. In a dose-finding trial of erlotinib plus the MET and VEGFR inhibitor cabozantinib (XL-184), 53 patients were evaluable and had a response rate of 8%. Interestingly, the two patients with confirmed *MET* copy number gain had disease shrinkage^[52]. Preliminary results from a phase I study using dacomitinib plus crizotinib (a MET and ALK inhibitor) noted a response rate of 5% in 20 patients who had previously had response or prolonged stable disease on an EGFR TKI^[53]. Further study of the infrequent *MET* amplification cohort will be of interest using this combination.

Although the second-generation agents do not appear to effectively combat acquired resistance, a novel class of third-generation EGFR inhibitors has been recently identified that much more potently inhibits mutant EGFR with T790M than wild type EGFR. The first such described molecules demonstrated impressive preclinical effectiveness in a mouse model of T790M-dependent acquired EGFR resistance^[54]. Since that time, two compounds with similar affinity for mutant, including T790M, EGFR protein, but minimal binding of wild-type EGFR, have early phase I results reported: CO-1686 and AZD9291. In a preliminary report of the CO-1686 clinical trial, 56 patients with *EGFR* activating mutations and failure of prior EGFR TKI therapy have been enrolled to a dose escalation trial. Of the 9 patients with tumors testing positive for T790M who were treated at the highest dose, 6 responded, 2 had stable disease (with slight decrease), and 1 patient progressed on therapy^[55]. In a similarly designed dose escalation study of AZD9291, 35 patients were treated with doses ranging from 20-80 mg. Fifteen of 35 patients (43%) had a confirmed or unconfirmed partial response, including those with and without documented T790M mutation. The majority of patients had some degree of tumor control, and only 4 patients progressed initially on treatment. Interestingly, wild-type EGFR toxicity for both agents appears quite mild, with rash and diarrhea infrequently reported^[56].

FIRST-GENERATION ALK TKI

Activating EGFR mutations are not the only actionable genetic alterations in NSCLC. In 2007, Japanese researchers working with NSCLC cells discovered an inversion

in chromosome 2p resulting in a novel fusion gene comprised of portions of the echinoderm microtubule-associated protein-like 4 (*EML4*) gene and the anaplastic lymphoma kinase (*ALK*) gene, including its entire intracellular tyrosine kinase domain. The *EML4* fusion partner mediates ligand-independent dimerization of ALK, leading to constitutive kinase activity. *EML4*-ALK fusion protein was tumorigenic in mice, and the Japanese researchers detected this transcript in about 5 out of 75 (6.7%) tumors from NSCLC patients^[57]. Subsequent published series have suggested that the frequency of *ALK* gene rearrangement in unselected NSCLC patients is about 3% to 6%^[58-63]. Besides *EML4*, several other fusion partners of *ALK*, *e.g.*, *KIF5B* and *TFG*, have been identified^[64,65]. Similar to activating *EGFR* mutations, *ALK* gene rearrangements are associated with younger age, never or light smoking status, and adenocarcinoma histology; however there is equal distribution by sex^[66,67].

Crizotinib, an oral, small-molecule inhibitor of ALK and c-Met, was originally developed as a potential therapeutic agent for *ALK*-positive anaplastic large cell lymphoma (ALCL)^[68]. The drug has indeed demonstrated activity in *ALK*-positive ALCL^[69] as well as *ALK*-positive diffuse large B cell lymphoma and inflammatory myofibroblastic tumors^[70,71]. However, crizotinib has been most widely applied in the treatment of NSCLC with *ALK* gene rearrangements after marked activity was noted in the patient population in the phase I trial, leading to FDA approval of the drug^[72]. In a phase III study, 347 patients with locally advanced or metastatic *ALK*-rearranged NSCLC who had received one prior platinum-based regimen were randomized to crizotinib or chemotherapy with either pemetrexed or docetaxel. The median PFS was 7.7 mo in the crizotinib group, significantly superior to the 3.0 mo in the chemotherapy group (HR for progression or death with crizotinib, 0.49; 95%CI: 0.37-0.64; *P* < 0.001). Common adverse events associated with crizotinib were visual disorders, nausea, diarrhea, vomiting, constipation, and elevated liver enzymes^[73]. Rare cases of esophageal ulceration associated with crizotinib have also been reported^[74]. In August 2011, only four years after the discovery of *ALK* gene rearrangements in NSCLC, the FDA granted accelerated approval to crizotinib for patients with *ALK*-positive NSCLC^[75]. An ongoing clinical trial seeks to demonstrate superiority of crizotinib compared to first-line platinum/pemetrexed chemotherapy for *ALK*-rearranged (ClinicalTrials.gov identifiers: NCT01154140 and NCT01639001). Accrual is complete and results are awaited, though crizotinib is commonly used in the first line setting without this evidence.

OVERCOMING RESISTANCE TO CRIZOTINIB

Unfortunately, many tumors with *ALK* gene rearrangements eventually acquire resistance to crizotinib, frequently within one year, similar to *EGFR*-mutant NSCLC

developing resistance to erlotinib or gefitinib. Researchers from Massachusetts General Hospital and collaborators analyzed 18 patients with crizotinib-resistant NSCLC and discovered that almost one-quarter of the patients had either secondary mutations in the *ALK* tyrosine kinase domain or *ALK* fusion gene amplification. About half of the patients were found to have tyrosine kinase activity via EGFR or KIT, thus bypassing the inhibited ALK-mediated pathway^[76]. The L1196M mutation has been shown to be a gatekeeper mutation in the *ALK* kinase domain, conferring resistance to crizotinib^[76-79], similar to the *EGFR* T790M mutation that confers resistance to erlotinib. Besides *EGFR* mutations^[76,78], *KRAS* mutations have also been identified as a possible mechanism of crizotinib resistance in a separate series of crizotinib-resistant patients from the University of Colorado^[78].

Multiple second-generation ALK inhibitors have been developed with increased potency and potential to overcome acquired resistance to crizotinib, including ceritinib (LDK378), AP26113, and alectinib (CH/RO5424802). Ceritinib has recently been shown to have efficacy against crizotinib-naïve as well as crizotinib-resistant *ALK*-positive lung cancer. In a multicenter phase I study, 131 patients with advanced malignancies harboring a genetic alteration in *ALK*, including 123 patients with *ALK*-rearranged NSCLC, received ceritinib orally at doses of 50 mg to 750 mg once daily. Among the 88 NSCLC patients who received ceritinib at 400-750 mg daily, the ORR was 70%. In the subset of 64 crizotinib-resistant patients, the ORR was similar at 73%, with responses observed in patients with different crizotinib resistance mutations, patients without detectable mutation, and even patients with untreated CNS metastases. In all 123 NSCLC patients, the median PFS was 8.6 mo (95%CI: 4.3-19.3). Ceritinib appeared to have more toxicities than crizotinib, however, with the most common adverse events, including all grades, being nausea (72%), diarrhea (69%), vomiting (50%), and fatigue (31%)^[80]. The drug has advanced to phase III clinical trials, being compared *vs* chemotherapy for *ALK*-rearranged NSCLC in the first-line setting (ClinicalTrials.gov identifier NCT01828099) or in the third-line setting for patients previously treated with chemotherapy and crizotinib (ClinicalTrials.gov identifier NCT01828112).

Another promising second-generation ALK inhibitor is AP26113, which exhibits activity against all 9 clinically-identified crizotinib-resistant mutants, including the L1196M gatekeeper, in preclinical experiments^[81,82]. Like most other ALK inhibitors, AP26113 also inhibits ROS1, an actionable target to be discussed later in this review, and selectively inhibits *EGFR* T790M without affecting the native receptor^[83]. In a phase I / II multicenter study, 55 patients with advanced malignancies, including 47 with NSCLC refractory to available therapies, received daily doses of AP26113. Of the 24 patients who had *ALK*-positive solid tumors, 15 responded. Among *ALK*-rearranged NSCLC patients with prior crizotinib only, 12 out of 16 (75%) responded. The drug appeared to

have activity in the CNS as well. Four out of 5 *ALK*-positive patients with untreated or progressing CNS lesions had evidence of radiographic improvement in the CNS, including 1 patient resistant to both crizotinib and LDK378. The most common adverse events were fatigue (40%), nausea (36%), and diarrhea (33%), generally at CTCAE grade 1/2^[84].

A third ALK inhibitor in development is alectinib, previously known as CH/RO5424802. In a phase I / II study of 58 patients with *ALK*-rearranged NSCLC and no prior ALK inhibitor therapy, the overall response rate for alectinib in 46 patients on the phase II part of the study was 93.5% (95%CI: 82.1-98.6) with 2 CRs and 41 PRs. With a median follow-up period of 12.6 mo, 47 out of 58 patients were still on study treatment, and the median treatment duration had passed 10.3 mo^[85]. Alectinib has been shown to have activity post crizotinib as well. In a phase I study of alectinib in 37 patients with *ALK*-rearranged NSCLC who progressed after crizotinib and chemotherapy, partial response (PR) was observed in 48% of all patients and 59.5% of the subgroup of patients receiving doses of 460 mg or higher twice a day. Median PFS had not been reached, with the median duration on study ranging from 39 d to over 347 d^[86]. Sixteen of these *ALK*-rearranged NSCLC patients had CNS metastases. Although PFS had not been reached by June 2013, alectinib demonstrated rapid benefit in brain metastases in a number of patients, including those resistant to crizotinib^[87]. The most common side effects of the drug were fatigue, myalgia, cough, liver enzyme elevation, peripheral edema and rash^[86,87].

There are also other second-generation ALK inhibitors in earlier stages of clinical development. For example, X-396, a potent and highly specific ALK TKI, demonstrated preclinical activity against the most common *ALK* fusions as well as against secondary *ALK* mutations that conferred resistance to crizotinib^[88]. X-396 is currently in phase I development (ClinicalTrials.gov identifier NCT01625234). PF-06463922 is a promising next-generation ALK/ROS1 inhibitor that has potent and selective inhibitory activity against all known acquired crizotinib-resistant mutations. PF-06463922 is also capable of penetrating the blood brain barrier in preclinical animal models^[89]. The drug is also currently in phase I development (ClinicalTrials.gov identifier NCT01970865). ASP3026 is another potent ALK inhibitor that also has activity against crizotinib-resistant tumors in mouse model^[90]. ASP3026 is currently in phase I development (ClinicalTrials.gov identifier NCT01401504).

OTHER "ACTIONABLE" MOLECULAR TARGETS

The discovery of the oncogenic alterations involving *EGFR* and *ALK* and their inhibitors has revolutionized the treatment of non-small cell lung cancer over the past decade. However, *EGFR*-mutant and *ALK*-rearranged cancers make up less than one-fifth of all NSCLC cases

Table 1 Selected current targeted therapies for non-small cell lung cancer and their stages of development

Drug	Company	Stage of development in NSCLC
EGFR activating mutations		
Gefitinib	AstraZeneca	Approved
Erlotinib	Roche	Approved
Afatinib	Boehringer Ingelheim	Approved
Dacomitinib	Pfizer	Phase III
CO-1686	Clovis	Phase I / II
AZD9291	AstraZeneca	Phase I / II
ALK gene rearrangements		
Crizotinib	Pfizer	Approved
LDK378	Novartis	Phase III
AP26113	ARIAD	Phase II
Alectinib	Chugai	Phase II
X-396	Xcovery	Phase I
PF-06463922	Pfizer	Phase I
ROS1 gene rearrangements		
Crizotinib	Pfizer	Phase II (approved for ALK-positive NSCLC)
LDK378	Novartis	Phase II
PF-06463922	Pfizer	Phase I
HER2 activating mutations		
Trastuzumab	Genentech	Phase II
Afatinib	Boehringer Ingelheim	No HER2-mutant NSCLC specific trial
Neratinib	Puma Biotechnology	Phase II
BRAF activating mutations		
Dabrafenib	GlaxoSmithKline	Phase II

NSCLC: Non-small-cell lung cancer; ALK: Anaplastic lymphoma kinase.

in the United States. Several other potentially actionable molecular targets have recently been found.

ROS1 gene rearrangements, involving the receptor tyrosine kinase *ROS1* and partners *CD74*, *SLC24A2*, and *FIG*, are the driver oncogenes in a small subset of NSCLC^[91-93] also responsive to crizotinib^[91]. An expansion cohort of the phase I crizotinib study PROFILE 1001 included 40 patients with *ROS1*-positive NSCLC. In the 35 patients who were evaluated, the ORR was 60% with 2 complete response (CR), 19 PR, and 10 stable disease (SD) cases. Six-month PFS probability was 76% (95%CI: 55-88). Median PFS had not been reached when the results were reported at the World Conference on Lung Cancer in October 2013^[94]. Unfortunately, acquired resistance to crizotinib in *ROS1*-positive patients has also been reported. A patient with the *ROS1-CD74* fusion oncogene initially responded dramatically to two mo of crizotinib treatment but then progressed in the third month. Her tumor was found to have a novel G2032R mutation in the *CD74-ROS1* fusion junction that had not been observed before crizotinib treatment^[95]. Recently a promising *ROS1* inhibitor, foretinib, has been shown to demonstrate efficacy against *ROS1*-rearranged tumor cells, including crizotinib-resistant cells. Foretinib, which also inhibits other kinases including *MET* and *VEGFR2*, is being studied in phase I and II studies in a variety of cancers.

About 1% to 2% of NSCLC tumors have mutations in *HER2* exon 20^[96,97], which is not clearly associated with *HER2* amplification. Although anti-Her2 therapies are in-

effective in *HER2*-amplified NSCLC^[98,99], *HER2*-mutant NSCLC has been shown to be responsive to trastuzumab plus chemotherapy, with an overall response rate of 50% and median PFS of 5.1 mo in one case series^[96]. Afatinib, the ErbB family inhibitor approved for *EGFR*-mutant NSCLC as discussed earlier in this review, also has clinical activity against *HER2*-mutant NSCLC in a small case series^[96,100]. As HER family members signal via the PI3K-AKT-mTOR cascade, recent attempts have been made to inhibit both *HER2* and mTOR in *HER2*-driven cancers. In a phase I study of the combination of neratinib (a small molecule pan-HER inhibitor) and temsirolimus (an mTOR inhibitor), 7 patients with *HER2*-mutant NSCLC were treated, with 2 showing partial responses^[101]. An ongoing phase II study compares neratinib *vs* neratinib plus temsirolimus in patients with *HER2*-mutant NSCLC (ClinicalTrials.gov identifier NCT01827267).

BRAF activating mutations can be observed in 1%-3% of NSCLC^[102,103]. In one case series, about half of these *BRAF* mutations are the V600E mutation that is also seen in melanoma. Unlike *EGFR*, *ALK*, and *ROS1* genetic alterations that are associated with light or never smoking status, *BRAF* mutations in NSCLC are often reported in current or former smokers^[103]. In a phase II study, 17 patients with *BRAF* V600E-mutant NSCLC received dabrafenib, which had previously shown activity in *BRAF* V600E-mutant melanoma. Seven patients out of 13 (54%) evaluable patients had PR, with 1 patient having stable disease. The drug was generally well tolerated, and the median duration of treatment was 9 wk, with the longest duration of response being 49 wk when the results were reported at the 2013 ASCO Annual Meeting^[104]. An ongoing phase II study tests dabrafenib *vs* dabrafenib and trametinib, an inhibitor of MEK that is downstream of BRAF, in patients with *BRAF* V600E mutation-positive NSCLC (ClinicalTrials.gov identifier NCT01336634).

Other rare genetic alterations in NSCLC have been found and have potentially therapeutic agents include *MET* amplification, *FGFR1* amplification, *RET* translocations, and *MEK1* mutations. Investigations using inhibitors of these oncogenic pathways are ongoing, with anecdotal responses reported in some cases. A detailed discussion of these targets is beyond the scope of this review. Table 1 summarizes the targeted therapies for NSCLC that have already been approved or are still in ongoing clinical trials.

CONCLUSION

Until several years ago, the only therapeutic option for advanced NSCLC was cytotoxic chemotherapy. The discovery of activating *EGFR* mutations and the unprecedented efficacy of erlotinib and gefitinib in *EGFR*-mutant NSCLC ushered in an era of truly personalized cancer care. There is increasing evidence that targeted therapies yield better outcomes than traditional chemotherapy in appropriate patients. The Lung Cancer Mutation Consortium recently reported that an actionable

driver was detected in 64% of patients with pulmonary adenocarcinoma and that among the 938 patients the consortium tracked, the median survival was 3.5 years for the 264 with an oncogenic driver treated with genotype-directed therapy, 2.4 years for the 318 with an oncogenic driver with no genotype-directed therapy, and 2.1 years for the 360 with no driver identified ($P < 0.0001$)^[105].

With the advance of next-generation sequencing, one can foresee a future in which every single tumor will be sequenced at the time of diagnosis to find potential driver mutations that can be therapeutically targeted. While some rare patients have had astounding disease remission, defined as long-term complete responses to EGFR TKI therapy^[106,107], these patients are still usually receiving active therapy and therefore cannot truly be considered “cured”. Therefore, challenges remain on how to overcome the seemingly inevitable acquired resistance to these therapies. The optimal sequence for the use of multiple inhibitors of the same target and the efficacy and tolerability of combinations of inhibitors of various oncogenic pathways are being actively studied. In addition, the emerging promise of immunotherapies such as PD-1/PDL-1 directed antibody therapy opens the door for studies of potential synergy with these drugs and tyrosine kinase targeted therapeutics. Even if a cure for advanced lung cancer still remains out of reach, one can hope that in the near future advanced NSCLC may be controlled like other chronic diseases with well-tolerated and effective therapies.

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WJCO 5th Anniversary Special Issues (1): Lung cancer**Apoptosis block as a barrier to effective therapy in non small cell lung cancer**

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the treatment of non-small cell lung cancers with platinum-based combination chemotherapy. New biomarkers of prognosis as well as new therapies focusing on molecular targets are emerging helping to identify patients who are likely to benefit from therapy. These are as yet only available to the minority of patients. Drug resistance remains the major cause for treatment failure. Apoptosis block as a mechanism for drug resistance and potential routes to overcome this will be reviewed.

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Abstract

Lung cancer, is the most common cause of cancer death in men and second only to breast cancer in women. Currently, the first line therapy of choice is platinum-based combination chemotherapy. A therapeutic plateau has been reached with the prognosis for patients with advanced non-small cell lung cancer (NSCLC) remaining poor. New biomarkers of prognosis as well as new therapies focusing on molecular targets are emerging helping to identify patients who are likely to benefit from therapy. Despite this, drug resistance remains the major cause for treatment failure. In this article we review the role of apoptosis in mediating drug resistance in NSCLC. Better understanding of this fundamental biological process may provide a rationale for overcoming the current therapeutic plateau.

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Key words: Apoptosis; Lung cancer; Adjuvant therapy; Mitochondria; BAX; BAK; BCL2

Core tip: A therapeutic plateau has been reached with

INTRODUCTION

Lung cancer is the leading cause of cancer death in the United Kingdom accounting for 24% of all male related cancer deaths and 20% in females^[1]. Similar trends are seen in the United States with lung cancer representing the most common cause of cancer death in men and women, recently overtaking breast cancer in the latter^[2]. In the United States, more than 213000 new cases were diagnosed in 2007 with a total of 1.61 million cases worldwide in 2008^[2,3]. The majority of these cancers (75%-80%) are non-small cell lung cancers (NSCLC)^[4].

Surgery is the mainstay of treatment for early stage and localised disease (Stage I and II and selected IIIA). Multimodal therapy is the norm for regionally advanced disease in the form of adjuvant chemotherapy. Palliative chemotherapy forms the mainstay of treatment in patients with advanced metastatic disease^[5]. Five year survival rates following lung resection for NSCLC are I A-73%, I B-54%, II A-48%, II B-38%, IIIA-25%^[6]. The majority of patients have advanced disease at the time of

diagnosis and therefore are not surgical candidates. This is illustrated by the fact that in the United Kingdom only 14% of patients diagnosed go on to have surgical resection^[7]. In those patients that are surgical candidates, more than 50% will develop a recurrence. Adjuvant chemotherapy has been used with limited success to decrease the recurrence rates but this has only yielded a survival benefit of 5%-15%^[8].

For patients not suitable for surgery due to advanced disease or those who have suffered recurrence following resection, chemotherapy forms the mainstay of treatment with evidence that platinum based therapies are most effective in the first line setting^[9,10]. Despite this, in a recent trial of platinum combination therapies in advanced NSCLC, only 30% of patients showed objective disease response and a significant proportion suffered toxic side-effects such as neutropenia (27%), anaemia (10%), thrombocytopenia (13%), alopecia (21%) and nausea (4%). During the trial, deaths due to study drug toxicity were in the region of 1%^[11]. This demonstrates the major problem of drug resistance in NSCLC to standard platinum based therapies and the associated toxicities.

MOLECULAR TARGETED THERAPY

A recent major advance in the management of NSCLC has been the identification of activating mutations in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR)^[12]. These mutations, found in 10% of NSCLC specimens in the United States and Europe, are associated with increased sensitivity to EGFR tyrosine kinase inhibitors erlotinib and gefitinib^[13]. EGFR mutations are often accompanied by gene amplification^[14]. Mutation of the EGFR can lead to signal transduction independent of ligand stimulation, relaying signals to the PI3K-AKT-mTOR pathway involved in cell survival, or to the RAS-RAF-MEK-ERK pathway involved in cell proliferation^[15]. The fact that kinase inhibition leads to apoptosis in cells with mutant EGFR supports the notion that these cells are "addicted" to signalling *via* the mutant proteins^[16]. This explains the dramatic response to EGFR tyrosine kinase inhibitors in EGFR mutated NSCLC with associated improvement in survival^[17,18].

Molecular profiling in NSCLC has revealed a number of other mutations such as EML4-ALK translocation^[19]. This is associated with extreme preclinical sensitivity to respective ALK kinase inhibition in both preclinical and clinical settings^[20,21]. Additional molecular subclasses with associated somatic gene alterations have been discovered, predominantly in lung adenocarcinomas and include mutations of BRAF^[22], Her2^[23] and PIK3CA^[24]. The continual discovery of new molecular subclasses represents significant progress in the treatment of NSCLC but they should not necessarily be viewed as a panacea as resistance to these novel targeted therapies are being reported^[25]. The above molecular subclasses still only account for less than half all new cases of the disease. The majority of patients still rely on standard combination

chemotherapy of platinum based doublet regimens.

In addition to molecular tumour markers, there is increasing interest in developing biomarkers associated with NSCLC. These may be predictive for response to treatment or prognostic. An area of interest in our unit relates to the link between plasma fibrinogen and NSCLC. In early stage disease amenable to surgical resection, raised fibrinogen is associated with increased risk of incomplete resection and correlates with T stage of tumour^[26]. More recently, serum fibrinogen has been shown to be an independent predictor for both disease recurrence and overall survival in both resectable and advanced disease^[27,28]. The mechanism by which fibrinogen acts as a marker is not fully understood. Its role as an extracellular matrix, in promoting angiogenesis and metastasis has been proposed^[29-32].

APOPTOSIS AND DRUG RESISTANCE

Cisplatin (*cis*-diamminedichloroplatinum II) is the most effective platinum based therapy in the first line setting for NSCLC^[33]. It forms DNA-platinum covalent adducts resulting in inhibition of DNA replication, suppression of RNA transcription and protein translation, attempted DNA repair as well as disturbance of the cell cycle and the activation of apoptosis^[34].

Apoptosis serves as a natural barrier to cancer development^[35]. Normally the accumulation of genetic mutations required to drive uncontrolled cell cycle progression and tumorigenesis would result in triggering of apoptosis within a cell^[36]. The ability of cancer cells to evade apoptosis is, therefore, an essential hallmark of cancer^[37]. Defects in apoptosis, harboured by cancer cells, not only underpin tumorigenesis but also drug resistance^[38]. Resistance of NSCLC cells to a diverse range of cytotoxic therapies suggests a defect in apoptosis signalling^[39]. Further understanding of the mechanisms by which apoptosis resistance occurs and how this can be overcome will be important in order to administer a more rational approach to anticancer drug design and therapy.

APOPTOSIS

Apoptosis, a genetically encoded programme of cell death, was originally defined based on the morphological features observed as the cell died; nuclear condensation, nuclear and cellular fragmentation, membrane blebbing and phagocytosis of the dying cell in the absence of inflammation^[40]. The process of apoptosis is conserved in a wide range of multicellular organisms from worms to humans and plays a key role in normal development and homeostasis. The apoptosis phenotype is produced through the activity of cysteine-aspartic proteases, a family of cysteine proteases termed caspases. A hierarchical cascade of activation occurs which results in apoptosis. Two groups of caspases have been classified; the initiator caspases and the effector caspases.

The initiator caspases, caspase-8, -9 and -10, are ac-

tivated early in apoptosis signalling and have restricted cleavage targets, limited to self cleavage, the effector caspases and BID (caspase-8). In contrast, the effector caspases, caspase-3, -6 and -7, have hundreds of cleavage sites broadly distributed throughout the cell^[41]. The effector caspases are held in the cytosol as inactive dimers. The activating event, catalyzed by the initiator caspases, involves conversion to catalytically active enzymes by cleavage in the linker region between the large and small active subunits. This allows intramolecular rearrangements to form an enzymatically active dimer^[42]. Caspase-3 and -7 display highly similar substrate specificity and carry out redundant but essential functions in apoptotic cell death as mouse embryonic fibroblasts lacking both enzymes are resistant to intrinsic and extrinsic apoptotic stimuli^[43].

Caspase-8 and -10 are involved in the death receptor signalling pathway whereas caspase-9 is involved in mitochondrial apoptosis.

It is clear caspase activation is a critical step in the execution of apoptosis and is generally a terminal event for the cell. Regulation of the process occurs through the extrinsic, death receptor apoptosis pathway and the intrinsic mitochondrial apoptosis pathway.

EXTRINSIC APOPTOSIS PATHWAY

Apoptosis occurs via the extrinsic apoptosis pathway as a result of signalling through death receptors expressed on the surface of mammalian cells. In the 1970's it was identified that certain products of lymphocytes and macrophages caused the lysis of certain types of cells, especially tumour cells. This product was termed 'tumour necrosis factor (TNF)^[44,45]. It has been established that TNF has its effect via cell surface receptor binding and activation. At least 18 TNF family ligands and 29 receptors have been identified in humans^[46]. In cancer research, interest has grown around the use of TNF-related apoptosis-inducing ligand (TRAIL) and targeting its receptors, members of the TNF superfamily, since the observation that recombinant human (rh) TRAIL induces apoptosis in various tumour cells but not in normal cells^[47].

TRAIL activates apoptosis by binding to specific transmembrane receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5)^[48,49]. It can also bind truncated, non-functional receptors TRAIL-R3 and TRAIL-R4 known as decoy receptors (DcR1 and DcR2)^[50]. Upon binding TRAIL to the death receptors (DR4, DR5), trimerization of the receptors occurs and a complex is formed termed the death-inducing signalling complex (DISC). This is the key step for subsequent initiator caspase activation to occur.

INTRINSIC APOPTOSIS PATHWAY

The other major apoptosis pathway leading to caspase activation and cell death is the Intrinsic (mitochondrial) apoptosis pathway. As DISC formation is the key step

in extrinsic (death receptor) signalling so mitochondrial outer membrane permeabilization (MOMP) is the key requirement for caspase activation and apoptosis via the mitochondrial apoptosis pathway. As the name suggests, this apoptosis pathway is engaged primarily as a result of internal cellular stresses such as DNA damage, ER stress^[41].

MOMP during apoptosis is primarily controlled by the BCL2 family of proteins^[51]. The pro-apoptotic members, BAX^[52] and BAK^[53], contain BH1-3 domains. BAX and BAK are often referred to as effector proteins as there is an absolute requirement for their activation at the outer mitochondrial membrane for MOMP to occur^[54].

A diverse range of death signals caused by eg DNA damage, growth factor deprivation leads to a shift in the balance of anti- and pro-apoptotic BCL2 family members with signals produced to engage MOMP. Activation of a "multidomain" proapoptotic member, BAX or BAK, is an essential gateway to mitochondrial dysfunction required for cell death in response to these diverse stimuli^[54].

Upon receipt of a death signal, BAX translocates to the mitochondrial surface where BAK already resides^[55,56]. A detailed sequence of events from BAX translocation to the OMM, subsequent activation and MOMP has been described recently^[57]. The requirement for activated BAX and/or BAK for MOMP to occur is clear, the mechanism by which they bring about MOMP is not. The key feature of MOMP in bringing about caspase activation and apoptosis is cytochrome C release. This is clear from evidence that cells lacking cytochrome C fail to activate caspases in response to UV irradiation, serum withdrawal, or staurosporine^[58]. The primary function of cytochrome C is in oxidative phosphorylation as it is a key component of the electron transport chain and it is found loosely associated with the mitochondrial inner membrane. The other important mitochondrial intermembrane space protein released during MOMP is second mitochondria-derived activator of caspase (SMAC) also termed DIABLO (direct IAP binding protein with low pI). It binds XIAP's, antagonising their ability to inhibit caspases^[59,60].

APOPTOSIS BLOCK IN CANCER

Having outlined how apoptosis proceeds, the mechanisms by which cancer cells evade this process will be explored. Block in mitochondrial apoptosis has been broadly divided into three groups^[61]: (1) Class A block occurs when normal generation of proapoptotic activators by aberrant behavior is inhibited. The mechanisms by which aberrant behaviour, such as genomic instability and oncogene activation, generate death signals via BH3-only proteins is as yet poorly understood. This is an area of ongoing study; (2) Class B block occurs when there is a significant loss of the BCL2 family effectors, BAX and BAK; and (3) Class C block occurs when increased expression of an anti-apoptotic BCL2 family protein is present, thereby inhibiting or sequestering pro-apoptotic BH3-only proteins. In this scenario, the cell has generated

an appropriate BH3-only death signal but it is inhibited by opposing anti-apoptotic expression. Cells in this state are referred to as “primed for death” and are “addicted” to the overexpression of the anti-apoptotic protein^[62].

Apoptosis blocks such as described above may determine the sensitivity of a cancer cell to a wide range of diverse chemotherapy agents and explain frequently observed phenomenon of multidrug resistance^[51]. The fact that being primed for death (class C block) is apparently more common in tumours than in normal cells may help explain why chemotherapy is often more toxic to cancers^[51].

Having classified apoptosis block in tumours, a new technique for determining what type of block a given cell employs has been developed termed BH3 profiling^[61,62]. The basic method involves incubation of mitochondria isolated from tumour cells with a panel of BH3 peptides. By assessing the pattern of response, the type of apoptosis block present can be identified. This may be used in the future to select drugs targeting anti-apoptotic proteins as a strategy for improving efficacy of drug treatments.

EXPRESSION OF BCL2 FAMILY MEMBERS IN NSCLC

Much focus has been placed on the role of BCL2 as a prognostic predictor in NSCLC. Many studies over the last 15-20 years have assessed its expression in many differing solid tumours. Given its function as an antiapoptotic protein, it would be predicted that overexpression would result in a more aggressive and treatment resistant phenotype. The published data is mixed with regard to its role as a prognostic marker.

A meta-analysis from 2003 compiled 28 studies from 1993 to 1999 which report the expression of BCL2 in NSCLC and the prognostic value of its expression in the primary tumour^[63]. Immunohistochemistry was used to detect expression in all studies. Of the 28 studies included, 11 concluded Bcl-2 expression was a good prognostic marker, 14 concluded it was not prognostic for survival with only 3 linking Bcl-2 expression to poor prognosis. Having performed the meta-analysis, the authors concluded that Bcl-2 positive tumours had a significantly better survival than those with Bcl-2 negative tumours. Given its function in the apoptotic pathway this would appear a paradoxical conclusion. One theory suggests that loss of Bcl-2 expression correlates with tumour de-differentiation and therefore a more aggressive phenotype^[64]. As discussed above, increased expression of BCL2 would likely confer a class C block in apoptosis and as such these tumours would be primed for death perhaps explaining the increased survival. This should be interpreted with caution as a result of heterogenous treatment patients in these studies received. Given the highly complex interplay between Bcl-2 and its other family members in regulating apoptosis, it is not surprising that the study of one anti-apoptotic member yielded such a result. As knowledge about the interplay between Bcl-2

family members improves, other targets or combination of targets may be more relevant to study rather than a single protein in isolation.

The crucial role the proapoptotic multidomain proteins BAX and BAK play in mitochondrial outer membrane permeabilisation warrants further study as prognostic markers. Loss of both proteins would confer a class B apoptosis block. Many studies exist reporting the status of BAX and BAK expression in NSCLC but again only report in isolation and each is considered separately as prognostic markers.

Altered BAX expression is frequently reported in NSCLC^[65-68]. None of these studies conclude that altered BAX expression has significant value as a prognostic marker, although none have investigated the expression of BAK together with BAX. Fewer studies report the incidence of altered BAK expression^[69,70]. These studies report the incidence of BAK loss at 42% and 59%. They also include data on BAX expression with loss reported in 34% and 47%. Neither study reports the incidence of double loss. Both conclude that no prognostic value is attributed to BAX or BAK expression in NSCLC but again each protein is analysed in isolation. Data from the International Adjuvant Lung Cancer Trial (IALT) suggests a trend toward increasing chemosensitivity with increasing BAX level^[71]. The converse effect of BAX negativity was not reported.

STRATEGIES TO OVERCOME MITOCHONDRIAL APOPTOSIS BLOCK

Given the frequent loss of expression of each protein, it is likely a significant portion of patients with NSCLC will have BAX and BAK double loss and given the evidence that this results in a highly apoptosis resistant phenotype it would be important to assess the impact on double loss on both survival and response to standard chemotherapy and radiotherapy in NSCLC.

Given what is known about the mitochondrial apoptosis pathway, class B block in apoptosis is likely to prove resistant to a range of targeted therapies. BH3 mimetics would be predicted to be ineffective due to absence of effector proteins BAX and BAK. Alternative strategies will be required to treat these potentially multidrug resistant cancers. *In vitro* evidence exists to show that in the absence of BAX and BAK, detachment of hexokinase from mitochondrial VDAC can lead to cytochrome C release and therefore mediate cell death^[72]. This can be achieved using either a competitive peptide, or by inhibiting AKT (which in turn regulates hexokinase interaction with VDAC) and prove a rational approach to treating cancers exhibiting Akt activation or an imbalance in the expression of antiapoptotic and proapoptotic members of the Bcl-2 family.

CONCLUSION

NSCLC presents a major health burden worldwide. Plati-

num based chemotherapy is the mainstay of treatment in the clinic today, although denovo and acquired drug resistance has resulted in a therapeutic plateau since its introduction over 30 years ago. Novel targeted therapies are beginning to emerge that induce apoptosis in certain molecular subclasses. Apoptosis resistance underpins tumorigenesis and drug resistance. Understanding how apoptosis resistance occurs in NSCLC will allow tailoring of therapy and development of novel targets to overcome this problem.

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WJCO 5th Anniversary Special Issues (1): Lung cancer

Surgical strategies in the therapy of non-small cell lung cancer

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Core tip: Lung cancer represents the leading cause of cancer mortality worldwide. To date, surgical resection is the primary mode of treatment for stage I and II non-small cell lung cancer (NSCLC) and has become an important component of the multimodality therapy of even more advanced disease with a curative intention. In fact, individualized treatment options, based on clinical tumor stages, in NSCLC patients have been established in the last years that are displayed in this review.

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Abstract

Lung cancer represents the leading cause of cancer mortality worldwide. Despite improvements in preoperative staging, surgical techniques, neoadjuvant/adjuvant options and postoperative care, there are still major difficulties in significantly improving survival, especially in locally advanced non-small cell lung cancer (NSCLC). To date, surgical resection is the primary mode of treatment for stage I and II NSCLC and has become an important component of the multimodality therapy of even more advanced disease with a curative intention. In fact, in NSCLC patients with solitary distant metastases, surgical interventions have been discussed in the last years. Accordingly, this review displays the recent surgical strategies implemented in the therapy of NSCLC patients.

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INTRODUCTION

Lung cancer represents the leading cause of cancer mortality worldwide, accounting for around 1.4-1.6 million deaths each year^[1-4]. While this tumor entity has by far the first place in incidence and mortality among other forms of cancer in males, it is the fourth most frequently malignant tumor in women after breast, cervical and colorectal cancer but it is also the second most common cause of death in the female population^[1]. Improving the survival of lung cancer patients is a major challenge for modern multimodality oncological treatment strategies, considering that the 5-year survival remains less than 15% across all stages of disease with fewer than 7% of patients alive 10 years after diagnosis^[5]. Even with improvements in preoperative staging, surgical techniques,

neoadjuvant/adjuvant options and postoperative care, there are still great difficulties in significantly improving survival, especially in locally advanced disease while early tumor stages theoretically represent the most consistent possibility of modifying the outcome of non-small cell lung cancer (NSCLC)^[5]. Indeed, similar to other malignant tumors, the therapy of NSCLC is stage related. At this, surgical resection is the primary mode of treatment for stage I and II NSCLC and an important component of the multimodality approach to stage IIIA disease with a curative intention. Standard resections include removal of the lobe involved with tumor and systematic evaluation of ipsilateral hilar and mediastinal lymph nodes. Moreover, even in NSCLC patients with solitary distant metastases, surgical interventions have been discussed in the last years. Therefore, the current review displays the recent surgical strategies implemented in the therapy of NSCLC (Figure 1).

NSCLC STAGE I + II (T1-2 N0-1, T3N0)

NSCLC stage I + II (T1-2 N0-1)

Patients with stage I and II NSCLC account for only 25%-30% of all patients presenting with the diagnosis of this malignant tumor^[6]. For medically operable patients, lobectomy, the surgical resection of a single lobe, with mediastinal lymph node dissection (MLND) or systematic lymph node sampling (SLNS), is generally accepted as the optimal procedure for this early tumor stage^[7,8]. Although the role of surgery has not been validated through randomized trials, the favorable results reported in surgical series and the relative infrequency of long-term survival in patients treated without surgery established this therapeutic approach as the treatment of choice^[9,10]. The current literature describes the prognosis for stage I and II NSCLC, undergoing primary surgical resection, expressed in terms of 5-year survival rates, as 60% to 80% for stage I and 30% to 50% for stage II NSCLC^[11].

In general, lung resections for NSCLC are performed through a lateral thoracotomy. However, in patients with early stage NSCLC, video-assisted thoracoscopic surgery (VATS) seems to be an acceptable alternative to the well-known open lobectomy in terms of complications and oncological value in patients with early stage NSCLC^[12].

In fact, in the last years a number of retrospective studies have shown that VATS lobectomy is associated with fewer complications, and shorter length of hospital stay compared to open surgery^[13-20]. These advantages were also described in elderly patients with NSCLC^[13,14,19,21-23]. Furthermore, two meta-analyses and two systematic reviews revealed that patients undergoing VATS lobectomy have reduced perioperative morbidity, mortality and less postoperative pain as short-term benefits compared to patients receiving open surgical resection while the long-term results (survival and recurrence rates) were not significantly different between the two surgical procedures^[12,22,24,25]. In addition, a recent study by

Park *et al.* with more than 6000 NSCLC patients undergoing either VATS or standard open lobectomy, showed not only that VATS lobectomy had fewer complications (38% *vs* 44%) and a shorter length of hospital stay (5 d *vs* 7 d) compared to open surgical resection but rather that patients undergoing VATS at high volume hospitals had a shorter median length of hospital stay (4 d *vs* 6 d) compared with patients receiving surgical resection at low-volume hospitals^[26].

However, the quality and efficiency of the lymphadenectomy (LAD) in NSCLC patients undergoing VATS lobectomy was controversially discussed for a long period of time. A review about 770 patients (VATS: 450 patients, open resection: 320 patients) with cN0-pN2 NSCLC by Watanabe *et al.* examined the total number of lymph nodes, lymph node stations and mediastinal nodes resected by VATS *vs* open lobectomy, and found no difference in any of these categories^[27]. Moreover, data from the recent American College of Surgery Oncology Group Z0030 trial (*n*: 752 patients, VATS: 66 patients; open: 686 patients) has also confirmed the efficacy of LAD by the VATS procedure with demonstrating similar number of lymph nodes removed and lymph node stations assessed by both surgical techniques^[18].

A point for discussion regarding VATS lobectomy in NSCLC patients might be the issue of smaller resection margins compared to the open procedure, as one might suggest that the extent of the bronchial margin has clinically relevant impact on disease-free and overall survival in early-stage non-small-cell lung cancer. However, recent studies showed that the complete surgical resection (R0 resection) but not the extent of the resection margin itself is of prognostic impact^[28].

Based on these findings VATS lobectomy is an appropriate surgical technique in the therapy of patients with early stage NSCLC in terms of morbidity, oncological value and survival compared to open surgery^[12].

It is generally accepted that the treatment of choice in stage I and II NSCLC patients with low surgical risk is the primary surgical tumor resection by excision of the whole affected lobe. However, especially due to the progress and development in CT imaging, a growing number of ground-glass opacities and small tumors are being detected^[29]. Therefore, there is an increasing number of studies dealing with the subject of a sub-lobar resection in such tumor cases. In fact, several retrospective studies have compared sub-lobar resection with complete lobectomy in patients with early NSCLC. For example, in the currently largest study performed by Whitson *et al.*^[30], using the American Surveillance Epidemiology and End Results (SEER) database, with data from 14,473 patients with stage I tumors treated by anatomical segmentectomy or lobectomy, the prognostic impact of both techniques was assessed. The authors demonstrated that the latter approach provides a statistically significant 5-year survival advantage, even after adjustment for other prognostic factors including tumour size.

Nevertheless, sub-lobar resection might be considered especially in the following patients: (1) Compromised

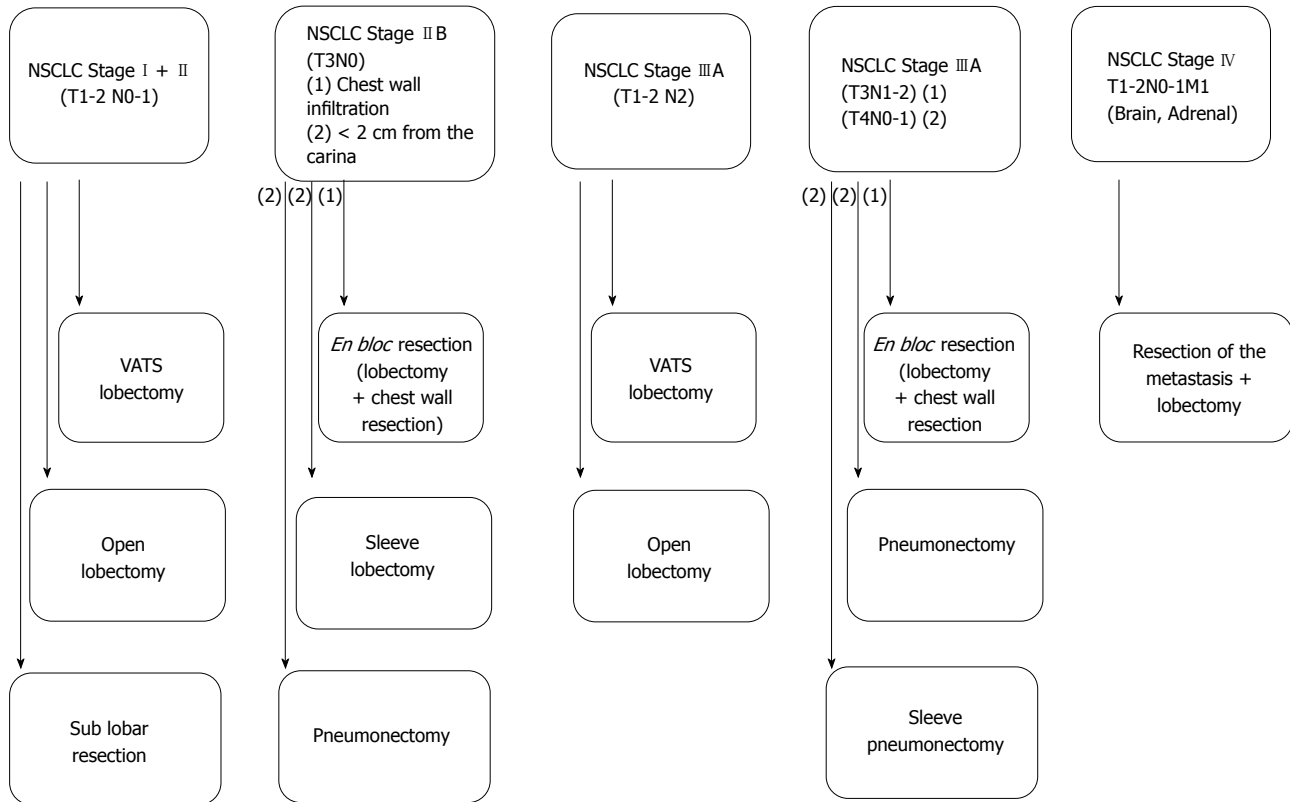


Figure 1 Clinical tumor stages in non-small cell lung cancer and possible surgical treatment options in operable patients. NSCLC: Non-small cell lung cancer; VATS: Video-assisted thoracoscopic surgery.

patients that might not tolerate a complete lobectomy; (2) Patients that could tolerate lobectomy but their tumor size itself might not demand a lobectomy.

As data from randomized trials (other than the LCSG trial^[31]) is still missing, currently it is not clear if sub-lobar resection is really safe enough in patients with contraindication for lobectomy and if it is associated with equivalent oncological long-term results. Perhaps there are some specific tumor characteristics in that a sub-lobar resection should be recommended in particularly. In addition, another point of discussion is the functional benefit achieved by sub-lobar resection compared to complete lobectomy. Interestingly, studies have shown that the difference in the mean reduction of the post-operative FEV1 and FVC ranges from 5%-6%, respectively 1%-6% (Table 1)^[31-33]. As long as it is not proven that the prognostic impact of sub-lobar resection in early stage NSCLC is really equivalent to complete lobectomy, the more invasive procedure should stay the treatment of choice. There are currently 2 randomised controlled trials that may clarify the role of limited resection, *i.e.*, the Cancer and Leukemia Group B 140503 trial (segmentectomy and wedge resection) and the Japan Clinical Oncology Group 0802/West Japan Oncology Group 4607L (segmentectomy) trial^[34,35]. In these trials, selection for limited resection includes tumors 2 cm or less in size, peripheral tumors close to the outer third of the lung and good functional status. Finally, sub-lobar resection should preferably be performed by a segment and not by a wedge

resection as current data in early stage NSCLC patients revealed that a segment resection is associated with a higher cancer-related survival and lower local recurrence rate compared with a wedge resection^[36].

Stage II B (T3N0M0)

Tumor invasion of the chest wall is an uncommon event in NSCLC with only around 5% of all patients. Interestingly, recent studies suggest that a long term survival rate after surgical *en bloc* R0 resection of 40%-50% in case of a T3N0M0 tumor stage can be achieved^[37]. Similar results (40% five-year survival rate in case of a T3N0 status) have been presented by Doddoli *et al.*^[38] in 212 patients with stage II B tumors. At this, the two most important prognostic factors affecting the survival of this patient subgroup are the complete tumor resection and the pathologic nodal status^[39,40].

STAGE III A (T1-2 N2, T3 N1-2, T4N0-1)

Stage III A (T1-2 N2)

Stage III NSCLC includes a heterogeneous population with disease presentation ranging from apparently resectable tumors with occult microscopic nodal metastases to unresectable bulky nodal disease. Patients with T1-2 pN2 tumor stage can be divided into 3 groups: (1) Patients with an unexpected intraoperative finding of a pN2 stage despite the preoperative staging. The reported incidence of an unexpected N2 lymph node status ranges from

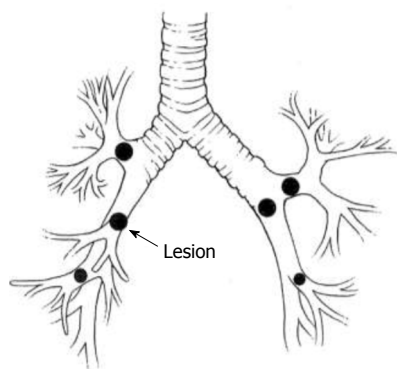


Figure 2 Common anatomical tumor locations for bronchoplastic pulmonary resection. Figure from Faber^[91].

4.7% to 26 %^[41-43]. In this subgroup of patients, the surgical resection should be continued, with a lobectomy and LAD. Cerfolio *et al*^[44] reported a 5-year survival rate in patients with unexpected N2 disease who underwent complete resection followed by adjuvant therapy of 35% while patients with single station N2 disease had even a better outcome^[44]; (2) Patients with preoperative evidence of N2 disease based on the CT or PET-CT findings and histopathological confirmation. In this group of patients a multimodality therapy is preferable^[45]. Interestingly, Ripley and Rusch have recently published a review about the role of induction therapy in NSCLC patients. The authors suggest in this review that a multimodality therapy should be the standard care for stage IIIA (N2) NSCLC patients with resection being offered to patients suitable for complete resection^[46]; and (3) Bulky N2 disease. Patients with extensive (bulky) N2 disease should receive a combined radiochemotherapy and only selected cases with good tumor down-sizing should be evaluated for surgical resection. However, for this group of patients just little data about the optimal therapy is currently available^[47].

Stage IIIA (T3N1-2, T4 N0-1)

In case of a chest wall infiltration (T3) a N2 lymph node status should not be considered as a contraindication for surgical resection in terms of lobectomy with partial chest wall resection and MLND or SLNS. The reported 5-year survival rate after complete resection is 21% in T3N2 disease patients^[48].

In case of T4 tumors with invasion of the main carina a sleeve pneumonectomy can be considered and has become an established procedure in carefully selected patients in experienced centers^[49]. While in patients with a complete resection and a post-operative N0 lymph node status the 5-year survival rate ranges between 25%-45%, it is less than 15% in patients with a post-operative N2 disease^[50].

LUNG CANCER SURGERY IN PATIENTS WITH SOLITARY BRAIN OR ADRENAL METASTASIS

Several studies have shown that patients with T1-2 tumors

Table 1 Functional benefit after sub-lobar resection

Ref.	Patients (L/SL)	Mo (follow-up)	Postoperative reduction of FEV1		Postoperative reduction of FVC	
			L	SL	L	SL
Ginsberg <i>et al</i> ^[31]	85/71	12-18	-11%	-5%	-5%	+1%
Keenan <i>et al</i> ^[32]	174/54	12	-9%	-3%	-4%	-3%
Harada <i>et al</i> ^[33]	45/38	6	-16%	-11%	-14%	-9%

L: Lobar resection; SL: Sublobar resection; FEV1: Forced expiratory volume; FVC: Forced vital capacity.

and simultaneously solitary brain or adrenal metastases seem to benefit from a surgical resection^[51-55]. This special group of patients can undergo a removal of the solitary brain/adrenal metastasis followed by surgical resection of the primary lung tumor. For example, Read *et al*^[51] revealed in 92 NSCLC patients with solitary brain metastases that the 5-year survival rate after curative resection of the lung tumor and brain metastasis was 21%. Similar data were reported by Billing *et al*^[52] in a retrospective analysis of 28 NSCLC patients with solitary brain metastasis undergoing surgical removal of both tumor locations. Also in NSCLC patients with synchronous solitary adrenal metastasis a surgical treatment with resection of the adrenal and lung mass seems to be beneficial especially for patients with a negative lymph node status^[52,54,55].

BRONCHOPLASTIC LUNG RESECTION

Pneumonectomy has been associated with higher morbidity/mortality and a worse 5-year survival rate after resection for NSCLC, compared to lobectomy or less advanced resection techniques^[56-58]. This created the impression that limited anatomical resections in the form of single or double sleeve resections may be beneficial avoiding pneumonectomy related morbidity and mortality. Interestingly, two reviews indicate equivalent survival for patients undergoing either pulmonary sleeve resection or pneumonectomy^[59,60]. Furthermore, there was no difference in the recurrence rate but rather a survival benefit for patients with pN0/1 disease undergoing pulmonary sleeve resection. In fact, the most common anatomical locations for bronchoplastic pulmonary resection involve tumors of the right upper lobe and the left upper lobe orifices (Figure 2)^[61-63]. At this, the bronchial anastomosis is typically covered using pleura, pedicled pericardial fat or pedicled muscle flap to minimize anastomotic complications^[64]. Furthermore, patients undergoing sleeve resection may experience an improved postoperative quality of life compared to patients with a pneumonectomy which was described by Deslauriers *et al*^[65]. Finally, a recent study by Schirren *et al*^[66] showed even in patients with advanced nodal disease no significant advantage for pneumonectomy over sleeve resection.

LYMPHADENECTOMY

Although it is generally accepted that lymph node stag-

ing of NSCLC should be as accurate as possible, there is ongoing debate regarding the extent of mediastinal lymphadenectomy. Worldwide, surgeons use a variety of techniques including selective sampling (sampling involving only selected suspicious or representative lymph nodes), a SLNS (exploration and biopsy of a standard set of lymph node stations in each case) and MLND, which involves the removal of all node-bearing tissue within defined landmarks for a standard set of lymph node stations^[67]. As data have shown that the accuracy of staging in NSCLC patients is more effective with either MLND or SLNS compared with selective sampling alone^[68-70], existing guidelines recommend either one of the methods at the time of surgical resection. Studies have shown that MLND leads to a significant reduction of local and systemic recurrence^[71,72]. Furthermore, another potential advantage of MLND is a more accurate tumor staging through the detection of skip- and micro-metastases^[71,73-75]. Nevertheless, some researchers argue that the improved outcome after MLND is in fact just a stage migration phenomenon (“Will Rogers phenomenon”)^[76,77]. In a retrospective study by Doddoli *et al*^[72] the effect of MLND *vs* SLNS on overall survival of patients with stage I NSCLC was assessed and the working group demonstrated that MLND was a favorable independent prognostic factor. Similar results were reported by Lardinois *et al*^[71] showing a longer disease-free survival in stage I NSCLC patients who underwent MLND *vs* SLNS (60.2 ± 7 *vs* 44.8 ± 8.1 mo).

EXTENDED RESECTIONS, ANGIOPLASTY AND MARGINAL RESECTIONS USING EXTRACORPOREAL MEMBRANE OXYGENATION

It is hypothesized that the need for extended resection in patients with locally advanced NSCLC is associated with a higher morbidity and mortality rate. However, evidence suggests that the mortality rate is not significantly increased after extended resections^[78]. For example, Izbicki *et al*^[78] showed in patients undergoing extended resection for stage T3 or T4 NSCLC tumors that the study patients with no residual tumor had a 3-year survival rate of 33%. In addition, Spaggiari *et al*^[79] reported a 3-year probability of survival of 39% after extended pneumonectomy with partial resection of the left atrium for advanced lung cancer. Even a study from Hillinger *et al*^[80] revealed that patients with T4 tumors (infiltration of great vessels, trachea, esophagus, vertebral bodies, *etc.*) showed an increasing 5-year survival rate from 15 to 35% after radical resection with acceptable preoperative mortality if treated in experienced centres. Extended surgical resections seems also appropriate for patients with locally advanced lung cancer with limited invasion of the carina, left atrium, superior vena cava, or pulmonary artery^[81]. Furthermore, in case of advanced tumor removals with the need of additional pulmonary resection, extracorporeal

membrane oxygenation (ECMO) support is a considered to be a safe alternative to cardiopulmonary bypass after pneumonectomy or even carina resection after pneumonectomy^[82,83]. In the event of a tumor infiltration into the pulmonary artery, a major anatomical lung resection with angioplastic procedures may achieve a similar oncological radicality with sparing distal lung parenchyma. The long term outcomes in these cases are significantly influenced by the nodal status and are comparable to those of conventional lobectomy^[84,85]. Therefore, lung resections with bronchovascular reconstruction are invaluable for patients with central tumours, although they do demand more skill than pneumonectomies. The acceptable clinical results and oncological reliability promote this type of interventions, which always have to be considered for any central tumour in order to avoid pneumonectomy and its complications^[86].

DISCUSSION

Thoracic surgery is a dynamic field with many scientific and technical changes occurred in the last years. A prominent example is the use of less invasive approaches for major resection of NSCLC patients. In fact, it has been proven in early stage NSCLC patients that VATS lobectomy as a routinely performed technical procedure is a method with equal or even better long-term results compared to the well-established open surgical resection.

Sub-lobar resection has its value in the surgical therapy of NSCLC patients with limited lung function. Especially for patients with decreased pulmonary function or comorbid disease and clinical stage I NSCLC a sub-lobar resection should have priority over a conservative treatment. Possible criteria for sub lobar lung resection in early stage NSCLC patients seem to be: (1) limited lung function; (2) tumor diameter ≤ 2 cm; (3) N0 lymph node status; and (4) Frozen section with tumor free edge resection. In case of a sub-lobar resection, segmentectomy should always be preferred rather than a wedge resection. However, a lot of reports about sub-lobar resection combine segmental and wedge resection which seems in our opinion not appropriate as segmentectomy offers the potential advantage of complete resection of lymphatic and vascular drainage.

The ongoing debate about the efficacy of LAD during VATS lobectomy originates mainly from the lack of strict technical standards. The guidelines of the American National Comprehensive Cancer Network recommend the complete dissection of at least three mediastinal lymph node stations^[87]. The European Society for Thoracic Surgery (ESTS) has published similar guidelines advising the removal of at least three lymph node stations including the subcarinal station^[88].

Another issue is the debate about the role of radiation therapy in postoperative N2 disease. Even though some studies suggest that postoperative radiation in case of N2 disease can improve local control, it remains controversial whether it has a prognostic effect^[89]. Based on

the National Cancer Institute of Canada and Intergroup Study, the postoperative radiation may be considered in selected patients to reduce the risk of local recurrence, if any of the following are present: (1) involvement of multiple nodal stations; (2) extracapsular tumor spread^[90].

Finally, multimodality treatment options have been implemented for patients with locally advanced or limited metastatic disease offering more patients a potential complete surgical tumor resection as the operative therapy remains the most important and successful option to really cure NSCLC patients.

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MicroRNAs as lung cancer biomarkers

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that have accumulated in the last ten years on the use of miRNAs as lung cancer biomarkers.

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Key words: Lung cancer; Non-small cell lung carcinoma; MicroRNAs; Biomarkers

Core tip: Biomarkers able to stratify for the subtype of lung cancer, prognosticate the course of disease, or predict the response to treatment are in high demand. In the last decade, microRNAs (miRNAs), measured in resected tumor samples have emerged as biomarkers, due to the ease of their detection and in their extreme specificity. Moreover, miRNAs present in sputum, in plasma, in serum or in whole blood have increasingly been explored in the last five years as less invasive biomarkers for the early detection of cancers.

Abstract

Lung cancer is the leading cause of cancer mortality worldwide. Its high mortality is due to the poor prognosis of the disease caused by a late disease presentation, tumor heterogeneities within histological subtypes, and the relatively limited understanding of tumor biology. Importantly, lung cancer histological subgroups respond differently to some chemotherapeutic substances and side effects of some therapies appear to vary between subgroups. Biomarkers able to stratify for the subtype of lung cancer, prognosticate the course of disease, or predict the response to treatment are in high demand. In the last decade, microRNAs (miRNAs), measured in resected tumor samples or in fine needle aspirate samples have emerged as biomarkers for tumor diagnosis, prognosis and prediction of response to treatment, due to the ease of their detection and in their extreme specificity. Moreover, miRNAs present in sputum, in plasma, in serum or in whole blood have increasingly been explored in the last five years as less invasive biomarkers for the early detection of cancers. In this review we cover the increasing amounts of data

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INTRODUCTION

Lung cancer is the leading cause of cancer mortality worldwide^[1]. About 270000 individuals were predicted to die of lung cancer in the European Union in 2013^[2]. This high mortality is due to the poor prognosis of the disease caused by a late disease presentation, tumour heterogeneities within histological subtypes, and the relatively limited understanding of tumour biology. Most lung cancer patients are diagnosed at an advanced stage of disease, and, although a small subsets of these patients can be treated with new drugs offering improved survival and reasonable quality of life, the majority of patients can only be treated with palliative chemotherapy. Overall survival remains poor, and many patients die within a few months

of diagnosis.

Lung cancer is not one, but rather a group of diseases: small cell lung carcinomas (SCLCs) are high grade neuroendocrine tumours (NET), metastasize earlier and are initially more chemosensitive than the so called non-small cell lung carcinomas (NSCLC). Over the past two decades it has become clear that NSCLC itself is a clinically and biologically heterogeneous group of lung cancers, and should not be treated as a single disease entity. The two main subgroups of NSCLC are adenocarcinoma (AD) and squamous cell carcinoma (SCC), with a remaining third class of carcinomas devoid of histological features of adeno- or squamous- differentiation, named LCC (large cell carcinoma)^[3]. The appearance of these tumours at light microscopy differs substantially, suggesting that their aetiology and biology differ as well. Importantly, the histological subgroups of NSCLC respond differently to some chemotherapeutic substances^[4-7] and side effects of some therapies appear to vary between subgroups^[8].

Beside SCLC, NETs include a spectrum of tumors from the low-grade typical carcinoid (TC) and intermediate-grade atypical carcinoid (AC) to the high-grade large-cell NE carcinoma (LCNEC). The distinction between these various entities may be sometimes difficult on histological grounds, but is of great therapeutic relevance.

Biomarkers able to stratify for the subtype of lung cancer, prognosticate the course of disease, or predict the response to treatment are in increasing demand. Lung cancer subtyping has traditionally relied on the histopathological observation of resected specimens, bronchoscopic biopsies, fine needle aspirations or sputum, which represent samples with decreasing invasiveness for the patient, but also of increasing challenge for the pathologist, as proportionally fewer tumour cells are captured^[9-11]. Recently, the introduction of several immunohistochemical markers has rendered lung cancer subtyping more accurate and clinically useful^[12].

In the last decade, microRNAs (miRNAs), measured either from tumour samples or in biofluids, have emerged as biomarkers for tumor diagnosis, prognosis and prediction of response to treatment. In the following pages we will review the increasing amounts of data that have accumulated in the last ten years on the use of miRNAs as lung cancer biomarkers.

MIRNAS AND CANCER

microRNAs (miRNAs or miRs) are small non-coding single-stranded RNAs, 19-25 nucleotides (nt) in length, acting as negative regulators of gene expression at the post-transcriptional level. More than 1000 miRNAs are transcribed from miRNA genes in the human genome. A single miRNA is able to modulate hundreds of downstream genes by recognizing complementary sequences in the 3'UTRs of their target mRNAs. It has been estimated that in humans about 30% of messenger RNAs (mRNAs) are under miRNA regulation, but this percent-

age is likely to grow in the future, as studies have shown that miRNAs can also bind target sequences located in the 5' UTR or in the open reading frame (ORF)^[13]. The biological functions of miRNAs are diverse and include several key cellular processes, such as differentiation, proliferation, cellular development, cell death and metabolism.

In a seminal paper the Croce laboratory showed in 2002 that the genes for miR-15 and miR-16 are deleted or down-regulated in the majority of chronic lymphocytic leukaemia cases^[14]. Scott Hammond, Gregory Hannon and collaborators demonstrated that miRNAs can modulate tumour formation and implicated the mir-17-92 cluster as a potential human oncogene in another fundamental paper^[15], in which they demonstrated that enforced expression of the mir-17-92 cluster acts with c-myc expression to accelerate tumour development in a mouse B-cell lymphoma model. Since then, evidences have accumulated to indicate that miRNAs play a role in the onset and progression of several human cancers^[16]. The transcription or processing of some miRNAs is altered in neoplastic tissues, in respect to their normal counterparts. miRNAs whose levels increase in tumors are referred to as oncogenic miRNAs ("onco-miRs"), sometimes even if there is no evidence for their causative role in tumorigenesis. On the other hand, miRNAs down-regulated in cancer are considered tumor suppressors. From the mechanistic point of view, it is important to understand how these variations may contribute to tumor progression.

In 2005, the Horvitz and Golub labs demonstrated the potential for miRNAs as diagnostic tumor markers, when they were able to indicate the tumor embryonic origin using miRNA expression profiles, successfully classifying poorly differentiated tumors, among which lung tumors^[17]. Subsequently, the Croce lab performed a large-scale miRNome analysis on 540 samples including lung, breast, stomach, prostate, colon, and pancreatic tumors, and identified a solid cancer miRNA signature composed by a large portion of over-expressed miRNAs. While some miRNAs were commonly dys-regulated in the six cancer types, several other miRNAs were associated to a particular type of cancer^[18]. The utility of miRNAs levels as diagnostic and prognostic biomarkers became clear already from these first studies^[19]. Moreover, the effectiveness of miRNAs as biomarkers for tracing the tissue of origin of cancers of unknown primary origin was demonstrated by Rosenfeld and colleagues^[20], who constructed a tissue classifier based on the measurement of 48 miRNAs on a microarray, to identify the tissue origin of metastatic tumors. These results were translated into a qRT-PCR platform, to develop a diagnostic test for the identification of tumour tissue origin^[21]. The classifier has been further implemented in a second-generation custom microarray based on the measurement of 64 miRNAs^[22] the usefulness of which as a diagnostic tool was very recently confirmed^[23].

A different 47-miRNA signature for the identification

of cancers with unknown primary tissue-of-origin was identified by other authors using a different microarray platform^[24].

MIRNAS AND LUNG CANCER

As far as lung cancers are concerned, the role of miRNAs in lung carcinogenesis was indicated as early as 2004, when the Croce lab demonstrated that more than half of the miRNA genes then known were located in cancer-associated genomic regions or in fragile sites and that several miRNAs located in this deleted regions have low expression levels in lung cancer cell lines as well as in chronic lymphocytic leukaemia samples^[25]. In the same year, Takamizawa and colleagues reported reduced expression of the let-7 microRNA in human NSCLC lung cancers^[26], followed by several independent studies^[27-29]. The let-7 family was later shown to have an onco-suppressor activity in NSCLC tumor development in mice xenografts^[30]. One of the consequences of let-7a down-regulation in lung cancer has been demonstrated to be the upregulation of RAS protein^[27]. A single nucleotide polymorphism (SNP) in a let-7 complementary site of KRAS mRNA was found to be associated with increased risk of NSCLC in moderate smokers^[31]. Based on *in vitro* experiments and analyses of patient samples the authors concluded that this SNP alters the ability of let-7 to regulate translation of KRAS, leading to overexpression of KRAS and increased lung cancer risk.

Other miRNAs may also interact with RAS. For instance, Wang *et al.*^[32] found that miR-451 is downregulated in NSCLC, and that low expression correlated with poor survival. The authors were able to show that miR-451 inhibits the expression of ras-related protein 14 (RAB14), suggesting that lower expression of miR-451 may allow this oncogene to escape regulation.

The oncogenic miR-17-92 cluster is markedly overexpressed in lung cancers, especially with SCLC histology and enhances cell proliferation *in vitro*, therefore possibly playing a role in the development of lung cancers^[33]. On the other hand, deletion of the miR-17-92 cluster, in mice, is lethal and causes lung and lymphoid cell developmental defects^[34].

The tumor suppressor protein p53 is mutated in a large number of lung cancer cell lines and tumour specimens from patients with lung cancer^[35,36]. There is growing evidence that p53 regulates the expression of several miRNAs^[37-42]. p53 directly regulates the expression of miR-34 family members, and the upregulation of these miRNAs result in the downregulation of genes associated with cell cycle control^[37] and promotion of apoptosis^[40] in cultured lung cancer cells. Further miRNAs, including miR-125a, have more recently also been linked to p53-regulated apoptosis in lung cancer cells^[41].

METHODS FOR THE QUANTIFICATION OF MIRNAS

The main advantage of the use of miRNAs as biomarkers

resides in the ease of their detection and in their extreme specificity. miRNAs are stable molecules well preserved in formalin fixed, paraffin embedded tissues (FFPE) as well as in fresh snap-frozen specimens, unlike larger RNA molecules as mRNAs^[43].

A range of methods has been used for the isolation and profiling of miRNAs. Purification of total RNA is obtained either through several commercially available column filtration protocols, implemented to guarantee recovery of miRNAs, or via the extraction of RNA by variously named "Tri-reagents" (acid phenol in combination with guanidinium-thiocyanate and chloroform), also available from vendors. Given that the interest is focused on the quantification of specific miRNAs in different conditions, the method of choice should exclude any bias in the purification of miRNAs from the samples. Importantly, the Kim laboratory has recently reported that, differently from what everybody in the field has thought for decades, in Tri-reagents-based RNA purification protocols, short structured miRNAs with low GC content are lost when a small number of cells are used^[44]. The finding raises warning flags about comparisons of miRNA levels between populations of cells at different densities.

Sequencing-, microarray- and quantitative reverse-transcription polymerase chain reaction (qRT-PCR)-based methods are currently used for miRNA profiling.

Next generation sequencing (NGS)^[45], providing accurate and sensitive miRNAs measurements, allows the identification of miRNAs differently expressed in tumor samples and matched healthy tissue. Furthermore, NGS enables scientists to discover novel miRNAs, as opposed to microarrays and qRT-PCR methods, which only can detect already known miRNAs. NGS techniques are rapidly evolving in power and multiplexing capacities, but they remain expensive and labor-consuming, both in sample preparation and data analysis.

The majority of miRNA profiling studies have been carried out so far using microarrays and have provided signatures consisting in few to several (5-30) distinct miRNAs^[46]. With time, it has become clear in the field that the use of microarrays for miRNA profiling presents with major problems of cross-hybridisation between members of miRNA families and discrepancies in comparing results obtained with different microarray platforms^[47-50]. A common strategy is to validate the microarray data by qRT-PCR, which warrants high sensitivity and specificity.

Additionally, the analysis of a great number of genes including ones whose changes are not directly or indirectly associated with cancer, in clinical settings is not necessary. Hence, the large amount of data obtained by microarray and NGS profiling needs to be transposed into clinical trials by developing an easily performed and serviceable assay that can analyze the cancer-specific miRNAs for cancer diagnosis and prognosis. Such an analysis has been so far relying on qRT-PCR assays.

Several methods for the analysis of miRNAs by qRT-PCR have been devised^[60] and companies are providing

multiwell plate-based qRT-PCR assays that promise to substitute microarrays in the high-throughput profiling of miRNAs. Additionally, qRT-PCR is the most easily performed and cost-effective technique and it is therefore the method of choice when it comes to measuring the levels of the restricted number of miRNA biomarkers in cancer samples, in the clinical setting.

qRT-PCR measures the relative levels of variable target miRNAs in comparison with one or more stably expressed reference genes (sometimes called housekeeping or endogenous control genes). This normalization is required to allow for variability in RNA quantity and quality and/or in the efficiency of cDNA synthesis. Despite miRNAs having been intensively studied in cancer research in the last years, suitable reference genes for relative quantification of miRNA levels in qRT-PCR assays have not been satisfyingly identified. Ribosomal RNAs (rRNAs) have been used as a reference RNA in miRNA studies^[26,61]. However, concerns have been raised regarding the use of rRNAs in normalization, as they can be expressed at much higher levels than the target RNA, making it challenging to quantify an rRNA and a rare transcript in the same RNA dilution. Moreover, there is evidence of rRNA deregulation in apoptosis^[62] and cancer^[63]. The most commonly used reference RNA in miRNA qRT-PCR experiments is the small nuclear RNA U6 (U6 snRNA)^[55,64]. However, using U6 snRNA to normalise miRNA levels is controversial because being much bigger in size (106-107 nt) it might differ from miRNAs with respect to efficiency of its extraction, reverse transcription and PCR amplification. U6 snRNA has been analysed as a reference gene for miRNA studies in several papers, and most of them came to the conclusion that the variance of U6 expression across tissues is high, therefore making U6 not a suitable reference gene for miRNA quantification^[65-67]. Further reference genes commonly used are snoRNAs but they too might be dys-regulated in cancer^[68]. Several authors have suggested to use as reference a combination of miRNAs whose levels do not vary in the specific tumor tissue under investigation. For example, in fresh-frozen lung cancer samples, Peltier and Latham^[65] suggest the use of a combination of miR-191 and miR-103.

The use of unvalidated and different reference genes makes it difficult to compare papers describing miRNA-expression cancer profiles and might represent one of the major reasons for the discrepancies in published studies regarding differentially expressed miRNAs in specific cancers.

A recent addition to the detection methods for miRNAs is droplet digital PCR (dPCR)^[69], a method especially useful for low abundance miRNAs. In droplet dPCR, single cDNA molecules are partitioned evenly among hundreds of individual droplets in which they are amplified to generate binary calls. With this method an absolute readout of total DNA copy number can be obtained, avoiding the need for an endogenous reference gene.

MIRNAS FROM RESECTED SPECIMENS

MiRNAs as biomarkers for the diagnosis of lung cancer and for stratifying lung cancer subtypes

In 2005, the Horvitz and Golub labs used a bead-based flow cytometric miRNA expression method to identify complex profiles consisting of approximately a hundred of dys-regulated miRNAs, able to classify 11 different tumor types, among which lung tumors^[17]. Subsequently, the Croce lab used a custom-made oligonucleotide miRNA microarray to compare lung carcinomas to normal tissue, and identified a group of three downregulated and 35 upregulated miRNAs. Among these, miR-21 was commonly up-regulated in the six cancer types analyzed (lung, breast, stomach, prostate, colon, and pancreatic tumors) and miR-17-5p, miR-128b, miR-155, miR-191 and miR-199a-1 were up-regulated in at least other two cancer types^[18].

In 2006 Yanaihara *et al.*^[28] compared the miRNAs expression profiles in 104 pairs of lung cancer tissues and corresponding non-cancerous lung tissue, by the same custom-made oligonucleotide miRNA microarray used by Volinia and colleagues. They identified a unique profile made of 43 differently expressed miRNAs (Table 1) allowing the distinction of lung cancer from the non-cancerous lung tissue. Of the 15 upregulated and 28 downregulated miRNAs, miR-21 and miR-205 are located in a chromosomal region amplified and miR-32, miR-126-5p and miR-126-3p in a region deleted in lung cancers, respectively. The authors next validated the microarray results by a solution hybridization detection method and by qRT-PCR, confirming that miR-21 and miR-205 are frequently up-regulated and miR-126-5p is often down-regulated in lung cancer tissues when compared with the corresponding noncancerous lung tissues.

Comparison analyses between ADC *vs* noncancerous tissues and SCC *vs* noncancerous tissues revealed 17 and 16 miRNAs with statistically different expression, respectively (Table 1). Six miRNAs (miR-21, miR-155, miR-191, miR-126-5p, miR-210 and miR-224) were shared in both histological types of NSCLC. Yanaihara and colleagues also directly compared the two most common histological types of NSCLC, identifying two miRNAs (miR-99b and miR-102) that were higher in ADC and 4 miRNAs (miR-202, miR-203, miR-205 and the precursor of miR-204) that were higher in SCC. However, the authors do not explore further the issue of distinguishing ADCs from SCCs, in this paper.

Decreased expression of miR-107, miR-185 and let7a, and the overexpression of miR-31a, was observed by qRT-PCR in lung cancer tissues and cell lines, compared to normal lung tissue^[70]. Landi *et al.*^[71] reported 34 miRNAs that significantly differentiated SCCs from ADCs in male smoker patients, of which 2 were downregulated and 32 upregulated in ADC *vs* SCC. Raponi *et al.*^[72] used Ambion microarrays to profile total RNA from 61 SCC samples and 10 matched normal lung samples and identified 15 miRNAs that were differentially expressed

Table 1 MiRNAs from resected samples as biomarkers for the diagnosis of lung cancer

MiRNA	Scope	Sample	Ref.
mir-21, mir-191, mir-210, mir-155, mir-205, mir-24-2, mir-212, mir-214, mir-17-3p, mir-106a, mir-197, mir-192, mir-146, mir-203, mir-150, (UP) mir-126-5p, mir-143, mir-192-prec, mir-224, mir-126, mir-30a-5p, mir-140, mir-9, mir-124a-1, mir-218-2, mir-95, mir-145, mir-198, mir-216-prec, mir-219-1, mir-125a-prec, mir-26a-1-prec, mir-199b-prec, let-7a-2-prec, mir-27b, mir-32, mir-29b-2, mir-220, mir-33, mir-181c-prec, mir-101-1, mir-124a-3, mir-125a (DOWN)	Lung cancer <i>vs</i> normal	Solid (Not specified)	[28]
mir-21, mir-191, mir-155, mir-210, mir-24-2 (UP) mir-126-5p, mir-126-3p, mir-219-1, mir-95, mir-192-prec, mir-220, mir-216-prec, mir-204-prec, mir-188, mir-198, mir-145, mir-224 (DOWN)	ADs <i>vs</i> normal	Solid (Not specified)	[28]
mir-205, mir-191, mir-210, mir-17-3p, mir-203, mir-155, mir-21, mir-214, mir-212, mir-197 (UP) mir-224, mir-126*, mir-140, mir-29b, mir-143, mir-30a-5p (DOWN)	SCC <i>vs</i> normal	Solid (Not specified)	[28]
miR-31 (UP) miR-107, miR-185, let-7a (DOWN)	Lung cancer tissue <i>vs</i> normal	Solid (Not specified)	[70]
miR-26a, let-7g, let-7f, miR-98, miR-29a, let-7c, miR-30b, let-7i, let-7b, miR-29b, miR-26b, let-7a, miR-146b-5p, miR-195, miR-29c, miR-30d, miR-20a, miR-17, miR-19b, miR-106a, miR-16, let-7d, miR-106b, miR-181a, miR-498, miR-103, miR-107, miR-191, mir-663, miR-491-5p, let-7e, mir-654-5p (UP) miR-453, miR-509-3p (DOWN)	AD <i>vs</i> SCC; male smokers patients	Solid, formalin-fixed, paraffin-embedded	[71]
miR-17-5p, miR-20a, miR-20b, miR-93, miR-106a, miR-106b, miR-182, miR-183, miR-200a, miR-200c, miR-203, miR-210, miR-224 (UP) miR-125a, let7e (DOWN)	SCC <i>vs</i> normal	Solid, Snap-frozen	[72]
miR-30a, miR-140-3p, miR-182, miR-210, miR-486-5p	Stage I-III <i>vs</i> normal	Solid, Snap-frozen	[73]
miR-182, miR-200c, miR-141, miR-375, miR-7, miR-429, miR-200a, miR-370, miR-200b, miR-382 (UP) miR-126, miR-451, miR-195, miR-486-5p, miR-214, miR-199a-5p (DOWN)	Primary lung tumors <i>vs</i> metastases	Solid, formalin-fixed, paraffin-embedded	[74]
miR-205, miR-21 (relative expression)	AD <i>vs</i> SCC	Solid, formalin-fixed, paraffin-embedded	[75]
miR-21, miR-155 (UP)	LCNECs and SCLCs <i>vs</i> TCs and ACs	Solid, formalin-fixed, paraffin-embedded	[79]
miR-205, miR-27a, miR-29a, miR-29b, miR-34a (DOWN in NSCLC) miR-25, miR-375 (UP in NSCLC)	SCLC <i>vs</i> NSCLC	Solid, formalin-fixed, paraffin-embedded	[80]
miR-29a, miR-29b, miR-34a, miR-375 (DOWN in SQ) miR-205, miR-25, miR-27a (UP in SQ)	SCC <i>vs</i> AD	Solid, formalin-fixed, paraffin-embedded	[80]
miR-7, miR-21, miR-29b, miR-106a, miR-125a-5p, miR-129-3p, miR-205, miR-375 (relative expression)	Carcinoid, SCLC, and squamous and nonsquamous NSCLC	Solid, Fresh Biopsy	[81]
miR-21, miR-155, miR-7 (UP)	Tumor <i>vs</i> normal	Solid, fine-needle aspirate (FNA)	[82]
miR-21, miR-155 (UP)	NSCLC <i>vs</i> normal	Sputum	[96]
miR-205, miR-210, miR-708 (relative expression)	SCC <i>vs</i> normal	Sputum	[97]
miR-21, miR-200b, miR-375 and miR-486 (relative expression)	AD <i>vs</i> Normal	Sputum	[98]
miR-31, miR-210 (relative expression)	Stage I NSCLC <i>vs</i> normal	Sputum	[99]
miR-31, miR-210 (Relative Expression) + computed tomography	Stage I NSCLC <i>vs</i> normal	Sputum	[100]

AD: Adenocarcinoma; SCC: Squamous cell carcinoma; LCNEC: Large cell neuroendocrine carcinoma; SCLC: Small cell lung carcinoma; TC: Typical carcinoid; AC: Atypical carcinoid; NSCLC: Non-small cell lung cancer.

between normal lung and SCC (Table 1). Two of these miRNAs were down-regulated in SCCs (miR-125a and let7e) while the remaining 13 miRNAs were upregulated (miR-17-5p, miR-20a, miR-20b, miR-93, miR-106a, miR-106b, miR-182, miR-183, miR-200a, miR-200c, miR-203, miR-210, miR-224). More recently, a 5-miRNA classifier was identified by microarray analysis (miR-30a, miR-140-3p, miR-182, miR-210, miR-486-5p) that could distinguish stage I-III SCC from normal lung tissues^[73]. This classifier had an accuracy of 94,1% in a training cohort (34 patients) and 96,2% in a test cohort (26 patients).

A panel of 16 miRNAs has been reported to differentiate between primary lung tumors and metastases to the lung of various origin (Table 1)^[74]. This miRNA profile was identified using microRNA microarray data generated from FFPE samples, and was confirmed by qRT-PCR. The panel includes miR-182, which was most strongly

over-expressed in the lung primary tumors, and miR-126, which was over-expressed in the metastatic tumors.

Researchers have also aimed at finding one or few miRNAs that can be used as a convenient tool for lung cancers diagnosis. Lebanony *et al.*^[75] used a microarray to measure miRNA levels in AD and SCC FFPE samples, and verified their findings by qRT-PCR. They identified miR-205 as a highly specific marker for SCC, when combined with the measured miR21 levels. The finding was confirmed by other papers^[76,77]. Moreover, an algorithm for accurate classification of NSCLC cases, diagnosed as LCC on purely morphologic grounds, was proposed by integrating immunohistochemical markers (Δ np63, DSC3, and napsin A) with miR-205 and miR-21 measurement^[78].

Evaluating by qRT-PCR FPPE specimens from NETs, Lee *et al.*^[79] found that the levels of miR-21 and

miR-155 were significantly higher in high-grade NET carcinomas (LCNECs and SCLCs) than in carcinoid tumors (TCs and ACs).

Two microRNA panels yielded high diagnostic accuracy in discriminating SCLC from NSCLC (miR-29a and miR-375) and in differentiating SCC from AD (miR-205 and miR-34a) in FFPE surgical lung specimens^[80]. Moreover, the same microRNA panels accurately differentiated SCLC from NSCLC and SCC from AD in bronchial brushing specimens.

Gilad *et al.*^[81] reported a single assay for the classification of the four main types of lung cancer (Table 1): carcinoid, SCLC, and squamous and nonsquamous NSCLC, based on the expression of eight miRNAs (miR-7, miR-21, miR-29b, miR-106a, miR-125a-5p, miR-129-3p, miR-205, miR-375). The assay was effective both on resected and on cytologic (fine-needle aspiration (FNA) and bronchial brushing and washing) lung cancer samples.

A recent study has also evaluated miRNAs in FNA NSCLC biopsies^[82]. miR-21, miR-155, and miR-7 showed a higher level in tumoral FNA than in normal FNA specimens, while let7a showed a lower level. A direct comparison of FNAs with resected specimens from the same patients indicated that the measured miRNAs had the same trend in the two types of specimens.

miRNAs as biomarkers for lung cancers prognosis

Reduced expression of the let-7 family has been correlated with poor postoperative survival in NSCLC^[26]. In a later study, AD patients with high expression of either miR-155, miR-17-3p, miR-106a, miR-93, or miR-21 and low expression of either let-7a-2, let-7b, or miR-145 were found to have a significantly worse prognosis (Table 2)^[28]. Overexpression of the precursor of miR-155 and reduced expression of let-7-a was especially predictive of poor survival.

Analyzing frozen resected specimens from NSCLC patients, Yu *et al.*^[83] identified a five-microRNA signature that can predict the survival and relapse of patients with lung cancer (Table 2). Two of these miRNAs (miR-221 and let-7a) were protective (*i.e.*, their down-regulation correlated with poor survival and high relapse probability), while the other three (miR-137, miR-182-3p, miR-372) were risky, and their up-regulation was predictive of poor survival and high relapse probability. The authors also demonstrated that miR-221, miR-137, miR-182-3p and miR-372 can alter the invasive ability of lung cancer cells in culture.

In the already described work by Raponi *et al.*^[72]. Twenty miRNAs were identified as having a significant association with overall survival in lung SCC patients (Table 2). Among these miRNAs, miR-146b alone was found to have the strongest prediction accuracy as the group with high miR-146b expression had significantly worse overall survival.

The p53-dependent miR-34 family was observed to be down-regulated in surgically resected NSCLC tumor

samples compared with normal tissue, and low levels of miR-34a expression were correlated with a high probability of relapse^[84]. MicroRNA expression profiles were also identified that may predict recurrence of localized stage I NSCLC after surgical resection^[85]. These profiles included miR-124-5p, miR-146b-3p, miR-200b-5p, miR-30c-1-3p, miR-510, miR-585, miR-630, miR-657 and miR-708 (Table 2).

High miR-16 levels, measured by qRT-PCR in resected NSCLC samples, were reported as a prognostic factor for poor disease-free survival and poor overall survival^[86]. Low miR-145 and high miR-367 are associated with shorter time to relapse (TTR) in resected NSCLC^[87]. Noteworthy, p53 regulates miR-145 expression, which, in turn, inhibits the translation of SRY-related HMG box (SOX)2 and octamer-binding transcription factor (OCT)4. These transcription factors control the expression of the miR-302-367 cluster.

In a panel of 27 miRNAs which were observed by microarray analysis to be deregulated greater than two-fold in NSCLC resected samples compared to normal lung tissue, Gao *et al.*^[88] identified three miRNAs whose levels (confirmed by qRT-PCR) were related to clinicopathologic characteristics or patient prognosis: low levels of miR-143 were significantly correlated with smoking status, high miR-21 expression and low miR-181a expression were associated with poor survival.

The lower expression level of a 5-miRNA signature (miR-25, miR-34c-5p, miR-191, let-7e, and miR-34a) correlated with poor overall survival among SCC patients (Table 2)^[71] and high expression of miR-31 was associated with poor survival in Chinese SCC patients^[73].

Eight miRNAs were confirmed to be significantly differentially expressed in NSCLC FFPE samples from patients with brain metastases compared with patients without brain metastases (Table 2)^[89]. In particular, in this work, the over-expression of miR-328 and miR-330-3p was indicated as a marker for patients at risk for brain metastases, and a role for miR-328 in conferring migratory potential to NSCLC cells was suggested.

miRNAs as biomarkers to predict response to therapy in lung cancer

miRNAs have also been used as biomarkers predictive of patient's response to therapy. miR-21 expression was significantly increased in platinum-based chemotherapy-resistant NSCLC patients and increased miR-21 expression was associated with the shorter disease-free survival^[90]. A single nucleotide polymorphism in *miR-196a-2* gene was reported to be associated with severe toxicity after platinum-based chemotherapy of advanced NSCLC patients in a Chinese population^[91].

Recent therapeutic advances for the treatment of NSCLC include the use of epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) including gefitinib and erlotinib. MiRNA-128b was shown to directly regulate EGFR translation and miR-128b LOH was found to be frequent in NSCLC samples and cor-

Table 2 MiRNAs as biomarkers for the prognosis and to predict response to therapy

miRNA	Experiment	Scope	Sample	Ref.
let-7a family (DOWN)	lung cancer tissue <i>vs</i> normal	NSCLC poor postoperative survival	Solid, (not specified)	[26]
mir-155, mir-17-3p, mir-106a, mir-93, mir-21 (UP)	AD <i>vs</i> SCC	AD poor survival	Solid, (not specified)	[28]
let-7a-2, let-7b (DOWN)				
miR-221, let-7a (DOWN) miR-137, miR-182-3p, miR-372 (UP)	NSCLC <i>vs</i> normal	NSCLC poor survival	Solid, snap frozen	[83]
miR-146b, miR-191, miR-155, miR-15a, miR-511, miR-100, miR-10a, miR-21, miR-126 (UP) miR-206, miR-299-3p, miR-122a, miR-513, miR-184, miR-453, miR-379, miR-202, miR-494, miR-432, miR-370 (DOWN)	SCC <i>vs</i> normal	SCC Overall Survival	Solid, snap frozen	[72]
miR-34a (DOWN)	NSCLC <i>vs</i> normal	NSCLC Probability of relapse	Solid, (not specified)	[84]
mir-124-5p, mir-146b-3p, mir-200b-5p, mir-30c-1-3p, mir-510, mir-585, mir-630, mir-657, mir-708 (relative expression)	stage I NSCLC <i>vs</i> normal	Stage I NSCLC recurrence	Solid, formalin-fixed, paraffin-embedded (FFPE)	[64]
miR-16 (UP)	NSCLC <i>vs</i> normal	NSCLC poor survival	Solid, (not specified)	[86]
miR-143 (DOWN)	Lung cancer tissue <i>vs</i> normal	smoking status	Solid, snap frozen	[88]
miR-21 (UP) miR-181a (DOWN)	Lung cancer tissue <i>vs</i> normal	NSCLC poor survival	Solid, snap frozen	[88]
miR-25, miR-34c-5p, miR-191, let-7e, miR-34a (DOWN)	AD <i>vs</i> SCC	SCC survival	Solid, formalin-fixed, paraffin-embedded (FFPE)	[71]
miR-31 (UP)	SCC <i>vs</i> normal	SCC poor survival	Solid, snap frozen	[73]
miR-325, miR-326, miR-328, miR-329-2-pre, miR-330-3p, miR-500a-3p, miR-370, miR-650-pre (UP)	BM – NSCLC <i>vs</i> BM + NSCLC	NSCLC, risk for brain metastasis	Solid, formalin-fixed, paraffin-embedded (FFPE)	[89]
miR-21 (UP)		Platinum-based chemotherapy-resistant NSCLC patients	Solid, snap frozen	[90]
miR-196a-2 SNP	Treatment <i>vs</i> genotype	Severe toxicity after platinum-based chemotherapy of advanced NSCLC patients	Genomic DNA from peripheral leukocytes	[91]
miR-128b (UP)		NSCLC samples correlated with clinical response and survival following gefitinib treatment	Microdissected primary surgically resected NSCLC tumors	[92]
miR-30b, miR-30c		Regulated by EGFR and hepatocyte growth factor (MET) receptor tyrosine kinase	Solid, Lung tumor tissue samples	[93]
miR-30b, miR-30c		Prognostic predictors in NSCLC patients who underwent first line treatment with TKIs	Solid, formalin-fixed, paraffin-embedded (FFPE)	[94]

NSCLC: Non-small cell lung cancer; AD: Adenocarcinoma; SCC: Squamous cell carcinoma; BM: Brain metastasis.

related significantly with clinical response and survival following gefitinib treatment^[92]. miR-30b and miR-30c expression levels, which are regulated by EGFR and hepatocyte growth factor (MET) receptor tyrosine kinase^[93] have been reported to be prognostic predictors in NSCLC patients who underwent first line treatment with TKIs^[94].

MIRNAS IN SPUTUM AS NON-INVASIVE LUNG CANCER BIOMARKERS

An early diagnosis of cancer remains a challenge and, in this context, it is important to find a sensitive, non-invasive tool to detect early neoplastic changes. One relatively non-invasive source of miRNAs for the diagnosis of lung cancers is sputum^[95]. The Jiang laboratory demonstrated that endogenous miRNAs are stably present in sputum specimens. Using qRT-PCR, miR-21 and miR-155 were detected, of which miR-21 was significantly overexpressed in sputum of NSCLC patients as

compared with cancer-free subjects^[96]. Furthermore, elevated miR-21 expression was more sensitive (70%) than conventional sputum cytology (48%) in diagnosing lung cancer. The same research group defined miRNA signatures for different histologic types of lung cancer in studies of similar design^[97,98]. For the diagnosis of SCC, the combination of miR-205, miR-210 and miR-708 yielded 73% sensitivity and 96% specificity. A panel consisting of miR-21, miR-200b, miR-375 and miR-486 produced 81% sensitivity and 92% specificity in discriminating sputum of AD patients from controls. The authors found no association between miRNA expression and stage of lung cancer, suggesting that the miRNA signatures can be used as a tool in the detection of early lung cancer. The same group recently reported that combined quantification of miR-31 and miR-210 copy number by digital PCR in sputum of the cases and controls provided 65.71 % sensitivity and 85.00 % specificity for stage I NSCLC diagnosis^[99]. Very recently, the same authors also reported that combining miR-31 and miR-210 detection by qRT-

PCR and Computed Tomography they improved NSCLC diagnosis specificity^[100].

In an independent study, a five-miRNA profile (miR-21, miR-143, miR-155, miR-210, miR-372) performed by qRT-PCR on sputum samples detected NSCLC with 83.3% sensitivity and 100% specificity^[101].

CIRCULATING MIRNAS

First evidences

The finding that miRNAs have an exceptional stability in several tissues suggested that these tiny molecules were also preserved, detectable and quantifiable in the circulation and in other biofluids.

The first indication of circulating microRNAs with a potential as non-invasive diagnostic biomarkers for diffuse large B-cell lymphoma (DLBCL) and possibly other cancers was found by Lawrie *et al.*^[102]. These authors were the first to highlight that miRNAs could be reliably detected in serum and they demonstrated that high levels of miR-21, miR-210 and miR-155 could discriminate cancer patients with (DLBCL) from healthy individuals. Plasma miRNAs were observed to be present in a remarkably stable form that is protected from endogenous RNase activity^[103]. These papers established the measurement of tumor-derived miRNAs in serum or plasma as an important approach for the blood-based detection of human cancer. Further studies demonstrated that microRNAs are also preserved and detectable in other biofluids such as urine, saliva, cerebro-spinal fluid and amniotic fluid^[104] and their composition and concentrations are measurably different among these fluids^[105].

On the origin of circulating miRNAs

miRNAs can be released in the circulation by two different pathways: energy-free passive release or active and selective secretion in response to different stimuli. The first process does not need energy, it occurs after cell breakage in pathological conditions such as tissue damage, apoptosis, metastasis or inflammation but it does not play a major role in the generation of circulating miRNAs^[106]. miRNAs active secretion, differently from the passive leakage, is a process ATP- and temperature-dependent. It is similar to the release of hormones and cytokines, with or without cell stimulation^[106].

Circulating miRNAs can be packaged in microvesicles (MVs) or apoptotic bodies (ABs) or can be found as microvesicle-free miRNAs, associated with various multi-protein or lipoprotein complexes.

MVs are small vesicles derived from cells, generally including microparticles (MPs) and exosomes. These two kinds of MVs have quite different vesicular structures: exosomes (50-90 nm), with an endocytic origin, are released by fusion of multivesicular bodies (MVBs) with the plasma membrane^[107]. They have been identified in various body fluids such as blood, urine, malignant ascites, bronchoalveolar lavage fluid, synovial fluid, breast milk and saliva^[108]. MPs, on the other hand, are larger

than exosomes (> 100 nm diameter) and are shed from plasma membranes^[109]. Almost all cell types can release MVs under normal physiological or pathological conditions.

Larger in size than MPs, ABs are generated in response to apoptotic stimuli^[110] and implicated in tissue repair and angiogenesis.

It has been demonstrated that MVs and ABs are involved in the transport of circulating miRNAs^[111-114]. Gibbings *et al.*^[115] showed that miRNAs loading into exosomes is not a random event, but it is mediated by proteins as Argonaute protein Ago2, a part of RNA-induced silencing complex.

On the other hand, several studies suggested that a significant fraction of extracellular miRNAs resides outside of vesicles and acts in exosome-independent manner, by its association with RNA-binding proteins including Ago2 and Nucleophosmin 1 (NPM1) or lipoprotein complexes such as high-density lipoprotein (HDL). Arroyo *et al.*^[116] showed that miRNAs in plasma are predominantly free of exosomes or microvesicles. They demonstrated an association of miRNAs with Ago2 protein, showing that this binding protects and increases the stability of released miRNAs^[116]. Turchinovich *et al.*^[117] confirmed this hypothesis and demonstrated that also additional Argonaute proteins (Ago1, -3, -4) may be associated with cell-free circulating miRNAs. Wang *et al.*^[118] found that other RNA binding proteins, such as NPM1, can protect miRNAs from degradation, playing a role in the packaging and export of circulating miRNAs. Vickers *et al.*^[119] in 2011 revealed a potential new role for HDL in gene regulation and intercellular communication, showing that this lipoprotein transports and delivers miRNAs to recipient cells.

Circulating miRNAs as cancer biomarkers

It has been shown that miRNAs present in body fluids can reflect altered physiological conditions, representing new effective biomarkers^[104].

A perfect biomarker should have some important features: non-invasivity, specificity, early detection, sensitivity and ease of translatability from model systems to humans.

Proteins used as blood biomarkers (*e.g.*, troponin for cardiovascular conditions, carcinoembryonic antigen (CAE) for various cancers, prostate specific antigen (PSA) for prostate cancer, and aminotransferases for liver function) do not respect all these criteria and are difficult to use in this field. On the contrary, secreted miRNAs have many of these requisites: they are stable in various biofluids, the expression of some miRNAs is specific to tissues or biological stages, and their level can be easily detected by various methods.

However, several challenges have to be overcome in order to successfully use circulating miRNAs as cancer biomarkers. First, biofluids contain very low amount of RNA, and normal quantification methods are not suitable for these type of samples. Second, it is important

to avoid cellular contamination and hemolysis and third, biofluids contain inhibitors of reverse transcriptase and polymerase enzymes used for miRNAs quantification. All these factors are obstacles to consider when circulating miRNAs are isolated and quantified from biofluids such as plasma or serum. Another major challenge for the analysis of circulating miRNAs is the choice of an appropriate reference gene, since some of the small RNA species frequently used as reference genes (such as U6 RNA) are present in extremely low concentrations in serum and plasma as well as in other biofluids. Moreover, normalization controls used to remove variations and increase the accuracy of miRNAs quantification cannot ensure constant expression under all experimental conditions, underlining the importance of the selection of a proper reference gene.

Chen *et al.*^[120] reported that U6 and 5S rRNA are degraded in serum samples from lung cancer patients, and miR-16 is inconsistent, choosing to normalize the level of circulating miRNAs to total RNA. In a study on sera from Hepatitis B infected patients and matched controls, snRNA U6-1 is found to have high variability and snRNA U6-2 is not detectable. In this type of samples the combination of miR-26a, miR-221, and miR-22* is recommended as the most stable set of reference genes for circulating miRNAs evaluation^[121]. Similarly, U6-2 is inconsistent in serum from gastric cancer patients and healthy controls. In this study, authors recommend the combined use of miR-16 and miR-93 as suitable reference genes^[122]. On the contrary, in serum from uro-oncological patients, U6-2 is detectable and rather consistent^[123]. snoRNA U44 levels are similar in sera from breast cancer patients and from age-matched healthy women, differently from miR-16 and 5S rRNS that show remarkable variability in the same samples. Surprisingly, snRNA U6-1 serum levels are found consistently higher in breast cancer patients compared to healthy controls, not only confirming that U6 is not an appropriate reference gene, but also indicating an interesting new paradigm in cancer^[124]. Finally, Sourvinou and colleagues showed that a combined use of endogenous miR-21 and miR-16 and exogenous cel-miR-39, compensates differences in miRNAs recovery and differences in cDNA synthesis between samples. Using this normalization procedure and miR-21 as a biomarker, it seems possible to clearly discriminate healthy individuals from NSCLC patients^[125].

Circulating miRNAs as lung cancer biomarkers

The Jiang laboratory found that miR-155, miR-197 and miR-182 can be potential biomarkers for early detection of lung cancer with 81.33% sensitivity and 86.76% specificity (Table 3). The levels of these miRNAs in plasma of NSCLC patients are elevated compared with healthy controls^[126]. The same group demonstrated that another set of plasma miRNAs (miR-21, miR-126, miR-210, and miR-486-5p), had 86.22% sensitivity and 96.55% specificity in distinguishing NSCLC patients from the healthy

controls (Table 3). Furthermore, the panel of four miRNAs produced 73.33% sensitivity and 96.55% specificity in identifying stage I NSCLC patients. The miR panel had higher sensitivity (91.67%) in diagnosis of AD compared with SCC (82.35%)^[127]. Authors from the Jiang lab recently reported that quantification of the plasma miR-21-5p and miR-335-3p by digital PCR provided 71.8% sensitivity and 80.6% specificity in distinguishing lung cancer patients from cancer-free subjects (Table 3)^[128].

Tang *et al.*^[129] reported that higher plasma miR-21 and miR-155 and lower plasma miR-145 expression levels distinguish lung cancer patients from healthy smokers with 69.4% sensitivity and 78.3% specificity (Table 3). Levels of miR-361-3p and miR-625-3p might have a protective influence on the development of NSCLC, and the quantification of these miRNAs in serum could be useful for the diagnosis of NSCLC, in particular in smokers^[130]. A study reported that the expression of miR-146b, miR-221, let-7a, miR-155, miR-17-5p, miR-27a and miR-106a is significantly reduced in sera of NSCLC cases, while miR-29c is significantly increased (Table 3). Unexpectedly, no significant differences were observed in plasma of patients compared with controls^[131].

Bianchi *et al.*^[132] provided an evidence that some serum-circulating miRNAs are important to identify asymptomatic high-risk individuals with early stage lung cancer (Table 3). Between others, they highlighted the importance of let-7 family, members of miR-17-92 cluster, miR-126 and miR-486 in sera of NSCLC patients^[132].

Recently, qRT-PCR was used to assess miR-205-5p, miR-205-3p, and miR-21-3p expressions in serum and tissue samples (Table 3)^[133]. The relative expressions of miR-205-5p and miR-205-3p were significantly higher in NSCLC tissues compared with cancer-adjacent paired specimens. In the serum, significantly higher miR-205-5p, miR-205-3p, and miR-21-3p relative expressions were observed in the NSCLC group compared with healthy volunteers or patients diagnosed with a benign lung disease (pulmonary tuberculosis, pneumonia, chronic obstructive pulmonary disease, or interstitial pneumonia). The relative expressions of miR-205-5p and miR-21-3p in NSCLC tissues and serum were significantly correlated, while no significant correlation was observed for miR-205-3p. Expressions of miR-205-5p and miR-205-3p in SCC specimens were significantly higher than in lung adenocarcinoma specimens. Similarly, higher serum miR-205-5p and miR-205-3p levels were observed in SCC patients.

MiRNAs expression profile in whole-blood showed that miR-190b, miR-630, miR-942 and miR-1284 are present in a majority of the classifiers generated during the analyses to distinguish lung cancer cases from controls^[64]. In a different study, miR-22, miR-24, and miR-34a were found upregulated in RNA extracted from whole blood of NSCLC patients *vs* healthy controls (Table 3)^[134].

In a recent paper, Aushev *et al.*^[135] described a specific panel of miRNAs (miR-205, -19a, -19b, -30b, and -20a)

Table 3 Circulating miRNAs as biomarkers in lung cancer

MIRNA	Function	Scope	Sample	Ref.
miR-155, miR-197, miR-182 (UP)	Diagnostic	Lung cancer patients <i>vs</i> healthy controls	Plasma	[126]
miR-21, miR-210, miR-126, miR-486-5p (relative expression)	Diagnostic	NSCLC patients <i>vs</i> healthy controls	Plasma	[127]
miR-21-5p (UP) and miR-335-3p (DOWN)	Diagnostic	Lung cancer patients <i>vs</i> healthy controls	Plasma	[128]
miR-21, miR-155 (UP), miR-145 (DOWN)	Diagnostic	Lung cancer patient <i>vs</i> healthy smokers	Plasma	[129]
miR-361-3p, miR-625* (DOWN)	Diagnostic	Lung cancer patients <i>vs</i> healthy controls	Serum	[130]
miR-146b, miR-221, let-7a, miR-155, miR-17-5p, miR-27a and miR-106a (DOWN), miR-29c (UP)	Diagnostic	Early stage NSCLC <i>vs</i> healthy controls	Serum	[131]
miR-92a, miR-484, miR-486-5p, miR-328, miR-191, miR-376a, miR-342, miR-331-3p, miR-30c, miR-28-5p, miR-98, miR-17-5p, miR-26b, miR-374, miR-30b, miR-26a, miR-142-3p, miR-103, miR-126, let-7a, let-7d, let-7b, miR-22, miR-148b, miR-139 (DOWN), miR-32, miR-133b, miR-566, miR-432-3p, miR-223, miR-29a, miR-148a, miR-142-5p, miR-140-5p (UP)	Diagnostic	Asymptomatic NSCLC patients <i>vs</i> healthy smokers	Serum	[132]
miR-205-5p, miR-205-3p, and miR-21-3p (UP)	Diagnostic	NSCLC patients <i>vs</i> benign lung disease and healthy controls	Serum	[133]
miR-190b, miR-630, miR-942 and miR-1284 (relative expression)	Diagnostic	Lung cancer patients <i>vs</i> healthy controls	Whole-blood	[85]
miR-22, miR-24, and miR-34a (UP)	Diagnostic	NSCLC patients <i>vs</i> healthy controls	Whole-blood	[134]
miR-205, miR-19a, miR-19b, miR-30b, miR-20a (DOWN)	Diagnostic	Patients after lung cancer surgery <i>vs</i> healthy controls	Plasma	[135]
miR-7, miR-21, miR-200b, miR-210, miR-219-1, miR-324 (UP), miR-126, miR-451, miR-30a, miR-486 (DOWN)	Diagnostic	NSCLC patients <i>vs</i> healthy controls	Plasma	[137]
miR-101, miR-106a, miR-126, miR-133a, miR-140-3p, miR-140-5p, miR-142-3p, miR-145, miR-148a, miR-15b, miR-16, miR-17, miR-197, miR-19b, miR-21, miR-221, miR-28-3p, miR-30b, miR-30c, miR-320, miR-451, miR-486-5p, miR-660, and miR-92a (relative expression)	Diagnostic	NSCLC patients <i>vs</i> healthy controls	Plasma	[138]
miR-155, miR-197 (UP)	Prognostic	Lung cancer patients with metastasis <i>vs</i> patients without metastasis	Plasma	[126]
miR-486, miR-30d, miR-1, miR-499 (relative expression)	Prognostic	NSCLC patients <i>vs</i> healthy controls	Serum	[139]
let-7f, miR-30e-3p (DOWN)	Prognostic	NSCLC patients <i>vs</i> healthy controls	Plasma	[140]
miR-125b (relative expression)	Prognostic	NSCLC patients <i>vs</i> healthy controls	Serum	[141]
miR-21 (UP)	Response to treatment	Platinum chemotherapy-resistant patients <i>vs</i> non resistant patients	Plasma	[90]
miR-21 and miR-10b (UP)	Response to treatment	NSCLC patients with EGFR mutation <i>vs</i> patients without mutation	Plasma	[142]
miR-22 (UP)	Response to treatment	NSCLC patients <i>vs</i> healthy controls	Whole-blood	[134]

NSCLC: Non-small cell lung cancer.

decreasing in plasma of patients after SCC surgery (Table 3). Interestingly, high levels of these miRNA are found in tumor-specific exosomes^[135].

Also NGS has been used to depict the differential expression of miRNAs in peripheral blood of lung cancer patients detecting 76 previously unknown miRNAs and 41 novel mature forms of known precursors. In addition, the authors identified 32 annotated and seven unknown miRNAs that were significantly altered in NSCLC patients^[136].

A plasma-based 24-miRNA signature classifier with predictive, diagnostic, and prognostic value was described, whose use could reduce the false-positive rate of low-dose computed tomography (LDCT), thus improving the efficacy of lung cancer screening (Table 3)^[137,138].

Regarding the potential of circulating miRNAs as prognostic factors, the levels of miR-155 and miR-197 have been found higher in plasma from lung cancer patients with metastasis than in those without metastasis (Table 3)^[126].

Moreover, Hu *et al.*^[139] using NGS described that serum levels of miR-486, miR-30d, miR-1 and miR-499 are significantly associated with overall survival (Table 3). NSCLC patients and healthy controls differ in vesicle-related miRNAs in plasma: let-7f and miR-30e-3p levels decreased in plasma vesicles of NSCLC patients and the expression of these miRNAs is associated with poor outcome (Table 3)^[140]. Finally, serum miR-125b may represent a biomarker in NSCLC with an independent prognostic potential for overall survival (Table 3)^[141].

Circulating miRNAs have also been explored for their ability to predict response to treatment. miR-21 expression has trends similar in plasma and matched resected specimens and was significantly increased in platinum-based chemotherapy-resistant patients, in which increased miR-21 expression was associated with the shorter disease-free survival (Table 3)^[90].

The expression of miR-21 and miR-10b was much higher in plasma samples of patients with NSCLCs with EGFR mutation than without mutation (Table 3)^[142]. Patients who had up-regulated miR-21 expression had shorter overall survival, but a better response to gefitinib than patients who had low expression of the microRNA. Additionally, miR-10b is highly expressed in progressive disease compared with complete remission or stable disease.

Franchina *et al.*^[134] recently reported a correlation between high expression of miR-22 in whole blood and the lack of response in pemetrexed treated NSCLC patients (Table 3).

CONCLUSION

MiRNAs have increasingly been pointed as important players in carcinogenesis and cancer progression, but also as potential diagnostic and prognostic markers.

For the future, circulating miRNAs could open new opportunities in the field of diagnosis and prognosis in various types of human cancers. The difficulties found in traditional therapies, due to insufficient disruption of oncogenic pathways, drug resistance and drug-induced toxicity, require the development of novel therapeutic strategies. The ease, specificity and sensitivity of determining body fluid miRNAs profiles paves the way for several applications and provides hope to accomplish this task. However, from the technical and applicative point of view, there are still several limitations to consider. Further studies are necessary to find the best possible normalization control and to improve the technique, but also to establish panels of miRNAs specific to each type of tumor, taking into account early or advanced cancer stages, response to treatment, patient outcome and recurrence. After the complete validation for several candidates, miRNAs studies could open a new era in cancer treatments, providing improved targeted agents for the cure of patients^[143] and constituting the basis for the development of novel therapies.

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WJCO 5th Anniversary Special Issues (1): Lung cancer

C4.4A as a biomarker in pulmonary adenocarcinoma and squamous cell carcinoma

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carcinomas of the lung, *i.e.*, in bronchial hyperplasia/metaplasia and atypical adenomatous hyperplasia. In the stages leading to pulmonary squamous cell carcinoma, expression is sustained in dysplasia, carcinoma *in situ* and invasive carcinomas, and this pertains to the normal presence of C4.4A in squamous epithelium. In pulmonary adenocarcinomas, a fraction of cases is positive for C4.4A, which is surprising, given the origin of these carcinomas from mucin-producing and not squamous epithelium. Interestingly, this correlates with a highly compromised patient survival and a predominant solid tumor growth pattern. Circumstantial evidence suggests an inverse relationship between C4.4A and the tumor suppressor LKB1. This might provide a link to the prognostic impact of C4.4A in patients with adenocarcinomas of the lung and could potentially be exploited for predicting the efficacy of treatment targeting components of the LKB1 pathway.

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Key words: LYPD3; Non-small cell lung cancer; Prognosis; Solid growth pattern; Liver kinase B1; Precursor lesions; Atypical adenomatous hyperplasia; Metaplasia; Squamous differentiation; Ly6/Urokinase-type plasminogen activator receptor

Abstract

The high prevalence and mortality of lung cancer, together with a poor 5-year survival of only approximately 15%, emphasize the need for prognostic and predictive factors to improve patient treatment. C4.4A, a member of the Ly6/uPAR family of membrane proteins, qualifies as such a potential informative biomarker in non-small cell lung cancer. Under normal physiological conditions, it is primarily expressed in suprabasal layers of stratified squamous epithelia. Consequently, it is absent from healthy bronchial and alveolar tissue, but nevertheless appears at early stages in the progression to invasive

Core tip: C4.4A is a new biomarker with potential prognostic value in pulmonary adenocarcinoma. High levels of protein expression correlate with poor patient survival and a histological growth pattern of the solid type. Recent data suggest that C4.4A is negatively regulated by the tumor suppressor liver kinase B1 (LKB1), which is inactivated in a fraction of adenocarcinomas of the lung. Such an inverse association between C4.4A and LKB1 could possibly render C4.4A a candidate predictive biomarker for therapeutic intervention targeting components of the LKB1 pathway, such as mammalian target of rapamycin.

Jacobsen B, Kriegbaum MC, Santoni-Rugiu E, Ploug M. C4.4A

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INTRODUCTION

Lung cancer is the most prevalent and most mortal form of cancer, with an estimated 1.6 million new cases and 1.375 million deaths every year^[1]. Survival is highly dependent on stage at presentation, decreasing drastically from localized (52%) to regional (25%) and distant disseminated disease (4%), yielding an overall relative 5-year survival of only approximately 15%^[2]. Despite these discouraging statistics, there have, with the advent of personalized medicine, been some improvements in the treatment of lung cancer^[3]. This pertains predominantly to an improved knowledge on tumor biology, which has uncovered a molecular rationale for different treatment efficacies. In this context, molecular and histologic parameters for prediction of drug responsiveness, in particular the identification of subsets of lung cancer harboring distinct targetable oncogenic driver mutations, have guided choice of therapy.

A striking example can be seen with small tyrosine kinase inhibitors (TKIs), for which activating mutations in exons 19 and 21 of the epidermal growth factor receptor (EGFR) gene are predictive of therapeutic efficacy^[4-7]. Consequently, activating *EGFR* mutations are now a validated biomarker for decisions regarding first-line treatment of advanced non-small cell lung cancer (NSCLC)^[4,8]. Response to the anaplastic lymphoma kinase (ALK)-TKI crizotinib similarly depends on the presence of the *echinoderm microtubule-associated protein-like 4 (EML4)-ALK* fusion gene in a subset of pulmonary adenocarcinoma (AC)^[9,10]. In addition to *EGFR* and *ALK* tests, the potential of other biomarkers is being validated in clinical trials, *e.g.*, ROS1, human epidermal growth factor receptor 2/neu (HER2), BRAF and mesenchymal-epidermal transition proto-oncogene (MET) primarily in AC, and fibroblast growth factor receptor 1 (FGFR1) and discoidin domain receptor 2 (DDR2) in squamous cell carcinoma (SCC)^[11]. Regarding NSCLC histology, there have been tolerance issues coupled to the inhibitor of angiogenesis, bevacizumab, which can cause life-threatening hemorrhage and hemoptysis in patients with SCC, and is thus contra-indicated in this subgroup^[12,13]. Another drug, the antifolate pemetrexed, inhibits three enzymes of the folate metabolism, thymidylate synthase (TS), dihydrofolate reductase, and glycinamide ribonucleotide formyltransferase, the consequence of which is a reduction in *de novo* purine and pyrimidine synthesis, thus interfering with DNA and RNA synthesis. Overexpression of TS might be associated with a reduced efficacy of pemetrexed, and this can explain why it is more efficient in advanced AC than in SCC^[14,15]. The fact that tumor histology and molecular features can influence the choice of treatment implies that different NSCLC histologic subtypes should be considered

as distinct disease entities, which in turn comprise distinct molecular subsets that should be managed individually for successful outcome.

Earlier detection of lung cancer, as well as assessment of the malignant potential of a resected tumor for decisions regarding adjuvant treatment, represent other promising avenues for reducing the high mortality of the disease. Continuously increasing our knowledge on the mechanisms involved in pathogenesis is crucial to further improve the rational targeting strategy that has been adopted for lung cancer treatment. New biomarkers of early disease, prognosis and prediction of response to targeted therapy are relevant in this context, to ameliorate patient survival^[16-18]. The protein C4.4A is a potential new biomarker in NSCLC.

C4.4A – A UROKINASE RECEPTOR PROTEIN HOMOLOG

C4.4A is a membrane protein anchored to the cell surface via a glycosylphosphatidylinositol (GPI) moiety, showing predicted structural homology to the other multidomain members of the Ly6/urokinase-type plasminogen activator receptor (uPAR) (LU) protein family, *i.e.*, the uPAR, Haldisin, TEX101, CD177 and *LYPD4*^[19-23]. The genes encoding these proteins are clustered in a small region of chromosome 19q13 (Figure 1A). After posttranslational processing, C4.4A consists of 278 amino acids distributed in 2 N-terminal LU domains and a serine, threonine, proline-rich region C-terminally (Figure 1B).

C4.4A IN NORMAL DIFFERENTIATION PROCESSES

Under normal physiological conditions, C4.4A is predominantly expressed in suprabasal layers of stratified squamous epithelia such as those of the skin, hair follicles, esophagus, oral and nasal cavity, vagina and cornea (Figure 1C). It is furthermore found in the cuboidal epithelium of human term placenta and sweat ducts, in the pigmented epithelium of the retina and in Hassall's corpuscle in the thymus^[19,24]. Remaining epithelia, including the alveolar and bronchial compartments of the healthy lung (Figures 2A and 3A; Table 1), are devoid of C4.4A, suggesting a tight regulation of its expression, possibly by the CCAAT/enhancer binding protein β (C/EBP β) or estrogen^[25,26]. This is clearly visualized at transition zones such as those present in the rodent stomach and at the ano-rectal junction, where the distinct C4.4A expression at the squamous side is abruptly terminated at the columnar side (Figure 1D). The stringent membrane-associated expression pattern of C4.4A would be in line with a putative role of this protein in cell adhesion^[19,27,28], but its biological function is still to be delineated.

C4.4A IN PATHOLOGICAL CONDITIONS

C4.4A was originally identified by two independent differ-

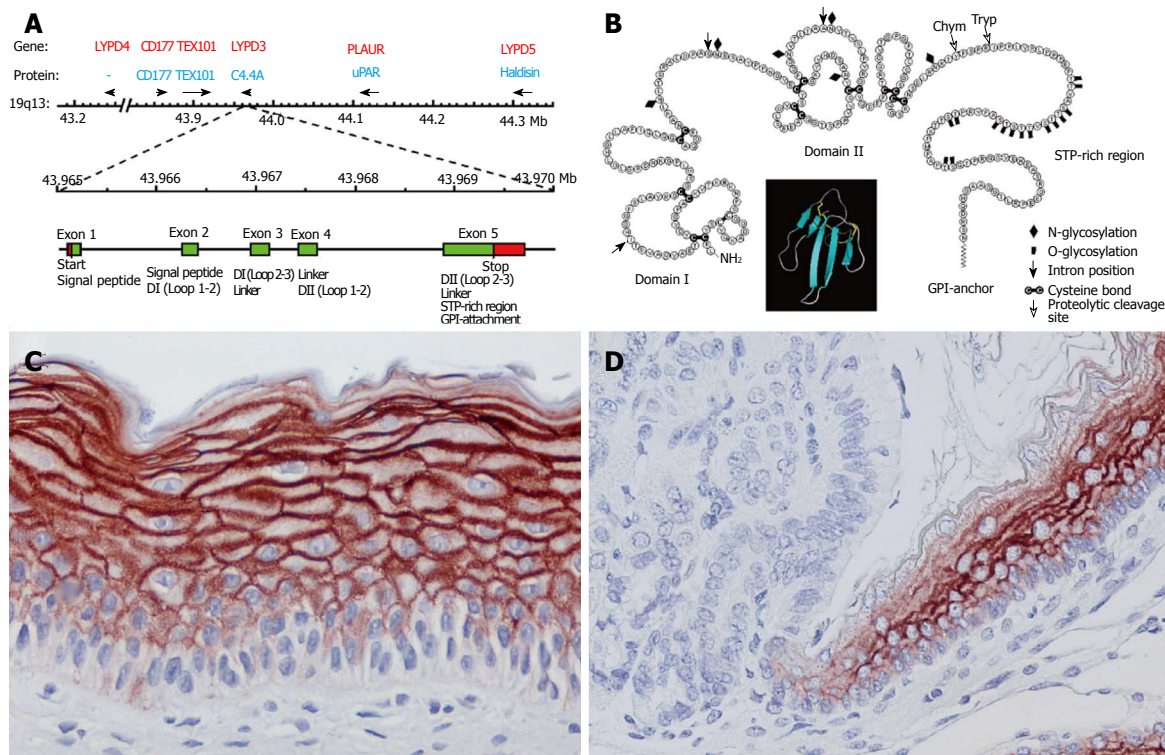


Figure 1 Structure and expression of the protein C4.4A. A: Schematic outline of the gene cluster encompassing multidomain members of the LU protein family, highlighting the gene encoding C4.4A (LYPD3) and its exon composition. Modified from Kriegbaum *et al.*^[20,24], 2011 and Jacobsen *et al.*^[20,24]. B: Cartoon of the structure of C4.4A. The insert represents the three-finger fold characteristic of LU domains (made in PyMOL, DeLano Scientific, using PDB coordinates 1NEA). Modified from Hansen *et al.*^[19]. C and D: C4.4A expression in suprabasal layers of squamous epithelium, exemplified by tissue-engineered human epidermis (C) and the transition between the glandular and non-glandular portions of the rodent stomach (D). C4.4A is detected by a polyclonal C4.4A antibody and visualized by NovaRED chromogen. D is reproduced with permission from BestPractice Onkologi, Denmark^[89].

Table 1 Expression and prognostic significance of C4.4A in pulmonary squamous cell carcinoma and adenocarcinoma								
		C4.4A reactivity				Multivariate survival analysis ⁴		Ref.
Premalignant lesions		0 ¹	+	++	+++			[34]
SCC	Normal	100%						
	Hyperplasia		59%	41%				
	Metaplasia			29%	71%			
	Dysplasia			20%	80%	Not available		
AC	Normal	100%						[33]
	AAH	17%	37%	37%	10%			
Invasive cancer		Median ²	Range	Lower quartile	Upper quartile	HR (95%CI)	P-value	
SCC		8	0-16	5	10		0.82	
AC		1 ³	0-16	0	6	1.65 (1.24-2.19)	0.0005	

¹0: Negative; +: Weakly/focally positive; ++: Positive; +++: Strongly positive; ²Refers to product of C4.4A intensity (0-4, where 0 = negative, 1 = very weak, 2 = weak, 3 = moderate and 4 = strong staining) and frequency (0-4, where 0 = 0%, 1 = 0%-25%, 2 = 26%-50%, 3 = 51%-75% and 4 ≥ 76% positive tumor cells); ³Median value for non-solid and solid AC is 0 and 4, respectively; ⁴Performed by the Cox proportional hazards model. SCC: Squamous cell carcinoma; AC: Adenocarcinoma.

ential antigen screens as a candidate metastasis-associated protein^[29,30], the first in a rat pancreatic adenocarcinoma cell line, and the second in an *in vitro* urothelial wound response model. Rösel *et al.*^[29] furthermore reported on the capability of C4.4A-positive but not C4.4A-negative tumor cells to penetrate a matrigel, in the absence but not in the presence of a monoclonal C4.4A antibody. C4.4A has indeed been implicated in a range of human cancers, including lung^[31-35], esophageal^[27], bladder^[30] and colorectal^[36], as evaluated by immunohistochemistry, *in situ* hybridization, PCR, North-

ern blotting or microarray screening. In colorectal cancer^[36], esophageal squamous cell carcinomas^[27] and bladder transitional cell carcinomas (Jacobsen *et al.*^[33], manuscript in preparation), C4.4A is upregulated at the tumor invasive front as compared to the tumor core, suggesting a possible association of C4.4A to the invasive process. Whether this can be further translated to a direct involvement in the ability of a tumor cell to metastasize as initially proposed has not been verified experimentally *in vivo*, but the expression of C4.4A seen in primary tumors is at least in the case of the esopha-

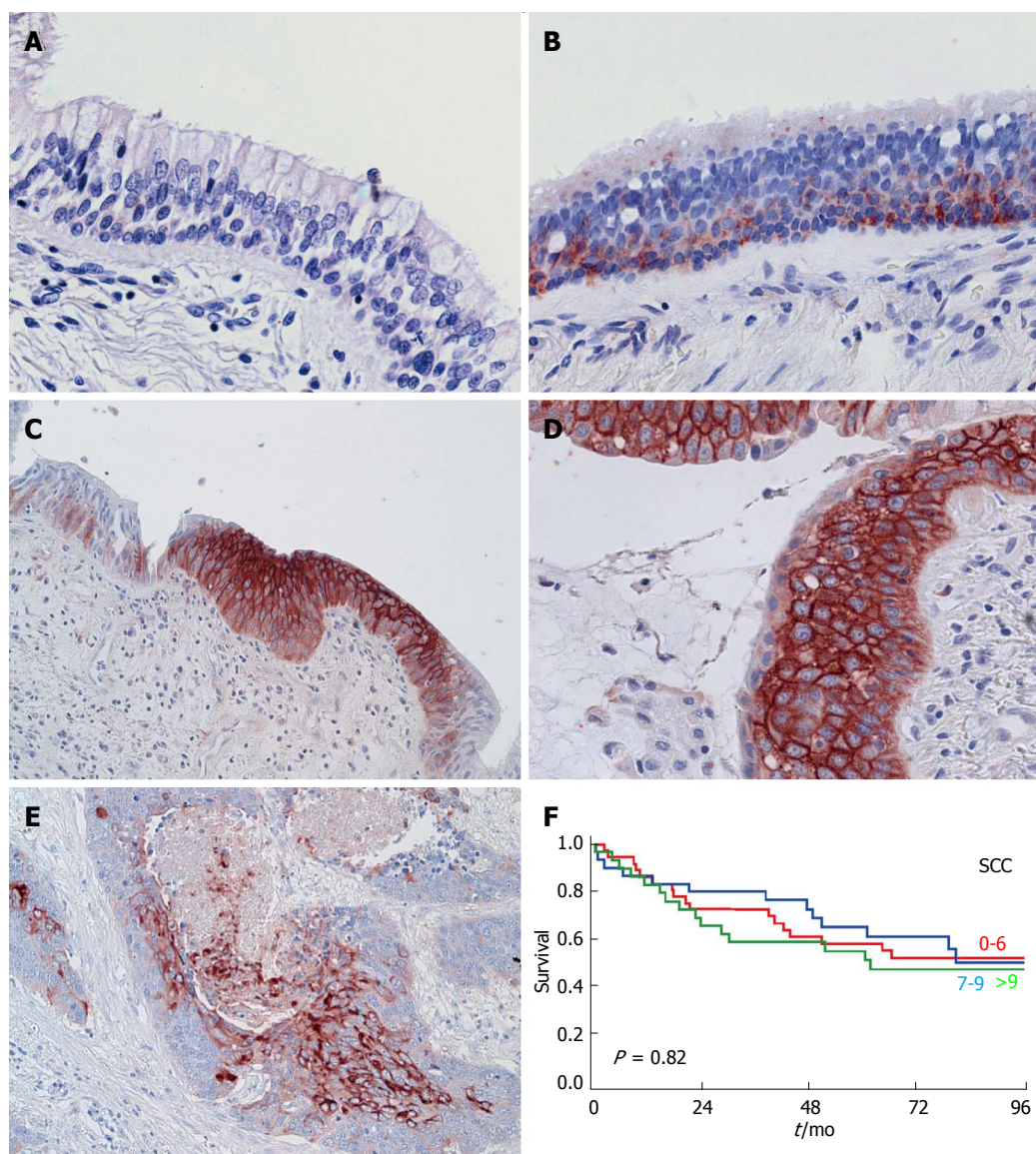


Figure 2 C4.4A in pulmonary squamous cell carcinoma. Panels A-E: C4.4A expression as detected by immunohistochemistry with a polyclonal antibody in normal bronchial epithelium (A), hyperplasia (B), metaplasia (C), dysplasia (D) and invasive squamous cell carcinoma (SCC) (E). A, B, D and C, E are reproduced with permission from Jacobsen *et al.*^[34], 2012 and BestPractice Onkologi, Denmark^[89], respectively. Panel F: Kaplan-Meier curves for the survival of SCC patients, which is independent of C4.4A scores, here stratified by tertiles (red: Lowest level of C4.4A; blue: Intermediate level of C4.4A; green: Highest level of C4.4A). Modified from Jacobsen *et al.*^[33], 2013.

gus and the lung recapitulated in corresponding lymph node metastases^[27,33].

One of the most well-studied diseases regarding C4.4A expression is NSCLC^[31,33,34]. The role of C4.4A in the progression to pulmonary SCC and AC is described in the following

C4.4A IN PULMONARY SQUAMOUS CELL CARCINOMA

Progression to malignancy through well-described premalignant lesions

Lung cancer is divided into two main histological types, where small cell lung cancer comprises around 15% of cases and NSCLC the remaining 85% of cases. The latter is further subdivided into AC, SCC and large cell carcinoma

(LCC), of which AC has become the most frequent type^[37-39].

Pulmonary SCCs most often originate in the bronchial compartment. There is a high degree of consensus concerning the stages that transform normal, pseudostratified columnar bronchial epithelium into invasive SCC^[40]. After an excessive proliferation phase resulting in hyperplasia of basal cells, metaplasia entails the transdifferentiation of bronchial cells resulting in the conversion of columnar epithelium to squamous epithelium. Dysplasia represents the first true premalignant stage and is followed by carcinoma *in situ* (CIS), which again can develop into malignant carcinoma. Morphologically, epithelial thickness, cell size and mitotic figures increase throughout this progression, which is also characterized by pleomorphism and loss of epithelial polarity^[37]. Molecularly,

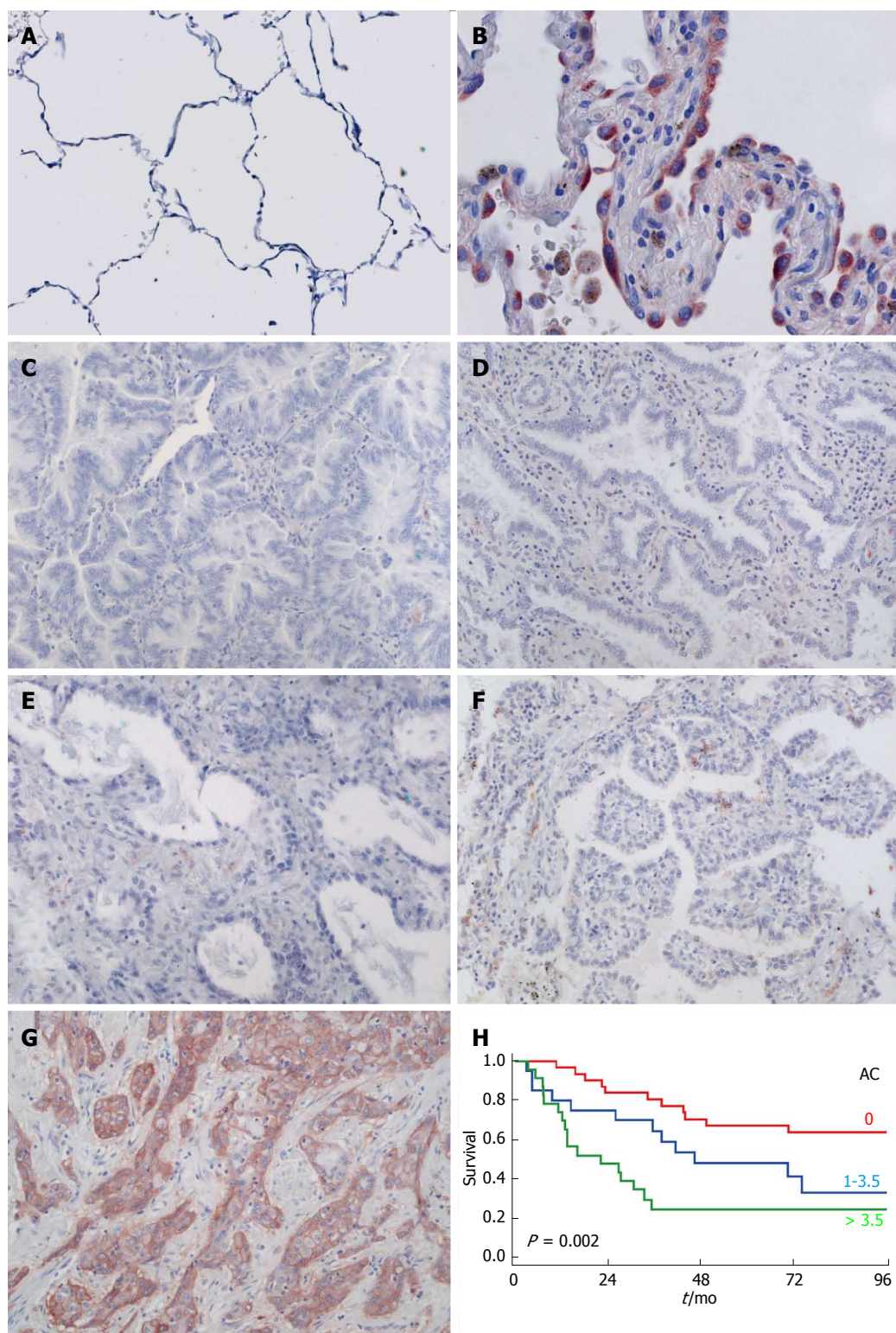


Figure 3 C4.4A in pulmonary adenocarcinoma. Panels A-E: C4.4A expression as detected by immunohistochemistry with a polyclonal antibody in normal alveoli (A; reproduced with permission from Jacobsen *et al.*^[34], 2012), atypical adenomatous hyperplasia (B), invasive AC with predominant mucinous lepidic (C), non-mucinous lepidic (D), acinar (E), papillary (F) and solid (G) pattern. Panel H: Kaplan-Meier estimates for the survival of AC patients, which is correlated with C4.4A scores, here stratified by tertiles (red: Lowest level of C4.4A; blue: Intermediate level of C4.4A; green: Highest level of C4.4A). Modified from Jacobsen *et al.*^[33], 2013.

activation of oncogenes and inactivation of tumor suppressor genes occur, along with allelic losses^[41-43].

Morphologically normal bronchial epithelium is devoid of C4.4A (Figure 2A), but expression appears

already at the stage of hyperplasia (Figure 2B) and becomes prominent upon metaplastic (Figure 2C) and dysplastic conversion (Figure 2D, Table 1)^[34]. The protein is consistently present in invasive SCC (Figure 2E), with

high levels detected in approx. 75% of patients in two different cohorts^[31,33].

C4.4A expression mirroring normal squamous differentiation

Given that hyperplasia and metaplasia are not true pre-malignant lesions, but rather regarded as reactive changes primarily caused by chronic irritation such as cigarette smoking, they do not by themselves indicate an increased risk of developing into a manifest carcinoma, as contrasted to severe dysplasia and CIS^[43-45]. A consequence of the very early appearance of C4.4A in basal cell hyperplasia/early metaplasia is that it cannot reflect the subsequent malignant transformation *per se* and therefore cannot be used as an early biomarker for pulmonary SCC. It thus reports on the differentiation status of the cells rather than on their malignant potential, and this provides an explanation for the presence of C4.4A in nearly all cases of invasive SCC^[31,33,34]. With consistent high levels of C4.4A in both premalignant and malignant lesions, C4.4A cannot differentiate between individual cases, and this is furthered into a lack of prognostic information in SCC patients (Figure 2F)^[31,33].

The detection of C4.4A in squamous metaplasia and dysplasia aligns excellently with the normal expression of C4.4A, which is closely linked to squamous differentiation of epithelium, as exemplified by murine embryogenesis, where C4.4A and the differentiation-specific cytokeratin 10 appear simultaneously in the nasal cavity (day E13.5) and in the interfollicular epidermis of the vibrissae (day E14.5)^[24]. Due to the stringent regulation of C4.4A in squamous epithelia, as convincingly shown at squamo-columnar junctions (Figure 1D), the appearance of C4.4A in basal cell hyperplasia in the bronchial compartment is on the other hand an unexpected finding. This could suggest that C4.4A reports on the induction of the metaplastic conversion process, where pseudostratified columnar epithelium is replaced by stratified squamous epithelium, even before the emerging squamous differentiation is evident morphologically. It has indeed been demonstrated that genetic aberrations such as mutations, deletions and overexpression of p53, and loss of heterozygosity (LOH) at chromosomes 3p and 9p (p16^{INK4a} locus) occur in histologically normal respiratory mucosa of smokers and lung cancer patients^[40,42,45-47]. Respiratory basal cells have been reported to be progenitors of squamous metaplastic cells and presumably preneoplastic epithelium^[37,40,42,48,49]. Delineating the function of C4.4A might give an indication of its role in the transdifferentiation process occurring in the bronchial mucosa, setting the stage for ensuing neoplastic transformation. This would increase our knowledge on the very early pathogenesis of pulmonary SCC. Considering the complete lack of C4.4A in normal bronchial epithelium, it might be used in conjunction with other histopathological, molecular and genetic markers in risk assessment for identifying malignant clones of the bronchial mucosa before these are manifest morphologically^[40], which could

suggest a need for a closer patient follow-up.

C4.4A IN PULMONARY ADENOCARCINOMA

From atypical adenomatous hyperplasia to invasive adenocarcinomas

Most ACs of the lung originate peripherally, which in contrast to the central airways is much more challenging to image, rendering a delineation of the various stages of transformation from normal alveolar epithelium to invasive AC corresponding to the development to invasive SCC difficult^[37,40,46]. On the basis of clinicopathologic and molecular studies, atypical adenomatous hyperplasia (AAH) has nevertheless been identified as a precursor lesion, which subsequently transforms into adenocarcinoma *in situ* (AIS), formerly known as bronchioloalveolar carcinoma (BAC)^[38,40,42,50-56]. AAH and AIS thus represent the counterparts of squamous dysplasia and CIS, respectively. Evidence for the stepwise development from AAH to AC comes from longitudinal case studies by low-dose computed tomography imaging^[57,58], the similarity of molecular aberrations^[40,59-62] and a common expression pattern of markers of the suggested progenitor cells, Clara cells and type II pneumocytes, in AAH and AC^[40,42,63] as well as mouse models of pulmonary AC, targeting KRAS/p53^[64] or EGFR^[65,66].

With a view to the preferential expression of C4.4A in squamous epithelium, as described above, one would not expect C4.4A expression in the alveolar compartment. This is indeed the case for normal alveoli (Figure 3A), but unexpectedly, the protein is present in approx. 25% of investigated cases of AC, albeit at much lower expression levels than in SCC, when scoring C4.4A semi-quantitatively as a product of intensity (0-4) and frequency (0-4) of immunohistochemical staining (median of 1 vs 8 on a scale up to 16) (Figure 3C-G, Table 1)^[31,33]. Interestingly, a small fraction of atypical type II pneumocytes in AAH lesions is also positive for C4.4A (Figure 3B)^[34]. This might be coupled to the cuboidal nature of these cells, as C4.4A also is found in other cuboidal epithelia such as the placenta and sweat ducts^[19] (and Krieglbaum, unpublished).

Prognostic impact of C4.4A in adenocarcinomas

As compared to known prognostic factors, such as stage, performance status, completeness of resection, tumor differentiation grade, nodal status, age and gender, the translational value of proposed lung tumor markers and gene expression signatures has been disappointing^[16,67]. It is therefore of interest to find new biomarkers or a combination thereof that could allow a more accurate survival prediction and aid in the decision making regarding adjuvant therapy. The prognostic impact of C4.4A in NSCLC has been investigated by a specific and reproducible semi-quantitative immunohistochemical protocol with substantial inter-observer agreement in two independent patient cohorts (40 ACs/56 SCCs from Denmark^[31]

and 88 ACs/104 SCCs from Germany^[33]). These studies showed that increasingly higher levels of C4.4A in ACs correlated with a decreasing patient survival, as evaluated by Kaplan-Meier analysis, where C4.4A scores were grouped according to tertiles (Figure 3H). Univariate and multivariate analysis of overall survival including C4.4A scores and the clinical covariates pathological stage, performance status, gender, age and treatment, likewise revealed C4.4A to be a significant prognostic factor (Table 1), together with pathological stage. The validation of the observations obtained in the first study in a second, larger patient population emphasizes the robust correlation between C4.4A levels and prognosis of AC patients. This has been further substantiated by another study based on quantitative real-time PCR, where the gene encoding C4.4A, *LYPD3*, was selected as one of 91 signature genes for survival prediction of pulmonary AC patients^[68]. It could be interesting to use patient material from previously conducted large clinical trials to retrospectively test for superiority of C4.4A to current prognostic factors, with a view to future validation in a prospective, randomized trial.

Considering the sequential development from AAH to AC, it is tempting to speculate whether a C4.4A-positive AAH eventually could develop into a C4.4A-positive AC with an ensuing compromised patient survival. If this were the case, this would entail that there are subpopulations of C4.4A-positive AAH cells with higher malignant potential than the C4.4A-negative counterparts, with the possibility to be used as an early marker of a supposedly more aggressive subtype of AC. In addition, considering that only a minority of AAHs progresses to AC, it would be interesting to assess whether C4.4A-positive AAH cases are more prone to this malignant progression than C4.4A-negative AAH. Addressing these questions would, however, require prospective, longitudinal studies and advanced imaging techniques not yet available for proper visualization of both AAH lesions and C4.4A, or alternatively following the various stages of malignant progression in a suitable mouse lung cancer model^[69].

Correlation of C4.4A expression with solid growth type

Pulmonary ACs encompass a histologically, molecularly and genetically very heterogeneous group of tumors. The new multidisciplinary classification suggests that AC should be described according to histological subtype, which can be mucinous or non-mucinous lepidic, acinar, papillary, micropapillary or solid^[38]. Gene expression profiling clusters the different AC types according to morphological and molecular characteristics, which emphasizes that ACs cannot be classified in one homogeneous group^[70,71]. Interestingly, patient survival also differs significantly with subtype, with 5-year relative survival rates of 86%-90% and 39%-70% when the lepidic and solid components, respectively, are predominant^[72,73].

Detailed analysis of C4.4A in the fraction of positive ACs, with focus on histological subtypes, has revealed that C4.4A expression is tightly correlated with the solid

growth pattern^[33]. Whereas the protein is almost absent in cases with a predominant mucinous/non-mucinous lepidic, acinar or papillary pattern (Figure 3C-F), the median value in cases with solid growth is 4 on a scale ranging from 0 to 16. This places the majority of these patients in the upper tertile of C4.4A scores in the AC group (> 3.5), with an ensuing poor prognosis as illustrated by Kaplan-Meier survival curves (Figure 3H). Given that a solid component in itself predicts poor patient survival^[72,73], it could be assumed that this clear interaction between C4.4A and solid growth would explain the prognostic impact of C4.4A. This is, however, refuted by multivariate overall survival analysis using the Cox proportional hazards model including growth pattern and the C4.4A/solid interaction in addition to classical clinical factors, where C4.4A and pathological stage are the only significant independent parameters. Despite the interaction between C4.4A and solid growth pattern, C4.4A is thus a stronger prognostic factor than solid growth^[33]. Given that AC patients with a predominant solid growth pattern seem to benefit from adjuvant radiotherapy, this might be exploited in clinical decision-making regarding this form of treatment^[74].

By comparing gene expression profiles of independent lung cancer patient populations obtained by DNA microarray analysis, Hayes *et al.*^[75] identified three AC subtypes that were termed bronchioid, squamoid and magnoid, with reference to their resemblance to bronchioalveolar carcinoma, SCC and LCC, respectively. AC tumors in a differentiation state close to the SCC phenotype thus seem to exist. This is further substantiated by a study of ACs of mainly the solid pattern with signet-ring morphology, which co-express thyroid transcription factor 1 (TTF-1) and p63, being markers of AC and SCC, respectively^[76]. In the WHO classification from 2004, carcinomas with histological and immunohistochemical evidence of double differentiation are termed adenosquamous carcinomas^[37]. The squamoid ACs are characterized by moderate or poor differentiation, solid growth, invasion, and a poor patient survival^[75,77]. The fact that C4.4A is primarily expressed in AC of the solid type, coupled to its close association to squamous differentiation and prognostic impact in AC would suggest that the C4.4A-positive ACs according to this classification are of the squamoid type.

Regulation of C4.4A by the tumor suppressor LKB1

The normal stringent control of C4.4A expression as seen in squamous epithelia is apparently disrupted in pulmonary AC. Whether the unexpected expression of C4.4A in pulmonary AC has functional implications in the pathogenesis of AC remains unknown. It might also be reporting on an erroneous regulation of a signaling pathway that when activated in the lung results in highly malignant tumors. Circumstantial evidence suggests the involvement of the tumor suppressor liver kinase B1 (LKB1). Firstly, C4.4A is negatively regulated by LKB1 in esophageal cancer cell lines, where *LYPD3* was up-

regulated upon loss of LKB1. The migratory and invasive potential of these cells was reduced when *LYPD3* subsequently was knocked down^[78]. Secondly, LKB1 reconstitution in the LKB1-deficient AC cell line H2126 downregulates C4.4A at the mRNA level^[79]. Thirdly, 17%-54% of pulmonary AC patients present with LKB1 inactivating mutations, of which the majority is found in poorly differentiated cases^[80-84]. Fourthly, LKB1 is lost in a fraction of AAH lesions^[85]. If there is an inverse relationship between LKB1 and C4.4A, C4.4A-positivity with its ensuing worsened patient prognosis in pulmonary AC might be explained by LKB1 inactivation in the given cases. This would imply that C4.4A potentially could be used as a biomarker for the efficacy of treatment targeting the LKB1 pathway, including mammalian target of rapamycin (mTOR) signaling, which is activated upon LKB1 inactivation^[86]. The potential of mTOR inhibitors as therapeutic agents in NSCLC is currently investigated in a range of clinical trials^[87,88].

One of the most recent genetic murine lung cancer models exploits a combination of LKB1 inactivation and KRAS oncogene activation to obtain metastatic tumors of a mixed phenotype, including AC, SCC and LCC^[79]. Crossing a C4.4A-deficient mouse with the KRAS/LKB1 model would allow a simultaneous investigation of the consequences of the absence or presence of C4.4A in the progression of both AC and SCC, revealing if C4.4A is functionally involved in or is a reporter of a molecular mechanism that could be targeted therapeutically.

CONCLUSION

C4.4A induction is an early event in the progression of two very distinct histological subtypes of lung cancer, *i.e.*, AC and SCC, where expression is seen in the presumed precursor lesions AAH and hyperplasia/metaplasia, respectively. Nevertheless, the differential prognostic impact suggests that the protein plays distinct roles in the pathogenesis of these two entities, and this underscores the high heterogeneity of lung cancer and the concept of AC and SCC as two different diseases.

In the progression to SCC, C4.4A is inherently linked to the phenotype that arises following the transdifferentiation process that converts pseudostratified columnar respiratory epithelium to squamous epithelium, completely in line with its normal expression. The appearance already in basal cell hyperplasia is interesting from a biological point of view, indicating that C4.4A is such an early marker of squamous differentiation that it is present even before the phenotype is morphologically manifest. However, given that C4.4A consistently is expressed in reactive lesions even preceding true premalignant lesions, is sustained in the subsequent stages of malignant progression and is invariably present in invasive carcinomas, it cannot possibly provide any information on the survival of SCC patients.

In sharp contrast to this, C4.4A is a strong, independent prognostic indicator in AC patients, and it therefore

has clinical relevance in this lung cancer subtype, especially in cases with a solid growth pattern, for which C4.4A is a surrogate marker. As such, whereas C4.4A follows a normal differentiation pattern in SCC, its expression in AC reflects an abnormal differentiation program. Clarifying whether this has implications for AC pathogenesis or is reporting on a dysregulated signaling pathway awaits the delineation of the biological function of C4.4A. Either way, it is tempting to speculate that there is a link between C4.4A-positive AAH lesions and ACs with a solid pattern, which would make C4.4A a very early biomarker for a particularly malignant form of AC. It is, however, at present unclear if AAH actually develops into overt solid AC^[38,46,53]. It is furthermore of interest to investigate the putative inverse correlation between LKB1 inactivation and high C4.4A expression and the hypothetical candidacy of C4.4A as a predictive biomarker for therapy aimed at components of the LKB1 pathway. This is in line with the goal of supplementing traditional NSCLC management according to the TNM classification with personalized medicine^[18], which depends on predictive biomarkers reporting on biological and molecular tumor characteristics that can identify the patient populations benefitting from a given targeted treatment.

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WJCO 5th Anniversary Special Issues (1): Lung cancer

Advances in adjuvant systemic therapy for non-small-cell lung cancer

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Abstract

Non-small-cell lung cancer remains a leading cause of death around the world. For most cases, the only chance of cure comes from resection for localised disease, however relapse rates remain high following surgery. Data has emerged over recent years regarding the utility of adjuvant chemotherapy for improving disease-free and overall survival of patients following curative resection. This paper reviews the clinical trials that have been conducted in this area along with the studies integrating radiation therapy in the adjuvant setting. The role of prognostic gene signatures are reviewed as well as ongoing clinical trials including those incorporating biological or targeted therapies.

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Key words: Carcinoma; Non-small-cell lung; Chemotherapy; Adjuvant; Radiotherapy; Biological therapy; Biomarker

Core tip: Improvement in survivals of patients with

resected non-small lung cancer has been rather modest. Adjuvant systemic chemotherapy with high dose cisplatin and vinorelbine has been established to be beneficial in improving disease free and overall survival in stage II and IIIA but not stage I patients. This benefit is observed also in elderly patients. Post-operative radiation therapy has a deleterious effect on early stage tumour but appears to improve survival and reduce local recurrence in higher risk stage III or N2 disease with modern techniques. The availability of targeted biologicals and biomarker development may allow future selection of high risk groups who benefit from adjuvant treatments.

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INTRODUCTION

Lung cancer remains the leading cause of cancer-related death, contributing 19.4% to the total number of cancer deaths worldwide in 2012^[1]. Between 1975 and 2008 the 5-year survival for this condition in the United States has only seen a modest rise from 12% to 17%^[2]. Non-small cell lung cancer (NSCLC) comprises approximately 80% of all lung cancers; despite surgery 5-year mortality ranges from 33% to 77% for stage I a and IIIa disease respectively, primarily from tumour recurrence^[3]. Over the last few decades adjuvant chemotherapy following curative surgery has been proven to decrease recurrence and improve patient survival, initially for node-positive breast cancer^[4] but subsequently to include other malignancies such as ovarian^[5], bowel^[6], gastro-oesophageal^[7] and pancreatic^[8] cancers. In addition, inhibitors of tumour growth signalling

pathways such as the anti-human epidermal growth factor receptor 2 (HER2) antibody trastuzumab for breast cancer^[9], are starting to be incorporated into adjuvant systemic therapies.

Patient selection for adjuvant therapy is mainly based on the odds of tumour recurrence. Generally, patients with high recurrence risk will derive the most benefit from adjuvant therapy. Conversely, patients who have a low risk of relapse may not derive a net benefit from adjuvant therapy, particularly if the marginal reduction in relapse risk is not offset against the risks and inconveniences of treatment. Adjuvant radiation can also confer a benefit although this advantage is generally restricted to preventing locoregional tumour recurrence. This article reviews the evidence for adjuvant therapy for NSCLC with a focus on systemic treatments.

CHEMOTHERAPY

Alkylating agents

Initial studies of adjuvant chemotherapy for NSCLC were not promising. In 1995 a meta-analysis of adjuvant chemotherapy by the NSCLC Collaborative Group showed that alkylating agents used in the earliest adjuvant studies had a detrimental effect on survival over surgery alone, with a HR of 1.15 ($P = 0.005$)^[10].

Early cisplatin trials

Despite the detrimental effect found with alkylating agents, the same meta-analysis suggested there may be a small survival benefit within the subgroup of trials that used cisplatin-based chemotherapy with a HR of 0.87 ($P = 0.08$); similarly there was a non-significant improvement in survival by 3% at 2 years, and 5% at 5 years, with treatment. The individual studies pooled to obtain this result were small, with sample sizes ranging from 28 to 332 patients.

Subsequent to this meta-analysis, the Eastern Cooperative Oncology Group (ECOG) 3590 study compared post-operative radiotherapy alone, to the same plus chemotherapy consisting of 4 cycles of cisplatin and etoposide on 488 patients^[11]. After a median follow-up of 44 mo, there was no improvement in median survival between the arms, being 38 mo for chemotherapy and 39 mo for observation. Only 69% of patients assigned chemotherapy received all 4 cycles of treatment, and this included patients who required treatment dose reductions.

The Big Lung Trial (BLT)^[12] was conducted to see if it could confirm the findings from the meta-analysis of a small survival advantage from adjuvant cisplatin-based chemotherapy. The design was pragmatic and clinicians could choose from 4 different cisplatin-based chemotherapy regimens; in addition chemotherapy could be administered either before or after surgery. This study closed early owing to slow accrual, reaching only 76% of the target of 500 patients. However even if full accrual were achieved, the sample size would still only have had 20% power to detect a 5% difference in survival.

The Adjuvant Lung Project Italy (ALPI) was the first large cisplatin-based adjuvant trial that had adequate statistical power to confirm the small benefits suggested by the 1995 meta-analysis^[13]. It recruited patients with resected stage I–III NSCLC between 1994 and 1999. Patients were randomised to receive 3 cycles of mitomycin, vindesine and cisplatin, or observation. Radiation was permitted in accordance with the policy of each treating institution.

After a median follow up of 64.5 mo, no advantage was seen in the chemotherapy arm for either overall survival or disease-free survival. Possible explanations for the negative result include the removal of 1 centre which recruited 108 of the total of 1209 patients owing to concerns over data integrity and low treatment compliance, with only 69% completing the 3 cycles of chemotherapy. Furthermore, about half of these patients required dose reductions. However, analyses adjusting for these effects still did not suggest that they were responsible for the negative result. Another possible explanation is the imbalance in radiation delivery favouring the control arm, as 26% of patients in the chemo arm had their radiation interrupted at an early stage compared to 11% of controls.

Recent cisplatin trials

The largest adjuvant lung cancer study to date is the International Adjuvant Lung Trial (IALT)^[14]. The study included 1867 patients with completely resected stage I–III NSCLC who were randomly assigned to 3–4 cycles of cisplatin-based chemotherapy or observation. This was a pragmatic study, with participating centres being allowed to choose from 4 chemotherapy regimens (details in Table 1). Post-operative radiotherapy was permitted in accordance to local institutional policy. At a median follow up of 56 mo, there was a statistically significant overall survival benefit from chemotherapy [hazard ratio (HR), 0.86; $P < 0.03$]. The 2-year survival rate was 70.3% in the chemotherapy group and 66.7% in the control group and at 5-years, this was 44.5% and 40.4%, respectively. Similarly, disease-free survival was significantly better in chemotherapy arm with a HR of 0.83 ($P < 0.03$).

An updated analysis of this study was published with a median follow up period of 7.5 years^[15]. The benefit seen earlier was maintained for overall survival (HR, 0.91; $P = 0.10$) and disease-free survival (HR, 0.88; $P = 0.02$). However there was an increase in late mortality seen after 5 years of follow-up (HR, 1.45; $P = 0.04$) with a positive test for interaction ($P = 0.006$). An increase in non-lung-cancer mortality was observed in the chemotherapy arm (HR, 1.34; $P = 0.06$), and probably explains the late mortality, as there was no evidence of an interaction between chemotherapy effect and the follow-up period. The statistically significant causes of non-cancer deaths were infections, circulatory and respiratory diseases.

The JBR.10 trial^[16] was a large North American

Table 1 Important randomised trials of adjuvant treatments for non-small cell lung cancer

Ref.	Size	Stage	Chemotherapy regimen	OS	DFS
IALT ^[14]	1867	I -III	Pragmatic study of 3-4 cycles of cisplatin (80-100 mg/m ² on D1) with either: vindesine 3 mg/m ² weekly; vinblastine 4 mg/m ² weekly; vinorelbine 30 mg/m ² weekly; etoposide 100 mg/m ² D1-3	4.1% absolute benefit at 5 yr (HR, 0.86; <i>P</i> < 0.03)	5.1% absolute benefit at 5 yr (HR, 0.83; <i>P</i> < 0.003)
NCIC JBR.10 ^[16]	482	I b-II	4 cycles of: cisplatin 50 mg/m ² D1 + 8; vinorelbine 25 mg/m ² D1, 8, 15, 22 in a 4-wk cycle	15% absolute benefit at 5 yr (<i>P</i> = 0.04)	12% absolute benefit at 5 yr (<i>P</i> = 0.08)
ANITA ^[18]	840	Stage I b-IIIa	4 cycles of: cisplatin 100 mg/m ² D1; vinorelbine 30 mg/m ² D1, 8, 15, 22 in a 4-wk cycle	8.6% absolute benefit at 5 yr (<i>P</i> = 0.17 for HR)	8.7% absolute benefit at 5 yr (<i>P</i> = 0.002 for HR)
JLCRG ^[24]	999	I	Daily UFT as tegafur 250 mg/m ² per day plus uracil, for 2 yr	3% absolute benefit at 5 yr (<i>P</i> = 0.04)	NR
NCIC BR19 ^[30]	1242	I b-IIIa	Gefitinib 250 mg daily for 2 yr	HR, 1.24 (<i>P</i> = 0.14)	HR, 1.22 (<i>P</i> = 0.15)
CALGB 9633 ^[16]	344	I b	4 cycles of carboplatin AUC 6 plus paclitaxel 200 mg/m ² , every 3 wk	HR, 0.83 (<i>P</i> = 0.12)	HR, 0.80 (<i>P</i> = 0.065)

¹Control arms treated with observation. OS: Overall survival; DFS: Disease-free survival; HR: Hazard ratio; NR: Not reported.

Intergroup adjuvant study of stage I b and II NSCLC. Chemotherapy consisted of 4 cycles of cisplatin plus vinorelbine, and radiation was not permitted. The results of this study strongly favoured chemotherapy, with a 5-year survival rate of 69% compared to 54% in the control arm (*P* = 0.03). The HR for death was 0.69 (*P* = 0.04). Similarly, 5-year disease-free survival rates were 61% and 49% for the study and control arms, respectively (*P* = 0.08).

The results of this study were updated after a median follow up of 9.3 years^[17]. Reassuringly, the improvement in overall survival from chemotherapy was maintained with a HR of 0.78 (*P* = 0.04) and 0.73 (*P* = 0.03) for overall survival and disease-free survival, respectively. Five-year survival rates were 67% in the chemotherapy arm and 56 % in the control arm. The benefit was confined to stage II patients, with a HR of 0.68 (95%CI: 0.5-0.92, *P* = 0.01) compared to 1.03 (95%CI: 0.7-1.52, *P* = 0.87) for stage I b patients where there is no benefit, in addition there was a trend for interaction with disease stage (*P* = 0.09).

The Adjuvant Navelbine International Trialist Association (ANITA) conducted a very similar study with equally similar results^[18], thus validating the strongly positive findings from JBR.10. In contrast to the JBR.10 study, patients with stage III NSCLC were included in the ANITA trial in addition to stages I b and II. Patients were randomised to receive 4 cycles of chemotherapy with cisplatin plus vinorelbine, or observation. After a median follow-up of 76 mo, there was an improvement in overall survival (HR, 0.8; *P* = 0.017) with chemotherapy. Median survival in the chemotherapy group was 65.7 mo compared to 43.7 mo in the control group. There was an absolute benefit from chemotherapy on overall survival of 8.4% at 7 years, however stage I b patients did not benefit from treatment with a HR for survival of 1.1 (95%CI: 0.76-1.57). In parallel with the JBR.10 findings, the ANITA study also found a trend towards an interaction between tumour stage and chemotherapy (*P* = 0.07).

Individual patient data from the 5 largest randomised trials of cisplatin-based chemotherapy were pooled in another meta-analysis in an attempt to verify the small but not statistically significant improvement in survival seen earlier by the Collaborative Group. The Lung Adjuvant Cisplatin Evaluation (LACE)^[19] reassuringly confirms a significant survival benefit (HR, 0.89; *P* = 0.005) and disease-free survival benefit (HR, 0.84; *P* = 0.001) from adjuvant chemotherapy. The absolute benefit at 5 years was 5.4% and 5.8% for overall and disease-free survival, respectively. There was no heterogeneity of chemotherapy effect between the trials. In parallel with the updated IALT analysis, there was a similar increase in non-lung-cancer deaths in the chemotherapy arm, particularly in the first 6 mo of follow-up, with a HR of 2.41 (*P* < 0.001). Of these 74 deaths seen in the chemotherapy arm, 18 were from chemotherapy toxicity. Another 40 deaths in this arm were from pulmonary/ cardiovascular events, compared to 21 in the control arm. The authors hypothesized that some of these excess cardiovascular deaths seen in the chemotherapy arm could be a result of cisplatin, particularly as the under-reporting of cardiovascular complications from cisplatin has been described in the literature^[20].

The NSCLC Meta-analyses Collaborative Group updated their previous results^[10] by performing 2 further meta-analyses^[21]. The first examined the benefit of adding chemotherapy to surgery. Similarly, the second analysis explored the benefit of chemotherapy, but in the setting of surgery plus radiotherapy as the control. Both these meta-analyses showed a benefit from adjuvant chemotherapy, with good concordance in survival HRs (for surgery, HR, 0.86; *P* < 0.0001; for surgery plus radiotherapy, HR, 0.85; *P* = 0.009) and also the absolute improvement in 5-year survival (4% for both).

Uracil-tegafur

The antimetabolite uracil-tegafur (UFT) has been

studied in Japan for the treatment of NSCLC. It is an orally administered combination of uracil and tegafur (a pro-drug of fluorouracil) and demonstrated single-agent activity against NSCLC as well as in combination with cisplatin^[22]. A small adjuvant study performed by the West Japan Study Group for Lung Cancer reported improved survival for the 2 arms that received adjuvant UFT-based chemotherapy *vs* observation^[23] and paved the way to a large confirmatory trial by the Japan Lung Cancer Research Group^[24]. Recruitment was restricted to patients with stage I adenocarcinoma and chemotherapy consisted of 2 years of UFT. With a median follow up of 72 mo the 5-year survival for the chemotherapy arm was 88% in the chemotherapy arm *vs* 85% in the observational group ($P = 0.047$). This advantage was driven by an 11% absolute improvement in 5-year survival for patients with T2 tumours ($P = 0.005$), whereas the difference in 5-year survival for patients with T1 tumours was only 1%.

Not all trials of adjuvant UFT have been positive, hence the second Collaborative Group meta-analysis^[20] pooled the results of 7 adjuvant studies that used tegafur/UFT as a single agent and found a survival benefit from treatment with a HR of 0.76 ($P = 0.001$). It also found a benefit for tegafur/UFT in combination with platinum, where the results of 8 pooled studies showed a HR of 0.79 ($P = 0.005$). Another meta-analysis restricted to studies using UFT alone showed a similar survival HR of 0.74 ($P = 0.001$)^[25]. The absolute improvements in 5- and 7-year survival were 4.3% and 7.0%, respectively, however it should be noted that there is considerable overlap in the studies pooled between these meta-analyses.

UFT has only been widely studied for NSCLC in Japan, where ethnic differences in pharmacogenomics and tumour biology may mean that these findings may not be generalisable to NSCLC patients worldwide^[26]. This suspicion is also heightened by the knowledge that its analog, fluorouracil, is generally considered to be ineffective against NSCLC. Lastly, adjuvant UFT has only been studied in a population that is made up almost exclusively of patients with stage I NSCLC, where the benefits from adjuvant cisplatin-based chemotherapy are still controversial. For these reasons UFT should not be considered a standard treatment for this setting, outside Japan.

TARGETED THERAPY

The epidermal growth factor receptor (EGFR) forms part of a signalling pathway that regulates a large range of cellular functions, including proliferation, invasion, angiogenesis and metastasis. It is commonly overexpressed in NSCLC. Trials of EGFR inhibitors showed activity in advanced NSCLC^[27-29] and in 2002 the NCIC instigated the BR19 study to compare the EGFR inhibitor gefitinib with placebo in the adjuvant setting^[30]. However, this study was closed prematurely in 2005, after the large ISEL study failed to show an improvement in overall survival from gefitinib for advanced NSCLC^[31].

By then it had only accrued 503 patients out of a target of 1242. With a median follow-up period of 4.7 years, this study did not find a benefit in disease-free survival (HR, 1.22; $P = 0.15$) or overall survival (HR, 1.24; $P = 0.14$).

In light of current knowledge that activating mutations in the EGFR gene strongly correlate with responsiveness to gefitinib^[32,33], it is possible that the negative result of this study is related to the unusually low incidence of EGFR mutations found in this cohort, being detected in only 4% of the 359 tumour samples tested. The results of ongoing adjuvant studies of EGFR inhibitors in selected patients with EGFR-mutant tumours are awaited with interest, and are discussed towards the end of this article under "Ongoing studies". Targeted therapies will no doubt shape the future of oncology and are discussed further in that section. Table 1 summarises the important individual studies covered in this section and provides further details on their respective treatment regimens.

CHOICE OF REGIMEN

The 2 trials showing the largest magnitude of benefit from adjuvant chemotherapy to date are JBR.10^[16] and ANITA^[18], with treatment providing similar improvements in median survival of 21 and 22 mo, respectively. The use of nearly identical doses of chemotherapy (high-dose cisplatin plus weekly vinorelbine for 4 cycles) in these trials provides further validation of the effectiveness of this combination. Both studies used 100 mg/m² cisplatin per cycle; in ANITA this was given as a single dose on day 1 whereas it was divided into 2 doses of 50 mg/m² given on days 1 and 8 in JBR.10. Both studies used vinorelbine 30 mg/m² for 16 weekly doses at the onset, however the dose was subsequently reduced to 25 mg/m² in JBR.10 owing to a high incidence of treatment-related adverse events following the commencement of this study. While the efficacy of chemotherapy in both studies were comparable, the early treatment-related mortality in the ANITA trial was relatively high at 2% justifying the decision by the JBR.10 investigators to reduce the dose is justified. The incidence of clinically significant toxicities from cisplatin and vinorelbine in both these studies can be found in Table 2.

The Lung Adjuvant Cisplatin Evaluation (LACE) meta-analysis^[19] confirmed the superiority of cisplatin plus vinorelbine over 2 other chemotherapy regimen subgroups ($P = 0.04$), but only when these subgroups were pooled together. There was also a trend in favour of a total cisplatin dose above 300 mg/m².

PATIENT SELECTION

The modest survival benefit obtained from ACT means that most patients will not personally benefit from the treatment. For instance, the 5% survival benefit from chemotherapy seen in the LACE meta-analysis would

Table 2 Incidence of treatment-related World Health Organization grade 3-4 toxicity from adjuvant cisplatin and vinorelbine

Trial	JBR.10 (%) ^[16]	ANITA (%) ^[18]
Anaemia	14	7
Thrombocytopenia	3	1
Neutropenia	85	73
Febrile neutropenia	9	7
Infection	11	1
Nausea/vomiting	27	10-17 ¹
Diarrhoea	2	<1
Constipation	5	3
Anorexia	15	10
Asthenia/fatigue	28	15
Peripheral neuropathy	3	3-7 ²
Creatinine elevation	(NR) ³	<1
Treatment-related mortality ⁴	0.8	2

¹Separately documented as nausea (10%) and vomiting (7%) in publication; ²Separately documented as hearing loss (2%), sensory neuropathy (2%) and motor neuropathy (3%) in publication; ³NR: Not reported; ⁴No WHO grade.

mean that 20 patients would need to be treated with chemotherapy to prevent 1 lung cancer death, whilst the other 19 patients would not derive any individual benefit from the treatment. Furthermore patients and clinicians also have to weigh up the risks and inconveniences of ACT. Cisplatin is considered to be strongly emetogenic, even though the current availability of 5HT₃ and substance P neurokinin-1 receptor antagonists have substantially reduced the magnitude of this side-effect. It also has the potential to cause permanent neuropathy that can be disabling in a small percentage of patients. Cardiovascular complications have been identified from cisplatin^[19] and as mentioned above an increase in cardiovascular-related mortality has been identified in the chemotherapy cohort of the LACE meta-analysis^[19]. For these reasons, it would be desirable to be able to accurately identify subgroups of NSCLC patients who would derive the greatest benefit from ACT.

Stage 1 disease

Hitherto all major adjuvant trials in NSCLC have used tumour stage as the criterion for selecting high-risk patients. Whilst adjuvant chemotherapy has become standard treatment for stages II and III NSCLC, it remains controversial for stage I tumours. The cohorts in the 2 meta-analyses of adjuvant UFT trials^[20,24] are almost exclusively made up of stage I lung adenocarcinomas within the Japanese population. Given their positive findings, they vindicate treatment with UFT in this population, however there are no studies confirming this approach to be effective outside Japan.

Stage 1A: Only a small number of stage I a patients have been entered into trials of adjuvant chemotherapy. In the LACE meta-analysis^[19] stage I a patients comprised 7% (347/4584) of the cohort; the HR for overall survival in this subgroup was 1.4 (95%CI: 0.95-2.06). The second

Collaborative Group meta-analysis^[20] collectively analysed 2058 cases with stage I a disease within their total cohort of 8447 patients; the HR for survival in this subgroup was 1.19 (95%CI: 0.84-1.68).

Stage 1B: Subset analyses of the IALT^[14], JBR.10^[16] and ANITA^[18] trials failed to show a benefit from chemotherapy for Stage 1b NSCLC. As discussed above, this is further reinforced by finding a trend towards an interaction between chemotherapy effect and disease stage in the updated JBR.10^[17] and ANITA trials. The LACE meta-analysis^[19] failed to show a benefit for stage I b tumours (HR, 0.93; 95%CI: 0.78-1.10), although the second Collaborative Group meta-analysis^[20] found survival HRs to be similar across stages I b-III NSCLC.

The Cancer and Leukemia Group B (CALGB) 9633 trial^[34] is the only adjuvant study of a pure population of stage I b NSCLC. It is also unique for its use of carboplatin instead of cisplatin. Three hundred and eighty-four patients were randomised to receive carboplatin plus paclitaxel, or observation. The preliminary results^[35] after a median follow-up of 34 mo were promising and showed an improvement in overall survival (HR, 0.62; $P = 0.028$) with chemotherapy. However the mature analysis after a median follow-up of 74 mo no longer showed a statistically significant survival benefit (HR, 0.83; 90%CI: 0.64-1.08³ $P = 0.12$) or disease-free survival benefit (HR, 0.80; 90%CI: 0.62-1.02, $P = 0.065$). Exploratory subgroup analysis of the CALGB 9633 results demonstrated a survival advantage from adjuvant chemotherapy for patients with tumours ≥ 4 cm in diameter (HR, 0.69; $P = 0.043$). Similar conclusions were found in the JBR.10 study where stage I b tumours ≥ 4 cm derived benefit from chemotherapy. In 2011 the IASLC re-classified stage I b tumours which are ≥ 5 cm to be stage II^[36]. Given that at least half of the stage I b patients in both the JBR.10 and CALGB 9633 cohorts had tumours ≥ 4 cm, a significant proportion of these cases will now thus be considered to have stage II disease, casting further doubt on the benefit of adjuvant chemotherapy for currently diagnosed stage I b tumours.

Age

As the world population continues to age, the elderly will comprise an increasing proportion of patients with NSCLC. Despite this they are poorly represented in clinical trials; in the LACE meta-analysis^[19] only 9% of patients were 70 years and older, yet this is close to the median age of diagnosis for NSCLC. This meta-analysis did not show evidence of an interaction between age and chemotherapy benefit.

A retrospective study evaluated the effect of age on adjuvant chemotherapy delivery, toxicity and survival in the JBR.10 study cohort^[37]. Patients were dichotomised using a cut-off age of 65 to define the elderly. The survival HR for adjuvant chemotherapy in the older age group was 0.61 ($P = 0.04$) which was similar to the treatment effect in the overall study population (HR, 0.69). There was no evidence of an interaction by age group.

This benefit was observed despite the authors finding that the older patients received less and lower doses of chemotherapy. It was also reassuring that there was no evidence that the older cohort had increased toxicities from undergoing chemotherapy.

A population-based study in Ontario compared survival for resected NSCLC cases diagnosed between 2001 and 2006; in 2004 adjuvant chemotherapy was adopted across Canada's universal health insurance program^[38]. Cases were identified using the Ontario Cancer Registry. This study found that only 16.2% of patients 70 years and older received adjuvant chemotherapy, compared to 42.7% of patients younger than 70. Despite this a small increase in 4-year survival from 47.1% to 49.9% could still be observed over this time period for patients 70 years and over (HR, 0.87; $P = 0.0123$). The adoption of chemotherapy was considered likely to have contributed to this improvement. Hence current evidence favours treating fit elderly patients with adjuvant chemotherapy.

Risk assessment models

An interactive online tool that uses clinicopathological variables to calculate an individual patient's relapse risk for NSCLC is available at www.adjuvantonline.com. It also calculates the estimated benefit of adjuvant chemotherapy and communicates the benefit of adjuvant chemotherapy in a format that is easy for patients and clinicians to comprehend. However, it should be noted that the ADJUVANT! model has only undergone external validation for its ability to predict recurrence for breast cancer in North American cohorts^[39].

Histology

A differential response to chemotherapy based on histologic subtype has been observed in advanced NSCLC. In this setting a large randomised study^[40] compared cisplatin and gemcitabine with cisplatin and pemetrexed and did not find a difference in overall survival between the treatment arms. However a pre-specified analysis showed that patients with adenocarcinoma and large cell carcinoma histology in the pemetrexed arm had improved survival. Pooled together into a "nonsquamous" subgroup by histology, their survival HR was 0.81 ($P = 0.005$). The treatment-by-histology interaction analysis ($P = 0.0011$) also showed that overall survival for patients with nonsquamous histology was significantly improved in the cisplatin/pemetrexed arm compared with the overall survival for patients treated with cisplatin/gemcitabine, or patients with squamous histology. This differential response to pemetrexed by histology was confirmed by re-analysing an older randomised study comparing pemetrexed and docetaxel in previously treated advanced NSCLC^[41], plus another study comparing maintenance pemetrexed to placebo^[42].

Hitherto no randomised adjuvant study on NSCLC has directly tested the effect of optimising chemotherapy by tumour histology in a similar manner. However, in the ongoing TASTE trial^[43] that restricts entry to non-

squamous tumours, all patients allocated to chemotherapy will receive cisplatin and pemetrexed. This trial is discussed further under "Ongoing studies".

Biomarkers

Tissue biomarkers can provide additional prognostic information to the existing clinico-pathological tumour staging information, and thus help with patient selection for adjuvant therapy. For example it may identify a subgroup of high-risk stage I patients who could benefit from adjuvant chemotherapy. In this section it is useful to note the distinction between prognostic and predictive biomarkers: a prognostic biomarker only provides information about cancer outcomes, regardless of therapy; on the other hand a predictive biomarker only gives information about the effect of a therapeutic intervention, independent of relapse risk^[44].

Excision repair cross-complementation group 1: The International Adjuvant Lung Cancer Trial Biology (IALT Bio) study^[45] examined whether immunohistochemistry (IHC) of lung cancer tissue of patients in the IALT trial (discussed above) could be used to determine which patients would obtain a benefit from cisplatin-based chemotherapy. The excision repair cross-complementation group 1 ERCC1 enzyme is critical for repairing cisplatin-mediated DNA damage, and overexpression has been linked to platinum resistance in tumour cell lines that include NSCLC. Using IHC, ERCC1 expression was evaluated retrospectively in a cohort of 783 patients of the 1045 enrolled in the original clinical trial. It found that patients whose tumours were ERCC1 negative had better overall survival with chemotherapy compared to the control group, with five-year survival being 47% *vs* 39% respectively (HR, 0.65; $P = 0.002$). Disease-free survival was also better with chemotherapy in this group compared to controls (HR, 0.65; $P = 0.001$). With ERCC1 positive tumours however, no significant difference was seen in overall survival between the chemotherapy and observation groups.

A systematic review and meta-analysis was conducted in 2011 to look at the prognostic and predictive utility of ERCC1 in lung cancer^[46]. This found considerable methodological weaknesses in published studies with variations in ERCC1 cutoff, lack of proven correlation between quantitative reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) assays with IHC, non-standardised protocols and inadequate study sample sizes. It did not find ERCC1 expression to be prognostic in metastatic NSCLC, however there was tentative evidence that high levels of ERCC1 expression predicted resistance to platinum-based chemotherapy. Consequently such patients had shorter survival and a reduced likelihood of tumour response compared to those with low levels of ERCC1 expression. The authors concluded that ERCC1 should only be considered a predictive NSCLC biomarker but not prognostic. They also recommended that ERCC1 should not be used

routinely in clinical decision making until adequately powered prospective trials are conducted.

The ERCC1 Targeted trial was a prospective adjuvant study that used ERCC1 to optimise chemotherapy. It was prematurely terminated as the antibody used did not appear prognostic/predictive based on interim results^[47].

Gene signatures: Gene expression profiling aims to identify unique “signatures” within the tumour’s genome that can help predict relapse risk, response to treatment, or both.

A 15-gene signature was identified in the cohort entered into the JBR.10 trial (discussed above) that was both prognostic and predictive^[48]. Microarray profiling was performed on 133 frozen tumour specimens collected from 482 patients enrolled in this study, to separate patients into a high and low risk group. Subsequently RT-qPCR was used in the same study to verify the microarray signature in this cohort, plus 30 additional cases from the study that were not profiled by microarray.

The prognostic value of this signature was tested using the cohort of patients randomised to observation, by dichotomising patients into high-risk and low-risk subgroups. Overall survival was significantly different between these subgroups, with a HR of 15.02 ($P < 0.001$) for patients whose tumours presented the signature. This finding was validated using 4 separate microarray datasets, and also subsequently using PCR on additional patients from the JBR.10 observation cohort.

This signature also had predictive value; high-risk patients derived a survival benefit from adjuvant chemotherapy (HR, 0.33; 95%CI: 0.17-0.63, $P = 0.005$) that was not seen with low-risk patients (HR, 3.67; 95%CI: 1.22-11.06, $P = 0.0133$). Additionally, although subgroup analysis in the original (clinical) trial found no benefit from chemotherapy in stage I B patients, this gene signature identified patients from this subgroup that had a survival benefit with adjuvant cisplatin and vinorelbine (HR, 0.44; 95%CI: 0.18-1.09, $P = 0.07$). Recently the signature has been validated in another independent cohort of 181 stage I and II patients^[49]. Furthermore, this study found that it was prognostic for survival in both adenocarcinoma and squamous cell carcinoma subtypes, as well as a subgroup of 48 stage I a patients where the survival HR was 5.61 (95%CI: 1.19-26.45, $P = 0.014$).

Another signature incorporating 14 genes has been developed by a United States-Chinese group using formalin-fixed paraffin-embedded (FFPE) specimens in a cohort of 361 patients from the University of California San Francisco with resected non-squamous NSCLC^[50]. This signature separated patients into low, intermediate and high-risk groups in terms of 5-year mortality. It was validated using 2 separate cohorts. The first was from Kaiser Permanente Northern California ($N = 433$), where multivariate analysis confirmed a survival HR of 2.04 ($P = 0.0016$) and 1.66 ($P = 0.0436$) for the high- and intermediate-risk groups, respectively. Using a China Clinical Trials Consortium dataset ($N = 1006$),

corresponding survival HRs were 2.37 ($P < 0.0001$) and 1.87 ($P = 0.0354$). The strengths of this method is that it uses FFPE tissue and qPCR which are easily available in the clinical setting, while the large validation cohorts from diverse geographical and ethnic backgrounds which enables better generalisability of the results. A prospective study is planned to compare adjuvant chemotherapy *vs* observation in stage I patients identified as high risk, using this signature.

A 61-gene signature has been identified that is predictive of a benefit from the MAGE-A3 vaccine that is currently being studied in metastatic melanoma and early NSCLC^[51]. This signature, determined by RT-qPCR, was developed using tumour samples from the melanoma cohort, where it was found to correlate with improved survival. The genes in this signature correlate with immune pathways involved in tissue-specific destruction such as interferon stimulated genes, CCR5 ligand, specific chemokine genes and immune effector function genes. The MAGE-A3 vaccine is concurrently being studied in the adjuvant setting for NSCLC and this is discussed further under “Ongoing studies”. Hence this molecular study also tested the gene signature for its utility as a predictive marker for NSCLC, using tumour samples from the adjuvant studies. Its presence correlated with an improved disease-free interval with vaccine treatment with a HR of 0.42 (95%CI: 0.17-1.03, $P = 0.06$).

At the time of writing, no biomarker has been prospectively validated for the adjuvant management of NSCLC^[52]. We predict that it is highly likely that EGFR (discussed in this article under “Targeted therapy” and “Ongoing studies”), currently used routinely in clinical practice as a predictive biomarker for advanced NSCLC, will also become a standard predictive biomarker for adjuvant therapy in the near future.

Individual preferences

The Preferences for Adjuvant ChemoTherapy (PACT) study surveyed 122 patients^[53] following resection for early NSCLC, and also their 82 respective cancer clinicians^[54] (medical oncologists and thoracic surgeons) to gauge the minimum survival benefit deemed necessary for them to be willing to undergo adjuvant chemotherapy. It found that the median values for the benefit to justify having chemotherapy were an increase in survival of 9 mo, alternatively a 5% increase in 3- or 5-year survival. These median values were similar between patients and clinicians, however the ranges varied widely between respondents, and even more so within the patient cohort. Compared to similar surveys for breast and colon cancer by the same authors, comparatively larger benefits were deemed necessary to justify having chemotherapy in this NSCLC cohort.

INTEGRATING ADJUVANT RADIATION

Post-operative radiotherapy (PORT) for NSCLC has been explored in multiple clinical trials which have yielded

conflicting results. A meta-analysis that included 2128 patients enrolled in nine randomised trials evaluating PORT *vs* surgery alone in resected NSCLC was published in 1998 and showed a significant detrimental effect of PORT on survival (HR, 1.21) with a 7% absolute reduction in overall survival at two years from 55% to 48% ($P = 0.001$)^[55]. On sub-group analysis, this adverse effect was greatest for patients with stage I - II disease, whereas for those with stage III disease there was no clear evidence of an adverse effect. This overview was updated in 2005 and still showed PORT to be detrimental with an 18% relative increase in the risk of death^[56].

The results of this meta-analysis are subject to a number of important limitations. The study included patients enrolled from 1966-1997, many of whom were treated with older radiotherapy techniques (including Cobalt machines) no longer consistent with current standards. The staging evaluation was variable and the analysis included a large number of early stage patients (approximately 25% had N0 tumours) not expected to benefit from PORT. Up to 30% of patients came from a single study^[57] where large dose per fraction (2.5Gy) and high total doses (up to 60Gy) were allowed which may have contributed to excess toxicity and decreased survival. A retrospective analysis of 7465 patients with stage II or III NSCLC from the Surveillance, Epidemiology and End Results (SEER) database between 1988-2002 confirms the findings of the meta-analysis^[58]. It found that PORT significantly decreased the 5-year survival rate in patients with N0 or N1 disease. However, patients with N2 disease had significantly higher 5-year survival with PORT (27% *vs* 20%, $P = 0.0077$).

Subsequent to the meta-analysis, adjuvant chemotherapy has become standard treatment for high-risk NSCLC. Additionally, there have been significant technical improvements in radiotherapy planning and delivery in the modern era including CT-based conformal planning which have made it possible to deliver radiation more safely and with greater precision. Four D-CT planning and respiratory gating now make it possible to control for respiratory motion, potentially reducing pulmonary toxicity. It is difficult to know if these advances in radiation techniques will lead to better outcomes as there are no recent randomised studies published on this, however a recent meta-analysis of "modern" PORT for stage III NSCLC reported an estimated absolute improvement in overall survival by 13% at 5 years and reduction in local recurrence as first relapse to 10%^[59].

Hence the salient question to current practice is whether there is a role for adjuvant radiation using contemporaneous techniques, in conjunction with adjuvant chemotherapy. To answer this question, the investigators of the positive ANITA trial of adjuvant vinorelbine plus cisplatin (discussed above) have published a subsequent retrospective analysis on the effect of PORT from this study^[60]. PORT was permitted in this trial in accordance with local institutional policy. An improvement in survival was seen for patients with N2 disease who received radiotherapy

with a median survival 47 mo *vs* 24 mo in patients given adjuvant chemotherapy, and 23 mo *vs* 16 mo respectively for the observation cohort. Conversely, patients with N0 or N1 disease had a significant detriment in survival through the addition of PORT. Whilst these findings concur with the meta-analysis and SEER findings, the unexpectedly large improvements in survival found in this study should be interpreted with some caution, particularly taking into account the retrospective nature of the analysis, and that radiation was allocated in this study by institutional policy and not randomisation.

The Lung Adjuvant Radiotherapy (LungART) trial^[61] is an ongoing international phase III study comparing PORT with observation following surgery and chemotherapy and will directly answer the important question on whether there is a benefit from radiotherapy after resection for stage III NSCLC.

Whilst survival is a key outcome when considering PORT, loco-regional control remains an important endpoint. The PORT meta-analysis provided data regarding local recurrence rates (LRR) for all included trials. There were fewer local recurrences but more deaths for PORT compared with surgery alone (N = 195 for PORT local recurrences *vs* 276 for surgery alone). However when the data were adjusted for the reduced survival, radiation was in fact found to be detrimental for local recurrence, with a HR of 1.16 (95%CI: 1.05-1.29, $P = 0.005$) favouring surgery alone. One study found no difference in LRR for 728 patients with completely resected NSCLC randomised to receive PORT (HR, 0.85; 95%CI: 0.64-1.14) or observation^[57]. Another study reported a significant overall reduction in LRR at the bronchial stump and/or mediastinum in the group randomised to PORT^[62]. A number of other studies have also reported a reduction in LRR favouring PORT^[59,63,64]. PORT should therefore be considered in the setting of close or involved surgical margins where the rate of local recurrence is high^[65].

The optimal sequence for integrating adjuvant chemotherapy and radiotherapy has not been established. A sequential approach delivering chemotherapy followed by radiotherapy has generally been favoured, although this is not supported by any randomised data. Adjuvant concurrent chemoradiation has been investigated in a number of phase two trials in patients with stage II and III disease and although the approach appears safe, a clear survival benefit has not been demonstrated^[11,66,67]. Some clinicians are also concerned this approach might compromise the ability to deliver the recommended doses and cycles of chemotherapy. Adjuvant concurrent chemoradiation after complete surgery is therefore not considered a standard treatment, although it is currently recommended as an option after surgical resection in patients with N2 disease and positive margins in the current National Comprehensive Cancer Network (NCCN) guidelines^[68].

Until further data becomes available, PORT should only be considered for patients with completely resected stage IIIA-N2 disease, or those at high risk of local recurrence due to close/involved surgical margins.

It should not be offered to patients with completely resected N0 or N1 disease outside a clinical trial.

ONGOING STUDIES

The presence of an activating EGFR mutation has been found to be a strong predictor of a response to EGFR inhibitors^[31]. In the setting of advanced NSCLC, several randomised trials have now consistently shown that patients with EGFR-mutant tumours have high response rates to front-line EGFR inhibition compared to chemotherapy^[33,69,70]. Hence, despite the negative result for adjuvant gefitinib in unselected NSCLC patients in the BR19 study^[30] there is enthusiasm to re-evaluate adjuvant EGFR inhibition in a patient population selected for sensitivity to this treatment. In this context the RADIANT trial compares adjuvant erlotinib with placebo in patients with EGFR-expressing tumours, and has now completed accrual^[71]. Two large ongoing trials are comparing adjuvant gefitinib with chemotherapy in China^[72] and Japan.

Bevacizumab, the monoclonal antibody against vascular endothelial growth factor (VEGF), has been shown to have activity in advanced NSCLC when added to chemotherapy^[73,74]. ECOG has recently completed accrual into a randomised trial that examines the benefit of adding bevacizumab to cisplatin-based chemotherapy in the adjuvant setting^[57].

The Tailored Post-Surgical Therapy in Early Stage NSCLC (TASTE) trial^[43] examines whether customising adjuvant treatment using biomarkers will improve outcomes. Patients in the experimental arm are firstly tested for the presence of an activating EGFR mutation; patients with these mutations will receive erlotinib for 12 mo. Patients without the mutation will be further tested for ERCC1 overexpression; if this is detected they will not be given any adjuvant treatment. Remaining patients in this arm will receive chemotherapy with 4 cycles of cisplatin and pemetrexed, similarly to all patients randomised to the control arm.

Another pharmacogenomics-driven study is the International Tailored Chemotherapy Adjuvant (ITACA) trial^[75]. Patients in the experimental arm are treated according to tumour ERCC1 and thymidylate synthase (TS) expression; increased expression of the latter enzyme correlates with resistance to antifolate drugs such as pemetrexed^[76,77]. Chemotherapy in this arm is with cisplatin/pemetrexed (low ERCC1, low TS), cisplatin/gemcitabine (low ERCC1, high TS), pemetrexed (high ERCC1, low TS), or paclitaxel (high ERCC1, high TS). In the control group, patient/oncologist preference is used to determine which one of 3 possible cisplatin combinations will be administered.

The MAGE-A3 protein has been recently identified as a relatively “pure” tumour antigen, being otherwise expressed primarily during embryogenesis. In adult humans, only the testis and placenta express this antigen. A randomised phase II trial of a vaccine to this antigen

recruited NSCLC patients whose tumours expressed the MAGE-A3 gene^[78]. There was a non-statistically significant improvement in DFS; consequently a confirmatory phase III trial^[79] has now completed accrual.

A small Japanese adjuvant study of 103 patients examined the addition of autologous activated killer T cells and dendritic cells to chemotherapy and found a statistically significant improvement in two-year survival (93.4% *vs* 66%) as well as five-year survival (81.4% *vs* 48.3%) with the addition of immunotherapy^[80]. This very encouraging initial result using a novel approach will no doubt spur interest for similar studies in the future.

CONCLUSION

The survival benefit from adjuvant chemotherapy has been seen to slowly but steadily improve over the last few decades. It is now evident that cisplatin-based chemotherapy offers an absolute overall survival benefit in the order of 5% to 10%. Given that the 2 trials that have shown the highest survival benefit used 4 cycles of high-dose cisplatin in conjunction with vinorelbine, this would currently be considered the optimal treatment regimen. Whilst there is clear evidence of benefit from chemotherapy for patients with stages II and III disease; the benefit for stage I tumours remains controversial. The development of a number of gene expression profile signatures to further stratify patients into low or high risk categories following conventional clinicopathological staging, may allow clinicians to determine which patients will gain a likely benefit from adjuvant therapy in the future. However this strategy requires prospective validation in randomised clinical trials. Given that the presence of activating mutations of EGFR have been found to be strongly predictive of a response to EGFR inhibitors in the setting of advanced NSCLC, similar results are eagerly awaited in the adjuvant setting. Progress in improving the survival of patients with NSCLC has been slow, but the recent improved understanding of the different molecular subtypes of this malignancy as well as the availability of new biological agents to target pathogenic pathways will hopefully translate into ongoing meaningful increments in outcome.

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WJCO 5th Anniversary Special Issues (1): Lung cancer**Epidermal growth factor receptor tyrosine kinase inhibitors for non-small cell lung cancer**

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Abstract

First-generation epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs), including gefitinib and erlotinib, have proven to be highly effective agents for advanced non-small cell lung cancer (NSCLC) in patients harboring an activating EGFR mutation such as the exon 19 deletion mutation and L858R. Although those reversible small molecular targeted agents provide a significant response and survival benefit, all responders eventually acquire resistance. Second-generation EGFR-targeting agents, such as irreversible EGFR/HER2 tyrosine kinase inhibitors and pan-HER TKIs, may improve survival further and be useful for patients who acquired resistance to first-generation EGFR-TKIs. This review discusses novel therapeutic strategies for EGFR-mutated advanced NSCLC using first- and second-generation EGFR-TKIs.

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Key words: Epidermal growth factor receptor mutation; Epidermal growth factor receptor tyrosine kinase inhibitors; Non-small cell lung cancer; Secondary resistance

Core tip: Although gefitinib and erlotinib provide a significant response and survival benefit, all responders eventually acquire resistance. Second-generation epidermal growth factor receptor (EGFR)-targeting agents, such as afatinib and dacomitinib, may improve survival further and be useful for patients who acquired resistance to first-generation epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs). This review discusses novel therapeutic strategies for EGFR-mutated advanced non-small cell lung cancer using first- and second-generation EGFR-TKIs.

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INTRODUCTION

Epidermal growth factor receptor (EGFR) is the founding member of the ErbB family of 4 structurally related receptor tyrosine kinases, including EGFR (ErbB1), ErbB2, ErbB3 and ErbB4. The receptors of the ErbB family are activated after binding to peptide growth factors of the EGF family. Upon ligand binding, the ErbB receptors form either homo- or heterodimers and, after dimerization, auto- and transphosphorylation on tyrosine residues of the ErbB receptors occurs^[1]. Non-small cell lung cancer (NSCLC) tumors harboring specific EGFR mutations are dependent on EGFR signaling for uncontrolled proliferation and resistance to apoptosis^[2-4] (Figure 1). The 2 most frequent activating EGFR mutations, responsible for approximately 90% of this anomaly in the cell cycle, are the L858R point mutation and the exon

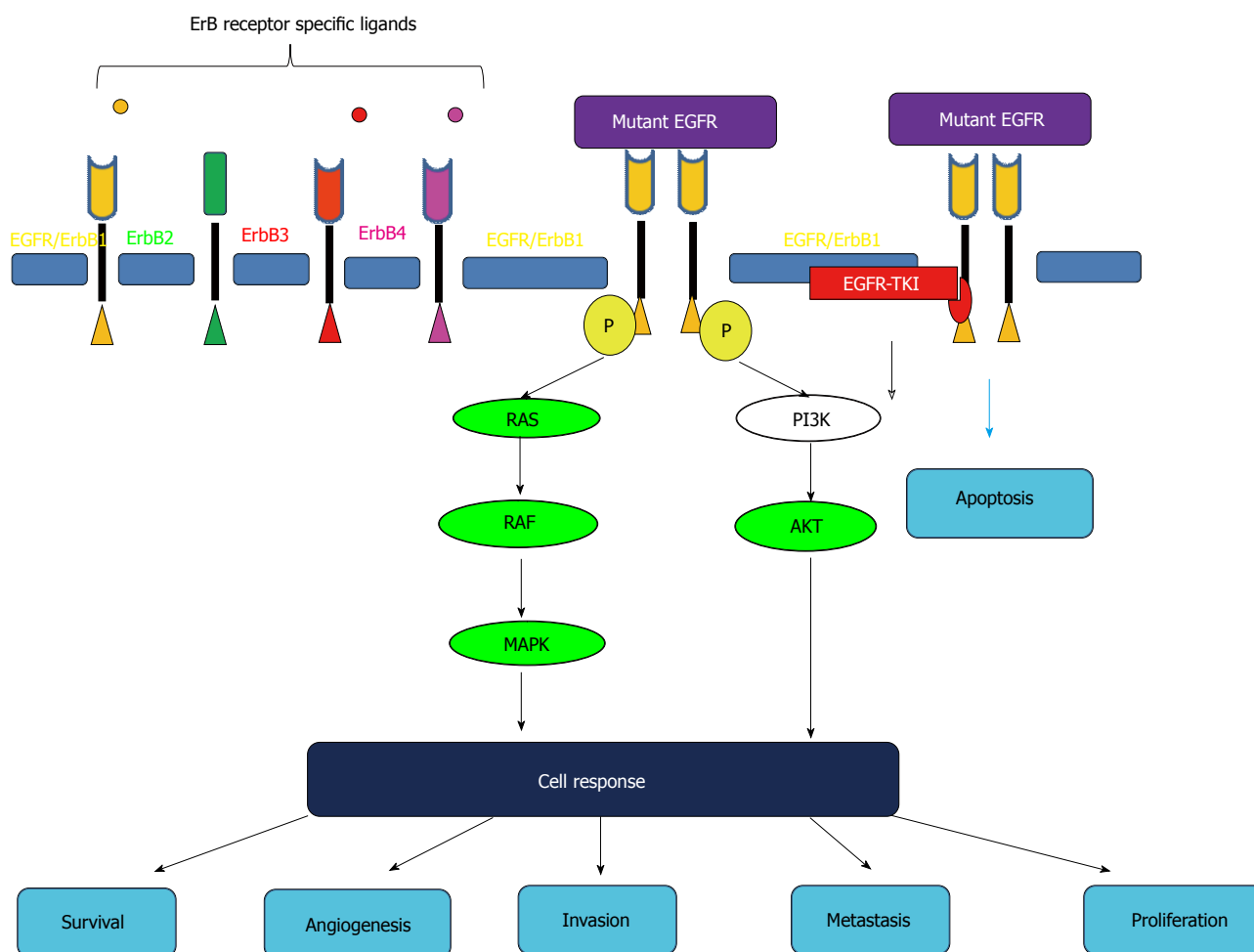


Figure 1 Cell-proliferative signaling pathways in lung cancer cell. The epidermal growth factor receptor (EGFR) family consists of 4 members: EGFR/ErbB1, ErbB2, ErbB3 and ErbB4. Specific ligands (e.g., EGF, TGF- α) binding to EGFR results in a conformational change of the receptor, exposing the dimerization domain and allowing for homodimerization with a second EGFR, or heterodimerization with another member of the EGFR family. Activation of the EGFR results in autophosphorylation of key tyrosine residues. These tyrosine phosphorylated sites leads to the activation of major downstream signaling cascades, including the Ras/Raf/MAPK pathway and PI3K/AKT pathway. These pathways act in a coordinated manner to promote cell survival. While wild type EGFR is activated in a ligand-dependent manner, mutant EGFR is constitutively activated. First- or second-generation EGFR-TKIs bind reversibly or irreversibly to the kinase domain and effectively inhibit EGFR tyrosine kinase by binding to the adenosine triphosphate-binding site of the enzyme and inhibit downstream signaling, leading to apoptosis of cancer cells.

19 deletion mutation^[5]. In the last decade, therapeutic agents targeting the EGFR signaling pathway, including 2 reversible EGFR tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib, have been clinically effective in treating lung cancer patients harboring activating EGFR mutations^[6-12].

Despite the great efficacy of first-generation EGFR-TKIs in patients with EGFR-mutated NSCLC, all responders eventually develop resistance to these agents. The treatment strategies for NSCLC patients who developed resistance to first-generation EGFR-TKIs are actively studied. Recently, second-generation EGFR-TKIs, including afatinib (BIBW 2992) and dacomitinib (PF-00299804), became available; these drugs are intended to further prolong survival in patients harboring activating EGFR mutation and may overcome the resistance to first-generation EGFR-TKIs. This article focuses on the EGFR-TKI-based strategy for patients with advanced NSCLC expressing activated mutant EGFR.

STANDARD PLATINUM-BASED CHEMOTHERAPY VS FIRST-GENERATION EGFR-TKIS AS A FIRST-LINE TREATMENT OF EGFR-MUTATED NSCLC

Efficacy and toxicity

Four previous randomized phase III trials assessing first-line treatment demonstrated a significantly higher response rate (RR) and longer progression-free survival (PFS) in patients treated with first-generation EGFR-TKIs, including gefitinib and erlotinib, than in patients treated with standard platinum-based combination chemotherapy (Table 1). Although these trials met their primary endpoint with statistically significant longer PFS, no significant difference was observed in terms of overall survival (OS). No restrictions were imposed on treatment after the end of protocol therapy in these 4 trials and the majority of patients in the control arm received EGFR-

Table 1 Randomized phase III trials comparing first-generation epidermal growth factor receptor-tyrosine kinase inhibitors to platinum-based combination chemotherapy as a first-line treatment in patients with epidermal growth factor receptor-mutated non-small cell lung cancer

Ref.	Treatment	Number of patients	Age	Response rate	Median PFS (mo)	Median OS (mo)
WJTOG3405 ^[9]	gefitinib	86	< 75	62%	9.2	35.5
	CDDP + TXT	86		32%	6.3	38.8
					HR, 0.48; <i>P</i> < 0.001	HR, 1.64; <i>P</i> = 0.211
NEJ002 ^[10]	gefitinib	114	< 75	74%	10.8	30.5
	CBDCA + PTX	114		31%	5.4	23.6
					HR, 0.30; <i>P</i> < 0.001	HR, 0.89; NS
OPTIMAL ^[11]	erlotinib	82	≥ 18	83%	13.1	22.7
	CBDCA + GEM	72		36%	4.6	28.9
					HR, 0.16; <i>P</i> < 0.001	HR, 1.04 NS
EURTAC ^[12]	erlotinib	86	≥ 18	58%	9.7	19.3
	Platinum + TXT/GEM	87		15%	5.2	19.5
					HR, 0.37; <i>P</i> < 0.001	HR, 1.04; NS

EGFR: Epidermal growth factor receptor; NSCLC: Non-small cell lung cancer; PFS: Progression-free survival; OS: Overall survival; CDDP: Cisplatin; TXT: Docetaxel; PTX: Paclitaxel; GEM: Gemcitabine; CBDCA: Carboplatin; NR: Not reached; HR: Hazard ratio; NS: Not significant.

TKI therapy at least once.

In these 4 randomized phase III trials, severe adverse events or treatment-related toxicity leading to discontinuation of the therapy were significantly less prevalent in patients treated with first-generation EGFR-TKIs compared to standard chemotherapy. The most common adverse events in patients treated with first-generation EGFR-TKIs were cutaneous toxicity, including skin rash and dry skin, diarrhea and elevated transaminase level. Compared to chemotherapy, hematological toxicity, fatigue, alopecia and nausea were less prevalent in the experimental arm of first-generation EGFR-TKIs^[9-12].

Quality of life

Three randomized phase III trials comparing first-generation EGFR-TKIs to standard chemotherapy have shown EGFR-TKI to be superior to chemotherapy in quality of life (QoL) effects. Two randomized phase III trials of first-generation EGFR-TKIs, including the IPASS study^[7] and OPTIMAL study^[13], assessed QoL as a secondary endpoint using Functional Assessment of Cancer Therapy-Lung (FACT-L), Trial Outcome Index (TOI), or Lung Cancer-Specific Subscale (LCS; Table 2). Patients receiving first-line EGFR-TKIs experienced clinically relevant improvements in QoL compared to patients treated with standard platinum doublet chemotherapy in these studies. Among patients harboring activating EGFR mutations in the IPASS study, significant improvement of QoL was found in patients treated with gefitinib compared to patients treated with chemotherapy. Furthermore, rapid improvement of QoL both in terms of FACT-L and LCS was observed in patients with mutated EGFR. In the OPTIMAL study, patients with an improvement in QoL showed improved PFS compared with patients with stable or worsened QoL. Further significant correlations were observed between improved QoL and tumor re-

sponse with FACT-L, TOI and LCS.

In the NEJ 002 study, QoL was assessed by analyzing time to deterioration from baseline in the physical, mental and life well-being QoL scales. Time to defined deterioration in physical and life well-being significantly favored gefitinib over standard chemotherapy [hazard ratio (HR) of time to deterioration, 0.34; 95% confidence interval (CI), 0.23-0.50; $P < 0.0001$ and HR, 0.43; 95%CI: 0.28-0.65, $P < 0.0001$ respectively]^[14].

FIRST-GENERATION EGFR-TKIS FOR ELDERLY PATIENTS AND/OR PATIENTS WITH POOR PS (3-4)

In the WJOG 3405 study and the NEJ 002 study, patients of older age (≥ 75) and poor performance status (PS 2-4) were excluded. An earlier phase II trial demonstrated efficacy of gefitinib as a first-line treatment in elderly patients with activated mutant EGFR and/or patients with poor PS (3-4; Table 3)^[15-17]. Although each trial had a small sample size and was a single-arm phase II trial, high RRs (59%-74%) and long PFSs were observed. Inoue *et al.*^[15] reported utility of first-line gefitinib for extremely poor PS patients and approximately 80% of the patients enrolled this trial improved PS after initiation of gefitinib. Among them, some patients with PS = 4 experienced a dramatic improvement in systemic advanced disease shortly after initiation of gefitinib. No prospective clinical trials of gefitinib except for this study in advanced NSCLC patients with poor PS (3-4) have been conducted and there have been no randomized trials comparing EGFR-TKIs to chemotherapy as a first-line treatment of EGFR-mutated advanced NSCLC.

Although no randomized controlled trials of erlotinib in elderly patients harboring an activating EGFR

Table 2 Quality of life assessment (first-generation epidermal growth factor receptor-tyrosine kinase inhibitors vs chemotherapy)

Study	Treatment	Method ^a					
		FACT-L	P ^b	TOI	LCS	P	
IPASS ^[7]	Gefitinib	70.2%	< 0.0001	70.2%	< 0.0001	75.6%	0.000
	CBDCA + PTX	44.5%		38.3%		53.9%	
OPTIMAL ^[13]	Erlotinib	74.3%	< 0.0001	73%	< 0.0001	77%	< 0.0001
	CBDCA + GEM	31.5%		25.9%		31.5%	

^aEvaluable for quality of life population; logistic regression model with covariates; ^b6-point improvement (FACT-L and TOI); 2-point improvement (LCS), maintained ≥ 21 d. EGFR-TKIs: Epidermal growth factor receptor tyrosine kinase inhibitors; FACT-L: Functional assessment of cancer therapy-lung; TOI: Trial outcome index; LCS: Lung cancer subscale; CBDCA: Carboplatin; PTX: Paclitaxel; GEM: Gemcitabine.

Table 3 Phase II trials of gefitinib in elderly patients with activated mutant epidermal growth factor receptor and in patients with poorer performance status

Ref.	Number of patients	Age	PS	Response rate	Median PFS (mo)	Median OS (mo)
Inoue <i>et al</i> ^[15]	29	50 \leq	1-4 ^a	66%	6.5	17.8
Asami <i>et al</i> ^[16]	17	75 \leq	0-1	59%	12.9	27.4
Maemondo <i>et al</i> ^[17]	31	75 \leq	0-1	74%	12.8	33.8

^aPatients with PS (1-2) were all 80 yr or older. PS: Performance status; PFS: Progression-free survival; OS: Overall survival.

Table 4 Clinical trials of first-generation epidermal growth factor receptor tyrosine kinase inhibitors in patients with epidermal growth factor receptor-mutated non-small cell lung cancer who failed chemotherapy

Ref.	EGFR-TKI	Number of patients	Response rate	Time to progression (mo)
Sutani <i>et al</i> ^[21]	Gefitinib	23	74%	9.4
Han <i>et al</i> ^[22]	Gefitinib	17	64%	21.7
Cortes-Funes <i>et al</i> ^[23]	Gefitinib	10	60%	12.3
Kim <i>et al</i> ^[24]	Erlotinib	8	63%	NR
Ahn <i>et al</i> ^[25]	Erlotinib	78	58%	8.6

EGFR-TKIs: Epidermal growth factor receptor tyrosine kinase inhibitors; NSCLC: Non-small cell lung cancer; NR: Not reached.

mutation have been conducted yet, 18 years old or older patients were enrolled in the OPTIMAL study and the EUROTAC study. No negative effects of erlotinib, such as severe toxicity, lower response and shorter survival, were documented in elderly patients in these studies. Another phase II trial showed that erlotinib is effective and relatively well tolerated in chemotherapy-naïve elderly patients (≥ 70) with advanced NSCLC^[18]. EGFR mutations were detected in 9 of 43 patients tested and all patients harboring an EGFR mutation achieved either a partial response (PR) or stable disease (SD).

Reck *et al*^[19] examined a subpopulation of elderly patients (≥ 70) receiving first-line erlotinib ($n = 485$) in the TRUST study ($n = 6580$), an open-label phase IV trial of erlotinib in advanced non-selected NSCLC patients who had previously failed, or were considered unsuitable to receive, standard chemotherapy or radiotherapy^[20]. In this subpopulation, disease control rate (complete response plus PR plus SD), median PFS and OS were 79% (*vs* 69% for the overall TRUST population; $P < 0.0001$), 4.57 mo (*vs* 3.25 mo), and 7.29 mo (*vs* 7.9 mo) respectively. Nev-

ertheless, elderly patients with poor PS (2-3) had worse survival outcomes than those with good PS (0-1). Median PFS of PS 0-1, 2 and 3 were 5.58 mo, 3.15 mo and 1.81 mo respectively. Median OS of PS 0-1 and 3 was 10.38 mo and 2.07 mo respectively. Eighteen percent of elderly patients had an erlotinib-related adverse event (AE) and 20 patients (4%) developed severe toxicity [grade ≥ 3 ; *vs* 173 patients (3%) in the overall TRUST population]. Twenty-seven percent of elderly patients needed a dose reduction of erlotinib (*vs* 17% in the overall TRUST population). No molecular information, including EGFR mutation status, was examined in this study. Considering the results of these studies, investigators concluded that first-line erlotinib may be well tolerated and be considered for elderly patients with advanced NSCLC the same as non-elderly patients.

WHICH LINE OF TREATMENT IS BETTER FOR FIRST-GENERATION EGFR-TKIS IN PATIENTS WITH MUTANT EGFR?

Several investigators have assessed first-generation EGFR-TKIs as a second/third-line treatment in patients with NSCLC carrying activated mutant EGFR based on their small prospective or retrospective studies and subset analysis of phase III trials (Table 4)^[21-25]. As for response rate and time to progression, these results were similar to the results of a previous large phase III trial of first-generation EGFR-TKIs as a first-line treatment. Rosell *et al*^[8] reported that no significant difference was observed between chemotherapy-naïve and chemorefractory patients in terms of RR (73.5% *vs* 67.4%), PFS (14 mo *vs* 13 mo) and OS (28 mo *vs* 27 mo) with erlotinib in patients with activated mutant EGFR.

In contrast, lower RR with gefitinib was documented

in the NEJ 002 study in patients who failed first-line chemotherapy compared to patients treated with gefitinib as a first-line treatment (56% *vs* 74%). Several studies documented that heterogeneity in EGFR gene expression and mutations was observed in patients with NSCLC^[26-28]. Bai *et al*^[29] reported that chemotherapy may reduce EGFR mutation frequency in patients with NSCLC. In their study, samples were derived from 3 cohorts and 409 patients were reviewed. The decrease in EGFR mutation rate was statistically significant and patients whose EGFR mutations switched from positive to negative after chemotherapy had a better RR than patients with a reverse change among the patients who received first-line chemotherapy with matched pre- and post-chemotherapy blood samples. A similar decrease in EGFR mutation rate was observed in tissues after neoadjuvant chemotherapy in the second cohort (34.9% *vs* 19.0%, $P = 0.013$). In the third cohort, 38.0% of the tumors showed intratumor heterogeneity of EGFR mutations, whereas 62.0% were homogeneous, either with an EGFR mutation or no mutation. The authors concluded that chemotherapy may reduce EGFR mutation frequency in patients with NSCLC.

Lee *et al*^[30] reviewed 23 randomized controlled trials comparing EGFR-TKIs or EGFR-TKIs plus chemotherapy to chemotherapy or placebo, including 13 studies as a first-line treatment, 7 as a second-line treatment, and 3 as maintenance therapy ($n = 14570$). Data on PFS were available from 21 trials of EGFR-TKIs, including gefitinib (10 trials), erlotinib (10 trials) and afatinib (1 trial), compared to control treatment. EGFR-TKIs prolonged PFS in patients with mutated EGFR and an EGFR mutation was predictive of PFS in all settings. In EGFR mutation-positive patients, EGFR-TKI treatment was associated with a lower risk of disease progression in first-line settings (HR, 0.43; 95%CI: 0.38 to 0.49, $P < 0.001$) and in second-line or later settings (HR, 0.34; 95%CI: 0.20 to 0.60, $P < 0.001$). This study demonstrates that the magnitude of effect on PFS in patients with mutated EGFR is similar to that in patients receiving EGFR-TKIs as either a first- or second-line treatment (HR, 0.43 and 0.34 respectively). EGFR-TKI treatment, however, had no impact on OS in patients with mutated EGFR.

A recent systematic review of chemotherapy trials for NSCLC indicated that PFS advantage is unlikely to be associated with an OS advantage due to the increasing impact of survival post-progression on OS^[31]. Salvage therapy after disease progression may have a great influence on the prolongation of survival. In randomized phase III trials, including the IPASS study, the WJTOG 3405 study and the OPTIMAL study, a considerable percentage of enrolled patients was not treated with EGFR-TKIs as a salvage therapy because of a patient's refusal and deterioration of the general condition: IPASS (36%), WJTOG3405 (41%) and OPTIMAL (30%). Although a considerable number of patients did not receive EGFR-TKI therapy after failure of standard chemotherapy, no statistically significant difference was noted in terms of overall survival in each trial.

GEFITINIB OR ERLOTINIB AS A FIRST-LINE TREATMENT OF NSCLC POSITIVE FOR AN ACTIVATING EGFR MUTATION

No trials comparing erlotinib directly with gefitinib as a first-line treatment in patients with activated mutant EGFR have been conducted. A retrospective study showed that PFS showed no difference with either agent in patients harboring an EGFR mutation^[32]. Among 224 patients, including 124 treated with gefitinib and 100 treated with erlotinib who were reviewed, 75 patients received EGFR-TKIs as first-line therapy and 146 patients tested positive for an activating EGFR mutation. In patients harboring an EGFR mutation, median RR and PFS with gefitinib and erlotinib was 51%, 10.5 mo ($n = 94$) and 58%, 10.4 mo ($n = 52$) respectively. No statistically significant difference was observed in terms of RR and PFS between patients treated with gefitinib and those treated with erlotinib. HRs for PFSs were 0.32-0.54 in previous randomized phase III trials of gefitinib as a first-line treatment compared to standard chemotherapy, including the IPASS study, First-Signal study^[33], WJTOG 3405 study and the NEJ 002 study.

On the other hand, HRs for PFSs were 0.16-0.37 in a phase III trial of first-line erlotinib, including the OPTIMAL study and EUROTAC study. Schwander *et al*^[34] reported at the International Society For Pharmacoeconomics and Outcomes Research (ISPOR) 2011 Annual International Meeting that erlotinib shows better efficacy as a molecular targeted agent in first-line settings compared to gefitinib in patients with EGFR-mutated advanced NSCLC. Investigators compared PFS HRs of erlotinib *vs.* gefitinib using indirect treatment comparison (ITC) assessment based on the OPTIMAL study and the IPASS study. A significant PFS difference (ITC HR, 0.33; 95%CI: 0.19-0.58, $P = 0.0001$) was observed. Furthermore, this statistically significant PFS difference was also observed when comparing OPTIMAL with WJTOG 3405 (ITC HR, 0.48; 95%CI: 0.24-0.97, $P = 0.0395$) or with NEJ 002 (ITC HR, 0.53; 95%CI: 0.30-0.90, $P = 0.0307$).

Paz-Ares *et al*^[35] identified congress reports and papers reporting PFS for EGFR-mutated NSCLC treated with chemotherapy, erlotinib or gefitinib (phase II / III trials/retrospective analyses) in a literature search and checked for duplication and reported the results at the 2012 Annual Meeting of the European Society of Medical Oncology (ESMO). Data were included from 20 chemotherapy studies ($n = 984$), 27 erlotinib studies ($n = 735$) and 56 gefitinib studies ($n = 1843$). Longer PFS was seen with both EGFR-TKIs compared with chemotherapy across treatment lines. Pooled median PFS of all lines of therapy for erlotinib and gefitinib was 12.4 mo (95%CI: 11.6-13.4 mo; $n = 735$) and 9.3 mo (95%CI: 8.9-9.8 mo; $n = 1843$) respectively. Furthermore, in the studies where 90% or more of patients received EGFR-TKIs in first-line settings (predominantly first-line), pooled median PFS for erlotinib and gefitinib was 12.0 mo (95%CI:

Table 5 First-generation epidermal growth factor receptor tyrosine kinase inhibitor plus chemotherapy for unselected patients with non-small cell lung cancer

Ref.	Treatment	Number of patients	Response rate	Median PFS (mo)	Median OS (mo)
INTACT-1 ^[37]	CDDP + GEM + placebo	363	47%	6	10.9
	CDDP + GEM + gefitinib ^a	365	51%	5.8	9.9
	CDDP + GEM + gefitinib ^b	365	50%	5.5	9.9
INTACT-2 ^[38]	CDDP + PTX + placebo	345	29%	5.0	9.9
	CDDP + PTX + gefitinib ^a	345	30%	5.3	9.8
	CDDP + PTX + gefitinib ^b	347	30%	4.6	8.7
TRIBUTE ^[39]	CDDP + PTX + placebo	540	19%	4.9	10.5
	CDDP + PTX + erlotinib	539	22%	5.1	10.6
TALENT ^[40]	CDDP + GEM + placebo	586	30%	5.6	10.1
	CDDP + GEM + erlotinib	586	32%	5.4	9.9

^aDose of gefitinib is 250 mg; ^bDose of gefitinib is 500 mg. EGFR-TKIs: Epidermal growth factor receptor tyrosine kinase inhibitors; NSCLC: Non-small cell lung cancer; PFS: Progression-free survival; OS: Overall survival; CDDP: Cisplatin; GEM: Gemcitabine; PTX: Paclitaxel.

10.8-13.3 mo; $n = 354$) and 9.7 mo (95%CI: 9.0-10.5 mo; $n = 716$) respectively. In contrast, pooled PFS of all lines of therapy and predominantly first-line for chemotherapy was 5.6 mo (95%CI: 5.3-6.0 mo; $n = 984$) and 5.8 mo (95%CI: 5.5-6.2 mo; $n = 868$) respectively. The investigators concluded that patients with activated mutant EGFR derived a greater benefit from EGFR-TKIs than from conventional chemotherapy, especially when administered as a first-line treatment.

Retrospective analysis of AEs comparing gefitinib with erlotinib showed that erlotinib appeared to have higher toxicity than gefitinib at each approved dose^[36]. Among 142 patients with NSCLC, including 107 treated with gefitinib and 35 treated with erlotinib who were retrospectively reviewed, 70 patients had an activating EGFR mutation. In the study, a significantly higher rate of AEs, including rash, stomatitis, constipation and anorexia, was observed in the erlotinib group. This group also had a tendency to require a dose reduction due to AEs. Further comparison of the frequency of grade 2 AEs showed that rash was the main reason for a dose reduction in a significantly higher percentage of patients in the erlotinib group.

CHEMOTHERAPY PLUS FIRST-GENERATION EGFR-TKIS IN PATIENTS WITH MUTATED EGFR

An earlier large randomized phase III trial of chemotherapy plus first-generation EGFR-TKI in unselected chemotherapy-naïve patients with advanced NSCLC, including the INTACT-1 study (chemotherapy plus gefitinib)^[37], the INTACT-2 study (chemotherapy plus gefitinib)^[38], the TRIBUTE study (chemotherapy plus erlotinib)^[39] and the TALENT study (chemotherapy plus erlotinib)^[40], failed to show superiority to standard platinum doublet chemotherapy in terms of RR, PFS and OS (Table 5).

In the CALGB 30406 study, a randomized phase II trial comparing erlotinib plus chemotherapy (carboplatin plus paclitaxel) to erlotinib monotherapy in chemotherapy- and EGFR-TKI-naïve patients with advanced

NSCLC, activating EGFR mutations were detected in 40% (66 of 164) of the enrolled patients^[41,42]. The response rate, PFS and OS of erlotinib plus chemotherapy were: 70%, 14.1 mo and 31.3 mo; and 73%, 17.2 mo and 38.1 mo, respectively. Although statistical comparison between erlotinib monotherapy and erlotinib plus chemotherapy was not carried out in patients with mutated EGFR in this study, longer survival, including PFS and OS, was found in patients with mutated EGFR treated with erlotinib plus chemotherapy. The FASTACT-2 study, a randomized double-blind trial comparing chemotherapy to intercalated combination of chemotherapy (gemcitabine plus cisplatin or carboplatin) and erlotinib in untreated patients with advanced NSCLC, met its primary endpoint of PFS (median PFS 7.6 mo *vs* 6.0 mo, HR, 0.57; $P < 0.0001$)^[43]. Among patients with mutated EGFR, median PFS and median OS were significantly longer in patients treated with chemotherapy plus erlotinib (PFS: 16.8 mo *vs* 6.9 mo, HR, 0.25; 95%CI: 0.16-0.39, $P < 0.0001$; OS: 31.4 mo *vs* 20.6 mo, HR, 0.48; 95%CI: 0.27-0.84, $P = 0.0092$). In contrast, no significant difference in PFS and OS between patients treated with chemotherapy plus erlotinib and patients treated with chemotherapy plus placebo was noted in patients with wild-type EGFR. Serious AEs were observed in 34% of patients in the chemotherapy plus placebo group and 31% of patients in the chemotherapy plus erlotinib group. The number of adverse events that led to discontinuation of the therapy was not significantly different between the 2 groups.

No prospective studies of EGFR-TKI plus chemotherapy as a first-line treatment in patients with EGFR-mutated advanced NSCLC have been conducted. Indirect comparison of data available from the INTACT 1 and 2 studies, the TRIBUTE study and the TALENT study indicates that EGFR-TKIs plus chemotherapy were effective in reducing the risk of disease progression in patients harboring an activating EGFR mutation compared to chemotherapy alone (HR, 0.54; 95%CI: 0.30-0.95, $P = 0.049$)^[30]. In contrast, EGFR-TKIs plus chemotherapy were not more effective than EGFR-TKIs in reducing the risk of disease progression (HR, 1.42; 95%CI:

Table 6 Clinical trials of second-generation epidermal growth factor receptor tyrosine kinase inhibitors (afatinib, dacomitinib) against non-small cell lung cancer expressing activated mutant epidermal growth factor receptor

Ref.	Phase	Treatment	Number of patients	Response rate	PFS (mo)	OS (mo)
LUX-Lung 2 ^[46]	II	Afatinib	106 ^a	66%	15.0	32
LUX-Lung 3 ^[47]	III	CDDP + PEM	104	NE	6.9	NE
LUX-Lung 6 ^[48]	III	Afatinib	204	NE	13.6	NE
		CDDP+GEM	122	23%	5.6	NE
		Afatinib	242	67%	11.0	NE
Kris <i>et al</i> ^[51]	III	dacomitinib	46	74%	18.2	NE

^aOf the 129 patients enrolled in the study, 106 patients tested positive for activating epidermal growth factor receptor mutations, including the exon 19 deletion mutation and L858R. PFS: Progression-free survival; OS: Overall survival; CDDP: Cisplatin; PEM: Pemetrexed; GEM: Gemcitabine; NE: Not evaluated.

0.80-2.53, $P = 0.23$) in patients with mutated EGFR.

SECOND-GENERATION EGFR-TKIS

The second-generation EGFR-TKIs, including afatinib^[45] and dacomitinib^[45], are intended to improve efficacy of treatment in patients with activated mutant EGFR and to improve the outcome in patients who acquired resistance to first-generation EGFR-TKIs. Table 6 shows previous studies of second-generation EGFR-TKIs, including afatinib and dacomitinib, for patients with advanced NSCLC carrying activated mutant EGFR.

Afatinib is an irreversible pan-HER-TKI and binds to EGFR receptors carrying the T790M substitution, which is the mutation conferring resistance to first-generation EGFR-TKIs. The LUX-Lung 2 study was a multicenter phase II trial evaluating the efficacy of afatinib 40-50 mg daily as a first- or second-line treatment in patients with EGFR-mutated advanced NSCLC^[46]. Among 129 patients enrolled in the study, 23 patients tested positive for uncommon EGFR mutations and the other cases were positive for activating EGFR mutations, including the exon 19 deletion mutation and L858R. The response rate, median PFS and median OS in patients harboring an activating EGFR mutation were 66%, 15.0 mo and 32.0 mo respectively. The most severe AEs (grade 3-4) were diarrhea and skin-related events and approximately a quarter of patients who developed these AEs received 50 mg of afatinib as an initial dose. Nearly 70% (of the 99 patients who had an initial dose of 50 mg) had to have their dose reduced to 40 mg and more than a half of these patients needed a further dose reduction to 30 mg. In 30 patients with a starting dose of 40 mg, a dose reduction to 30 mg was needed in 11 (37%) patients.

The LUX-Lung 3 study was a randomized phase III trial comparing afatinib to standard platinum doublet chemotherapy as a first-line treatment in patients with advanced EGFR-mutated lung adenocarcinoma^[47]. In total, 345 patients harboring EGFR mutations were randomly assigned to treatment groups (230 to afatinib and 115 to chemotherapy) and an activating EGFR mutation such as the exon 19 deletion mutation and L858R was detected in 308 patients (204 in the afatinib group and 104 in the chemotherapy group). Median PFSs were 11.1 mo for

afatinib and 6.9 mo for chemotherapy (HR, 0.58; 95%CI: 0.43-0.78, $P < 0.001$) in the enrolled patients and 13.6 mo for afatinib and 6.9 mo for chemotherapy (HR, 0.47; 95%CI: 0.34-0.65, $P < 0.001$) in patients harboring an activating EGFR mutation. Compared to chemotherapy, afatinib significantly delayed deterioration of cancer-related symptoms, including cough and dyspnea (cough, HR, 0.60; $P = 0.007$; dyspnea, HR, 0.68; $P = 0.015$). The prevalence of AEs leading to discontinuation of the therapy was similar in both groups. The most frequent AEs were diarrhea (95%), rash or acne (89%), stomatitis or mucositis (72%), paronychia (57%) and dry skin (29%) in patients treated with afatinib. Afatinib controlled cough and dyspnea better than chemotherapy, whereas diarrhea, dysphagia and sore mouth were worse with afatinib. Global health status/QoL was also improved over time with afatinib compared to chemotherapy.

At the 2013 Annual Meeting of the American Society of Clinical Oncology (ASCO), Wu *et al*^[48] reported the results of LUX-Lung 6, a randomized phase III trial comparing afatinib to standard platinum doublet chemotherapy as a first-line treatment in Asian patients with advanced EGFR-mutated lung adenocarcinoma. There were 364 chemotherapy-naïve patients (242 treated with afatinib, 122 treated with cisplatin plus gemcitabine). Afatinib was administered daily at 40 mg. This study met its primary endpoint with significant longer median PFS compared to chemotherapy (13.7 mo *vs* 5.6 mo, HR, 0.26; $P < 0.0001$). The response rate was significantly higher in patients treated with afatinib (66.9% *vs* 23.0%, $P < 0.0001$). Severe AEs (grade 3-5) were noted in 36% of patients treated with afatinib. The most common AEs were rash/acne (14.6%), diarrhea (5.4%) and stomatitis/mucositis (5.4%) in patients treated with afatinib. AEs leading to discontinuation of treatment were reported in 5.9% of patients treated with afatinib and 39.8% of patients treated with chemotherapy. Patient-reported outcomes showed significantly better control of cancer-related dyspnea, cough and pain with afatinib.

Dacomitinib is an irreversible pan-HER inhibitor and binds irreversibly to the adenosine triphosphate domain of 3 kinase-active members of the HER family, including EGFR, HER2 and HER4. In preclinical studies, dacomitinib showed greater antitumor activity in first-

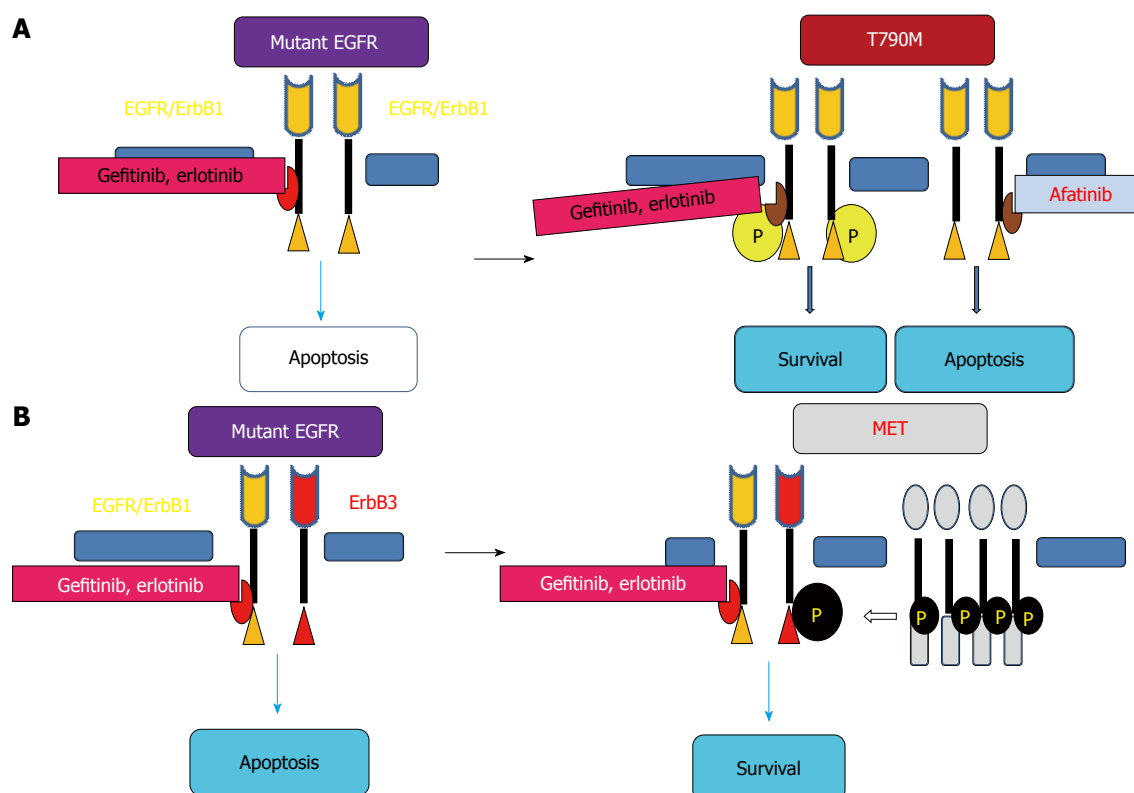


Figure 2 Major mechanisms of epidermal growth factor receptor resistance to epidermal growth factor receptor-tyrosine kinase inhibitors in lung cancer cell. Secondary epidermal growth factor receptor (EGFR) T790M mutation prevents binding of first-generation EGFR-tyrosine kinase inhibitors (TKIs), including gefitinib and erlotinib to EGFR, resulting in cancer cell survival (A). Afatinib inhibits the ATP-binding site of the tyrosine kinase associated with EGFR T790M, leading to apoptosis of cancer cell. MET amplification has been shown to confer resistance to EGFR-TKIs by activating phosphorylation of ErbB3 with activating of the PI3K/AKT pathway, resulting in cancer cell survival (B).

generation EGFR-TKI-resistant cell lines (including gefitinib and erlotinib) and in xenograft NSCLC models^[45,49]. In a randomized open-label trial comparing dacomitinib to erlotinib in previously treated patients with advanced NSCLC, 188 patients were randomly assigned to the 2 treatment groups^[50]. Although median PFS was significantly longer in patients treated with dacomitinib (2.9 mo *vs* 1.9 mo, HR, 0.66; 95%CI: 0.47-0.91, $P = 0.012$), no significant difference was noted in terms of median OS (9.5 mo *vs* 7.4 mo, HR, 0.80; 95%CI: 0.56-1.13, $P = 0.205$). Among all patients enrolled in the study, an activating EGFR mutation was detected in 30 patients (19 in the dacomitinib group, 11 in the erlotinib group). In patients with mutated EGFR, median PFS was 7.4 mo with either dacomitinib or erlotinib (HR, 0.46; 95%CI: 0.18-1.18, $P = 0.098$). AEs leading to treatment withdrawal were uncommon in both treatment arms. Common treatment-related adverse events were dermatological and gastrointestinal, predominantly grade 1 to 2, and more frequent with dacomitinib.

At the 2012 Annual Meeting of ASCO, Kris *et al.*^[51] reported the results of dacomitinib in chemotherapy-naïve patients with EGFR-mutated NSCLC. A total of 92 patients were enrolled in the study and 46 cases were positive for activating EGFR mutations. Among patients with mutated EGFR, RR was 74% (34 of 46 patients) and PFS at 4 mo after initiation of dacomitinib and PFS

were 95.5% (95%CI: 83.2-98.9%) and 18.2 mo (95%CI: 12.8-23.8 mo) respectively. For all 92 patients, common side effects (grade 3-4) were skin related toxicity (17%) and diarrhea (14%). Three patients (6.5%) with activated mutant EGFR discontinued the therapy because of drug-related toxicity.

TREATMENT AFTER A FAILURE OF FIRST-GENERATION EGFR-TKIS AGAINST EGFR-MUTATED NSCLC

Despite a good response and PFS benefits with first-generation EGFR-TKIs, the majority of responders ultimately develop resistance to the therapy after 9-14 mo^[7,9,11-12]. The most frequent secondary resistance to first-generation EGFR-TKIs is the EGFR T790M mutation (50%-60%) and the other mechanisms of resistance are amplification of the MET and HER2 genes, mutations in PIK3CA and BRAF, and conversion to small cell lung cancer^[52-54] (Figure 2). Approximately 30% of patients who acquired EGFR-TKI resistance have an unknown mechanism of resistance.

In the LUX-Lung 1 study, a randomized phase II b/III trial comparing afatinib to placebo in patients who failed first-generation EGFR-TKIs, 585 patients were randomly allocated to treatment groups (390 to afatinib

and 195 to placebo). Median overall survival was 10.8 mo (95%CI: 10.0-12.0 mo) in patients treated with afatinib and 12.0 mo (95%CI: 10.2-14.3 mo) in the placebo group (HR, 1.08; 95%CI: 0.86-1.35, $P = 0.74$). Median PFS was longer in the afatinib group than in the placebo group (3.3 mo *vs* 1.1 mo; $P < 0.0001$). The response rate was 7% (29 of 390 patients) in the afatinib group and 0.5% (1 of 195 patients) in the placebo group^[55].

Cetuximab is a chimeric human-murine monoclonal antibody that binds competitively and with high affinity to the EGFR receptor^[56]. In a study of cetuximab in NSCLC patients previously treated with EGFR-TKIs, the response rate, median PFS and median OS were 0%, 1.8 mo (95%CI: 1.6-5.4 mo), 7.5 mo (95%CI: 2.2-19 mo) respectively. Among 3 patients who harbored an activating EGFR mutation, 1 maintained its stable disease effect for approximately 6 mo^[57].

Janjigian *et al*^[58] reported safety and efficacy results of a cohort study of the combination of afatinib and cetuximab in patients with NSCLC who had acquired resistance to erlotinib or gefitinib. One hundred patients were enrolled in the study and received the therapy. An activating EGFR mutation was detected in 94% (94/100) and the EGFR T790M mutation was detected in 53% (53/100) of the patients. Ninety-six patients were evaluated for efficacy of the therapy. Twenty-nine patients (30%) had PR to the therapy. Seventeen (32%) of 53 patients harboring the secondary-resistance EGFR T790M mutation had PR. Treatment-related toxicity leading to discontinuation of the therapy was observed in 19% of the patients. The most common AEs associated with the therapy were skin rash (97%) and diarrhea (71%).

LUX-Lung 4 was a phase II trial of afatinib in Asian patients who failed gefitinib or erlotinib or both^[59]. Of the 62 patients enrolled in the study, 45 patients had activating EGFR mutations. The response rate, median PFS and median OS were 8.2% (95%CI: 2.7-18.1%), 4.4 mo (95%CI: 2.8-4.6 mo), and 19.0 mo (95%CI: 14.9 mo to not achieved) respectively. Among 2 patients harboring an EGFR mutation who acquired the T790M mutation, 1 patient had stable disease for 9 mo and another for 1 mo. The most common treatment-related AEs were diarrhea (100%) and rash/acne (92%). Twenty-nine percent of the patients enrolled in the study discontinued the therapy due to afatinib-related AEs.

Several investigators have suggested that erlotinib may have a stronger biological activity than gefitinib based on their own findings. Gefitinib (250 mg per day) is typically administered at 1/3 of its maximum tolerated dose, whereas erlotinib (150 mg per day) is administered at its maximum tolerated dose. *In vitro* data showed that the mean concentration of gefitinib in blood plasma is 0.24 µg/mL at the 300 mg daily dose and 1.1 µg/mL at 1000 mg/d. In contrast, median concentration of erlotinib at 150 mg/d was 1.26 µg/mL. Previous findings suggest that erlotinib (150 mg/d) has a higher biological dose of EGFR inhibition than gefitinib (250 mg/d)^[60]. In the results of previous retrospective studies of second-line

erlotinib after a failure of gefitinib in patients harboring activating EGFR mutations, RR and PFS were 3%-10% and 2-3 mo respectively^[61-63]. The investigators suggested that subsequent erlotinib may elicit a response and a survival benefit in patients with mutated EGFR, with good performance status, good response and shorter duration of gefitinib administration (less than 12 mo).

DISCUSSION

Our recommended first- and second-line therapeutic regimens, mainly based on the results of phase III studies, are shown in Figure 3. First- and second-generation EGFR-TKIs, including gefitinib, erlotinib and afatinib, and cytotoxic chemotherapy are optimal first-line therapies in patients harboring activating EGFR mutations. Chemotherapy is recommended as a second-line treatment after failure of first-line EGFR-TKIs, including gefitinib, erlotinib and afatinib, and second-line therapy using these EGFR-TKIs is recommended in patients who failed chemotherapy. Subsequent erlotinib therapy may be a reasonable treatment in specific patients who failed first-line gefitinib therapy.

Although the data from several trials are insufficient to definitively determine the optimal treatment for EGFR-TKIs in patients with EGFR mutations, EGFR-TKIs play a key role in the treatment of patients harboring EGFR mutations and non-administration of these agents could adversely affect survival. Therefore, EGFR-TKIs should be administered early in the course of treatment, as a first- or second-line therapy, so that a chance to administer these agents is not missed due to clinical deterioration or severe toxicity after cytotoxic chemotherapy. Physicians should select either chemotherapy or an EGFR-TKI according to the patient's clinical condition, including PS, age, organ function and complications in non-elderly patients harboring an activating EGFR mutation. For elderly patients (75 years or older) who should not receive chemotherapy and/or patients with poor performance status (PS 3-4), first-line treatment with gefitinib may be considered.

No QoL assessment is currently available comparing second-line EGFR-TKIs after failure of chemotherapy to second-line chemotherapy after failure of EGFR-TKIs in patients harboring an activating EGFR mutation, which is problematic. Furthermore, it is unclear which EGFR-TKI(s) are most desirable as an initial therapy and whether second-generation EGFR-TKIs can overcome acquired secondary resistance to first-generation EGFR-TKIs in NSCLC. Additionally, the appropriate timing for discontinuation of EGFR-TKIs after confirmation of tumor progression is not clear. Some retrospective studies suggest that continuation of EGFR-TKIs beyond disease progression may prolong overall survival of patients with mutated EGFR, with a good therapeutic response^[64,65]. Investigators concluded that EGFR-TKI responders should continue the therapy until the clinical condition and/or imaging findings are reversed to the condition at therapy initiation. Treatment assessment based on

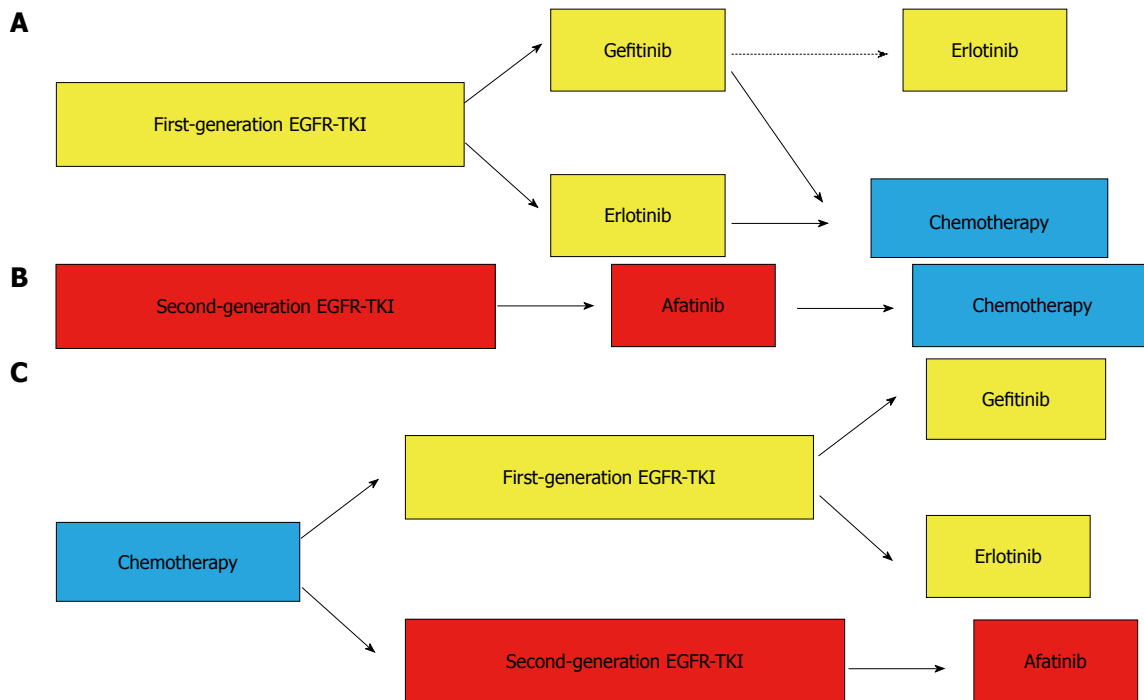


Figure 3 First- and second-line treatment strategies for activating epidermal growth factor receptor-mutated advanced non-small cell lung cancer. First- or second-generation EGFR-tyrosine kinase inhibitors (TKIs), including gefitinib, erlotinib and afatinib, are recommended as initial EGFR-TKI therapy (A and B). First-line gefitinib is recommended in patients with poorer performance status who cannot be treated with systemic chemotherapy. First- (C) or second-line (A and B) cytotoxic chemotherapy is recommended in chemotherapy naïve patients. Subsequent erlotinib may be useful in specific patients who failed gefitinib (A).

Table 7 Ongoing trials for advanced activating epidermal growth factor receptor -mutated non-small cell lung cancer

Line	Trial	Phase	Treatment	Primary endpoint
First	LUX-Lung 7 (NCT01466660)	II b	Afatinib <i>vs</i> Gefitinib	PFS/OS
	ARCHER-1050 (NCT01774721)	III	Dacomitinib <i>vs</i> Gefitinib	PFS
	Tamiya <i>et al</i> ^[65] (UMIN000005503)	II	CBDCA + TS-1 + gefitinib	PFS
	NEJ 009 (UMIN000006340)	III	CBDCA + PEM + gefitinib <i>vs</i> Gefitinib	OS
Second/third	WJOG (UMIN000002014)	III	Gefitinib <i>vs</i> Erlotinib	PFS
	IMPRESS (NCT01544179)	III	Continuation of gefitinib + CDDP + PEM <i>vs</i> CDDP+PEM	PFS
	JMTO12-01 (UMIN000007765)	II	Continuation of gefitinib + DOC/PEM <i>vs</i> DOC/PEM	PFS

EGFR: Epidermal growth factor receptor; NSCLC: Non-small cell lung cancer; PFS: Progression-free survival; OS: Overall survival; CBDCA: Carboplatin; PEM: Pemetrexed; CDDP: Cisplatin; DOC: Docetaxel.

response evaluation criteria in solid tumors (RECIST) may be unsuitable for EGFR-TKIs and a new treatment assessment that may impact survival is needed^[66]. Table 7 shows the ongoing trials for patients harboring activating EGFR mutations. The results of these studies will provide considerable information for EGFR-TKI selection for EGFR-mutated NSCLC. In the future, investigators need to assess the QoL of patients treated with EGFR-TKIs and to compare first- and second-line administration in the same study population.

CONCLUSION

In summary, the data reported suggest that activating EGFR mutations may play a key role in the efficacy of EGFR-TKIs. Administration of first- and second-generation EGFR-TKIs as first- or second-line therapy is an optimal strategy in patients with EGFR-mutated

advanced NSCLC. Second-generation EGFR-TKIs may be superior to first-generation EGFR-TKIs because of their stronger biological activity. Ongoing trials of EGFR-TKIs may identify an EGFR-TKI that is most applicable as an initial EGFR-TKI treatment. Furthermore, the results of these trials may establish new treatment guidelines for activating EGFR-mutated NSCLC and for NSCLC with acquired secondary resistance.

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WJCO 5th Anniversary Special Issues (1): Lung cancer

Chronic obstructive pulmonary disease as a risk factor for lung cancer

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Abstract

The association between chronic obstructive pulmonary disease (COPD) and lung cancer has long been a subject of intense debate. The high prevalence of COPD in elderly smokers inevitably strengthens their coincidence. In addition to this contingent coincidence, recent studies have revealed a close association between the two diseases that is independent of the smoking history; that is, the existence of COPD is an independent risk factor for the development of lung cancer. Molecular-based evidence has been accumulating as a result of the efforts to explain the underlying mechanisms of this association. These mechanisms may include the following: the retention of airborne carcinogens followed by the activation of oncogenes and the suppression of tumor suppressor genes; the complex molecular mechanism associated with chronic inflammation in the distal airways of patients with COPD; the possible in-

volvement of putative distal airway stem cells; and genetic factors that are common to both COPD and lung cancer. The existence of COPD in patients with lung cancer may potentially affect the process of diagnosis, surgical resection, radiotherapy, chemotherapy, and end-of-life care. The comprehensive management of COPD is extremely important for the appropriate treatment of lung cancer. Surgical resections with the aid of early interventions for COPD are often possible, even for patients with mild-to-moderate COPD. New challenges, such as lung cancer CT screening for individuals at high risk, are now in the process of being implemented. Evaluating the risk of lung cancer in patients with COPD may be warranted in community-based lung cancer screening.

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Key words: Chronic obstructive pulmonary disease; Airflow limitation; Inflammation; Lung cancer; Carcinogenesis; Cancer screening; Computed tomography screening; Early intervention

Core tip: This article reviews current perspectives on the epidemiological, clinical, and etiological problems associated with the coexistence of lung cancer and chronic obstructive pulmonary disease.

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INTRODUCTION

Smoking causes many diseases including lung cancer

Table 1 Major causes of death reported in patients with chronic obstructive pulmonary disease

Author	Publication year	Total No. of COPD	Total deaths	Cause of death (%) ¹				
				COPD ²	Lung cancer	Other cancer	Cardiovascular	Others
Vilkman <i>et al</i> ^[14]	1997	2237	1070	30	12	8	33	17
Garecka <i>et al</i> ^[11]	1997	135	70	61	4	4	6	24
Antonelli Incalzi <i>et al</i> ^[8]	1997	270	228	63	5	4	16	12
Zielinski <i>et al</i> ^[15]	1997	215	215	49	7	ND	37	7
Keistinen <i>et al</i> ^[12]	1998	2727	973	22	13	8	32	25
Pauwel <i>et al</i> ^[13]	1999	1277	18	6	33	6	39	17
Garcia-Aymerich <i>et al</i> ^[10]	2003	340	98	74	5	2	12	6
Celli <i>et al</i> ^[9]	2004	625	162	61	12	ND	14	13
Anthonisen <i>et al</i> ^[16]	2005	5887	731	8	33	21	22	16

¹Calculated from the data reported by each literature; ²Including respiratory causes, respiratory failure, respiratory infection. Pulmonary embolism was included in cardiovascular cause. ND: Not described; COPD: Chronic obstructive pulmonary disease.

and chronic obstructive pulmonary disease (COPD). These diseases usually affect relatively elderly individuals. Because of the rapidly aging populations of many developed countries, the number of patients with lung cancer and/or COPD is growing. Lung cancer is one of the leading causes of death in many countries and accounted for approximately 1.37 million deaths worldwide in 2008^[1]. As was extensively discussed in the Global Initiative of Chronic Obstructive Lung Disease (GOLD)^[2], accurate estimations of the prevalence and mortality of COPD are difficult. The prevalence of COPD in a specific region varies because of differences in the survey methods, diagnostic criteria and analytic approaches^[3,4], ranging from 7.8% to 19.7% of the total population^[2]. COPD is often a primary cause of death. However, COPD can also contribute to death from other diseases, such as pneumonia, ischemic heart diseases and heart failure, making it difficult to estimate the COPD-associated mortality rate. Meanwhile, the Global Burden of Disease Study has projected that COPD will become the third leading cause of death worldwide by 2020^[5].

Because of the high prevalence of both conditions and strong common risk factors associated with smoking, the coexistence of lung cancer and COPD is inevitably high. In addition, recent studies have uncovered a non-confounding association between COPD and lung cancer development, together with its molecular mechanisms^[6,7]. Investigating and resolving the problems of the coexistence of these two diseases is important in view of their clinical relevance; their interaction leads to worse outcomes. This article reviews the current perspectives on the epidemiological, clinical, and etiological problems associated with the coexistence of lung cancer and COPD.

COEXISTENCE OF LUNG CANCER AND COPD ASSESSED ACCORDING TO PULMONARY FUNCTION

Because of the high mortality associated with lung cancer, the proportion of deaths from lung cancer among patients with COPD can reasonably be considered to

represent the coexistence of lung cancer among patients with COPD. As shown in Table 1, the causes of death reported in patients with COPD vary significantly among studies, and the proportion of deaths from lung cancer ranges from 4% to 33%^[8-16], presumably because of different patient characteristics and analytical methods. Among their investigation, a prospective study identified 731 deaths that occurred over 14.5 years out of 5887 asymptomatic non-smoking individuals who had mild or moderate airflow limitations, with an average FEV₁/FVC of 65.0% ± 6.1% and an average age of 48.4 ± 6.8 years. The primary cause of death was lung cancer, representing 33% of the total deaths^[16]. Three other prospective studies disclosed a high incidence of lung cancer development or mortality among patients with COPD or individuals with airflow limitations^[17-19]. Skillrud *et al*^[17] prospectively observed 113 individuals who had a %FEV₁ of 70% or less by comparing them with 113 age-matched, sex-matched, occupation-matched, and smoking history-matched individuals with a %FEV₁ of 85% or more. The 10-year cumulative rates of lung cancer development were 8.8% and 2.0% in the former and latter groups, respectively, resulting in a statistically significant difference ($P = 0.024$)^[17]. Kuller *et al*^[18] reported an increase in lung cancer mortality according to a reduction in the FEV₁. The lung cancer mortality per 1000 person-years was 3.02 and 0.43 in the lowest and highest quintiles of the FEV₁, respectively, and this association was not weakened by adjustments for smoking factors^[18]. The relative risk of COPD or airflow limitations associated with lung cancer was therefore calculated to be 4.4 and 7.0 in the studies by Skillrud *et al*^[17] and Kuller *et al*^[18], respectively. In a large-scale prospective study involving 448600 lifelong nonsmokers who were cancer-free at baseline and who had a 20-year follow-up period, Turner *et al*^[19] again found a significant association between lung cancer mortality and pulmonary emphysema, with a hazard ratio of 1.66 (95%CI: 1.06-2.59)^[19]. Wasswa-Kintu *et al*^[20] conducted a population-based meta-analysis to reveal that even a relatively small decrease in the FEV₁ (approximately 90% of predicted) increased the risk of lung cancer by 1.3-fold in men (95%CI: 1.05-1.62) and 2.6-fold in women (95%CI:

Table 2 Odds ratios for coincidental lung cancer based on factors indicative of chronic obstructive pulmonary disease or interstitial lung diseases, according to smoking status¹

	Never smoker (n = 284)	Ex-smoker (n = 101)	Current smoker (n = 179)	Total (n = 564)
FEV ₁ /FVC < 70%	10.42 (2.82-38.55) ³	5.89 (2.02-17.22)	7.456 (3.48-15.95)	7.17 (4.03-12.74)
%VC < 80%	2.02 (0.42-9.86)	No data ⁴	2.25 (0.62-8.11)	4.73 (2.00-11.17)
LAA score ≥ 1 ²	0.98 (0.21-4.43)	3.43 (1.50-7.85)	5.07 (2.51-10.28)	3.63 (2.24-5.89)
Fibrosis score ≥ 1	6.39 (2.13-19.18)	3.94 (1.20-12.88)	5.45 (2.39-12.44)	5.10 (2.82-9.24)
GGA score ≥ 1	2.32 (0.80-6.71)	3.59 (1.09-11.80)	2.48 (1.06-5.77)	2.71 (1.52-4.81)

¹See the reference by Mizuno *et al.*^[24] for details; ²Low attenuation area (LAA) was assessed using Goddard's scoring system; fibrosis and ground glass attenuation (GGA) were assessed using Kazzeroni's scoring system; ³Numbers in parentheses indicate 95%CI; ⁴No data calculated because of insufficient number.

1.30-5.31), concluding that even a modest reduction in the %FEV₁ is a significant predictor of lung cancer, especially among women^[20].

COEXISTENCE OF LUNG CANCER AND COPD ASSESSED BY CT IMAGES

The National Lung Cancer Screening Trial (NLST) for annual low-dose CT screening reported a 20% decrease in lung cancer mortality among heavy smokers between the ages of 55 and 74 years^[21]. This great achievement has resulted in several established guidelines recommending low-dose CT lung cancer screening for individuals with defined conditions, and the time when screening will be implemented as a part of community health care is drawing closer^[22]. The more common use of chest CT examinations in asymptomatic individuals would increase the chance of detecting pulmonary diseases, including lung cancer and pulmonary emphysema. Because a definitive diagnoses of COPD and pulmonary emphysema are based on functional and pathological criteria, respectively, they cannot, by definition, be diagnosed using CT^[2]. Nevertheless, the presence of a substantially distributed low attenuation area (LAA) in the pulmonary fields strongly suggests pulmonary emphysema and COPD. In fact, Goddard *et al.*^[23] proposed a scoring system, which is used worldwide, to evaluate pulmonary emphysema using CT.

Six studies, consisting of 2 cohort and 4 case-control studies, evaluating the relationship between CT-based emphysema and the risk of lung cancer have been published^[24-29], and Zurawska *et al.*^[30] concisely reviewed these studies. The relative risk (RR) for lung cancer, according to CT-based emphysema assessed using semi-quantitative visual methods, ranged from 1.9 to 4.7, with a pooled RR of 2.34 (95%CI: 1.46-3.76). Using multivariate analyses adjusted for covariates including age, sex, and smoking status, the adjusted odds ratio for lung cancer, according to the CT-based detections of emphysema, ranged from 1.73 to 3.14. Mizuno *et al.*^[26] analyzed CT images from 947 healthy individuals who underwent a low-dose CT screening and 256 patients with lung cancer. After adjusting for sex and age, 423 matched healthy individuals and 141 patients with lung cancer were extracted for inclusion in multivariate analyses, with stratification according

to their smoking status. The results disclosed that many functional and CT image factors indicative of COPD (relative risks for lung cancer; 7.17 with FEV₁/FVC < 70% and 3.63 with LAA score ≥ 1) or interstitial lung diseases (relative risks for lung cancer; 4.73 with %VC < 80%, 5.10 with a fibrosis score ≥ 1 and 2.71 with a GGA score ≥ 1) were also risk factors for coincident lung cancer in a manner that was independent of the smoking status (Table 2). These observations suggesting a close association between COPD and lung cancer seem to imply at least three possibilities: first, airflow limitation itself is a risk factor for the development of lung cancer; second, some putative inter-individual variations in the sensitivity to harmful substances, such as active smoking, passive smoking, air pollutants, and cooking oil fumes, may link COPD and lung cancer; and third, some specific genotypes may predispose individuals to both COPD and lung cancer.

POSSIBLE MECHANISMS UNDERLYING THE COEXISTENCE OF LUNG CANCER AND COPD

A growing volume of evidence suggests that some molecular mechanisms may link COPD and lung cancer. A genome-wide association study has uncovered some putative common loci responsible for both COPD and lung cancer^[31]. Taking advantage of such modern technologies, we are soon likely to benefit from abundant information regarding the molecular mechanisms involving the pathogenesis of COPD and lung cancer. The current major possible explanations are outlined below.

Retention of airborne carcinogens because of airflow limitations may enhance carcinogenesis in the airways

In this speculative scenario, the airflow limitations that result from COPD suppress the clearance of carcinogens from the airway, leading to cancer in the airway. This classical scenario is now decorated with modern technical terms including DNA repair errors, oncogene activations, impaired tumor suppressor genes, oncogenic microRNA, the involvement of epithelial-mesenchymal transition, and a wide variety of epigenetic alterations.

Chronic inflammatory processes arising from COPD may enhance carcinogenesis in the airways

An association between persistent chronic inflammation and cancer has long been recognized. Examples include the relationships between cirrhosis and hepatocellular carcinoma, inflammatory bowel disease and colorectal cancer, burns and squamous cell carcinoma of the skin, and dental prostheses and lingual cancer. Recently, the relationship between chronic inflammation and cancer has been explained by a series of processes in which unscheduled necrotic cell death as a result of inflammation causes subsequent epithelial proliferation resulting in the suppression of immunity^[32]. Meanwhile, a volume of evidence has proven that complex inflammatory processes involving many types of immune cells, cytokines, matrix metalloproteinases, reactive oxygen species, and growth factors cause tissue damage and remodeling. This phenomenon ultimately results in the development of COPD^[2]. These factors also contribute to the enhancement of carcinogenesis^[33,34]. Parimon *et al.*^[35] observed a reduced lung cancer risk in patients using inhaled corticosteroids in a dose-dependent manner. This observation strongly supports the idea that the pathophysiology of COPD may enhance the development of lung cancer.

Putative lung stem cells activated by chronic inflammation in patients with COPD may transform to lung cancer stem cells

There is no evidence supporting the existence of lung stem cells in humans or cancer stem cells in human lung cancer^[36]. However, Kim *et al.*^[37,38] has reportedly identified bronchioalveolar stem cells in mice. If distal lung stem cells exist in humans, chronic inflammatory stimuli as a result of COPD might activate and transform these lung stem cells into lung cancer stem cells^[7].

Genetic backgrounds common to both COPD and the development of lung cancer may exist

A deficiency or decrease in alpha-1 antitrypsin is known to increase the risk of lung emphysema development. This condition, acting directly or indirectly through tissue damage to the lung, also increases the risk of lung cancer development^[39-42], although some controversy regarding this point still exists^[43]. Smoking is the strongest definitive risk factor for lung cancer development. Tobacco-derived pro-carcinogens are activated to become carcinogenic by a cluster of phase I enzymes. These activated carcinogens are then degraded and excreted by a cluster of phase II enzymes. Although not fully understood, some polymorphisms of these enzymes are known to lead to increased or decreased enzymatic activities, most likely causing inter-individual variations in the sensitivity to smoking^[44-49]. A better understanding of these mechanisms may identify individuals who are susceptible to lung cancer development when exposed to active or passive smoking. More recently, a decreased serum level of Crab (Clara) cell secretory protein (CC-16) has been found to increase the risk of both COPD^[50] and lung cancer^[51].

PROGNOSIS

As discussed above and illustrated in Table 1, a substantial proportion of patients with COPD die from lung cancer. The coexistence of lung cancer clearly worsens the prognosis of patients with COPD, but a discussion of how COPD worsens the outcome of patients with lung cancer and how this might be overcome is important.

The deterioration of pulmonary function together with the risk of acute exacerbation of COPD would, in theory, make invasive diagnostic procedures, surgery, radiotherapy and chemotherapy difficult in patients with lung cancer. A retrospective study by Sekine *et al.* reported an increased rate of postoperative complications, including pneumonia and the need for a tracheostomy, increased cancer recurrence, and a decreased 5-year survival rate in patients with completely resected stage IA non-small cell lung cancer (NSCLC) and coexisting COPD; the 5-year survival rates of patients with COPD ($n = 80$) and of those without COPD ($n = 362$) were 77.0% and 91.6%, respectively ($P = 0.0001$)^[52]. A recent retrospective study involving 902 patients with stage IA to IIB NSCLC treated with surgical resection also disclosed that 63.4% (572/902) of the patient population had self-reported, physician-diagnosed COPD, and the patients with COPD had a poorer 5-year progression-free survival rate (50.1% *vs* 60.6%, $P = 0.007$) and a poorer 5-year overall survival rate (54.4% *vs* 69.0%, $P = 0.0002$) than the patients without COPD^[53].

TREATMENT

Treatment algorithms can be complex when different diseases coexist in the same patient. It seems reasonable to prioritize the treatment of diseases with a more serious impact on the prognosis: for example, the treatment for stage III small cell lung cancer would be given preference over the treatment for a coincidental low-grade prostatic cancer in the same patient. The situation, however, is totally different in patients with coexisting COPD and lung cancer. It is very important to diagnose, evaluate, and manage both the COPD and lung cancer in a comprehensive manner for the following reasons.

To avoid the deterioration of quality of life because of COPD

COPD symptoms significantly overlap with those of lung cancer. Cough, sputum, shortness of breath, body-weight loss, appetite loss, and even depression may arise from COPD rather than from lung cancer, in some cases. The appropriate treatment for COPD may relieve these symptoms, leading to an improved quality of life (QOL).

To avoid the acute exacerbation of COPD during treatment for lung cancer

The acute exacerbation of COPD can be precipitated by upper respiratory tract viral infections, bacterial infec-

tions of the tracheobronchial trees, and other factors. Once acute exacerbation has occurred, the mortality rate is high. In patients with COPD and lung cancer, stress or immunosuppression as a result of surgery, radiotherapy, chemotherapy, or a terminally ill status can act as predisposing factors for an acute exacerbation. It is particularly important to understand that COPD exacerbations can often be prevented with the appropriate management of the disease^[2].

To decrease the complications associated with lung cancer treatment

Acute lung injury is a serious complication associated with lung cancer treatment, especially when patients suffer from symptomatic or asymptomatic idiopathic pulmonary fibrosis (IPF). Acute lung injury can occur in patients undergoing surgery, thoracic radiotherapy, or cytotoxic or molecular-targeted therapy^[54,55]. Although this complication seems to be more frequent in Japanese patients than in patients from other countries, the precise epidemiology and underlying mechanisms are not well known. In contrast to IPF, the presence of COPD is not recognized as a significant risk factor for acute lung injury associated with lung cancer treatment.

To ameliorate the outcome of the surgical resection of lung cancer

As a result of improvements in anesthesiology, surgical technique, and postoperative managements including rehabilitation, the curative-intent surgical resection of lung cancer in patients with COPD has become safer than previously expected^[56]. When a lung cancer is located within a severely damaged emphysematous region, its resection does not necessarily impair pulmonary functions to the extent that a lung volume reduction surgery for severe emphysema would restore the impaired pulmonary function. In a study with a limited number of patients, Kobayashi *et al.*^[57] demonstrated better postoperative pulmonary functions than expected, with improved pre-operative symptoms and pulmonary function, in patients with treatment-naïve COPD and lung cancer who were treated with inhaled tiotropium, a long-acting anticholinergic bronchodilator, starting 2 wk prior to surgery. As limited resections of lung cancer often result in poor outcomes, a curative-intent standard resection is recommended even for patients with mild to moderate COPD, with the aid of early interventions including drug therapy and rehabilitation for COPD^[56].

FUTURE DIRECTIONS

Low-dose CT screening effectively detects 10-fold more lung cancers at earlier stages compared with screenings using conventional chest X-rays^[58]. As mentioned earlier, the NLST, a randomized trial involving subjects with a high risk of lung cancer, confirmed a 20% reduction in the lung cancer mortality among subjects allocated to a low-dose CT screening group compared with an X-ray

screening group^[21]. This screening strategy targeting individuals at high risk was based on their smoking history and age. Sekine *et al.*^[59] emphasized the importance of the early detection of COPD for the purpose of lung cancer screening and proposed CT screening for individuals with suspected COPD as a strategy to target individuals at high risk. Based on a quick questionnaire that collected information about age, smoking history, and chronic respiratory symptoms, they identified 878 individuals fulfilling the defined criteria out of 89100 participants (1.0%). A total of 567 of the 878 participants (64.6%) underwent further evaluation consisting of CT and spirometry, resulting in the detection of COPD in 161 participants, with 38.5% of them requiring COPD treatment^[60]. Although the data regarding lung cancer detection are not yet available because the trial is still ongoing, this strategy for identifying a high-risk population based on suspected COPD could be an alternative to identification strategies based on age and smoking history. We envision that this new approach could enable us to evaluate and manage COPD and lung cancer comprehensively at early stages and from screening through specialized treatment.

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WJCO 5th Anniversary Special Issues (1): Lung cancer**Acetylcholine receptor pathway in lung cancer: New twists to an old story**

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Core tip: Genome-wide association studies revealed that allelic variation in the $\alpha 5\text{-}\alpha 3\text{-}\beta 4$ nicotine acetylcholine receptor (nAChR) cluster on chromosome 15q24-15q25.1 was associated with a higher risk for development of lung cancer. nAChRs are membrane ligand-gated cation channels whose activation is triggered by the binding of the endogenous neurotransmitter acetylcholine (ACh) or other biologic compounds including nicotine. nAChRs have been found on lung cancer cells, underscoring the idea that the non-neuronal nAChR signaling pathway has considerable implications for lung cancer. Several studies involving the design of nAChR antagonists with improved selectivity might identify novel strategies for the treatment of lung cancer.

Abstract

Genome-wide association studies revealed that allelic variation in the $\alpha 5\text{-}\alpha 3\text{-}\beta 4$ nicotine acetylcholine receptor (nAChR) cluster on chromosome 15q24-15q25.1 was associated with lung cancer risk. nAChRs are membrane ligand-gated cation channels whose activation is triggered by the binding of the endogenous neurotransmitter acetylcholine (ACh) or other biologic compounds including nicotine. nAChRs have been found on lung cancer cells, underscoring the idea that the non-neuronal nAChR pathway has important implications for lung cancer. Several studies focusing on the treatment with nAChR antagonists with improved selectivity might trigger novel strategies for the intervention and prevention of lung cancer. Here we review the genetic risk factors for lung cancer in the nAChR gene cluster, the roles of nicotine receptors, and the molecular mechanisms of acetylcholine receptor pathways to lead to more opportunities for intervention and prevention of lung cancer.

Niu XM, Lu S. Acetylcholine receptor pathway in lung cancer: New twists to an old story. *World J Clin Oncol* 2014; 5(4): 667-676 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i4/667.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i4.667>

INTRODUCTION

Lung cancer is a multifactorial disease, resulting from a combination of genetic, environmental, and psychological factors. The evidence that genetic factors influence lung cancer risk has been demonstrated by many studies, tracing back to the landmark study in 1963^[1]. This study demonstrated a 2.5-fold higher risk in smoking relatives of lung cancer cases compared with smoking relatives of controls^[1], fully suggesting the importance of genetic factors in lung cancer risk.

More recently, some large genome-wide association (GWA) studies identified an association between single-

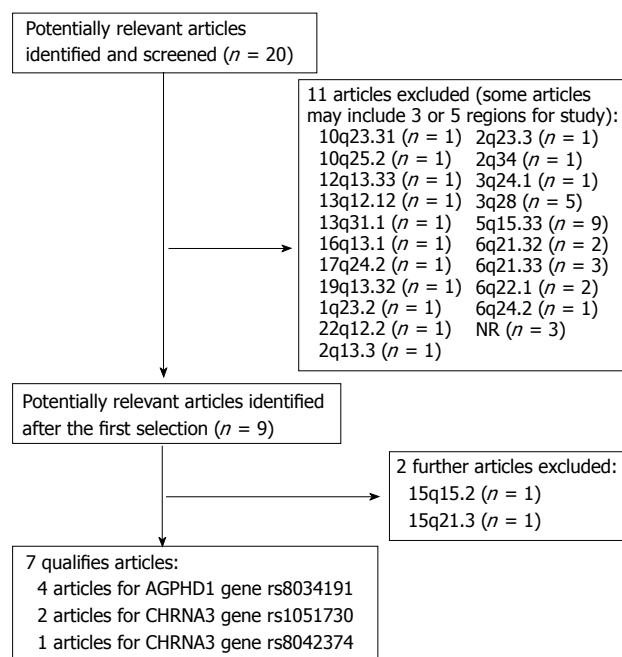


Figure 1 Flow diagram of search strategy and study selection^[67].

nucleotide polymorphism (SNP) variation at 15q24-15q25.1 and susceptibility to lung cancer^[2-11]. Genes mapping to this region of association include the $\alpha 5\text{-}\alpha 3\text{-}\beta 4$ nicotinic acetylcholine receptor (nAChR) cluster. nAChRs, encoded by the nicotinic acetylcholine receptor encoding (CHRN) genes^[2-4,12], are members of the Cys-loop superfamily of ligand-gated ion channels^[13], which are activated by endogenous agonist acetylcholine (ACh) and the exogenous compound nicotine^[14] and nitrosamines, potential lung carcinogens in cigarette smoke and foods.

CHRN genes are expressed in both neuronal and non-neuronal tissues, suggesting that nAChRs may play an important role in processes other than synaptic transmission. Indeed, apart from the classic role at neuromuscular junctions, nAChRs have also been implicated in the regulation of cellular processes such as proliferation^[15], cell-cell interaction, and cell death^[16-18]. A decreased survival correlation with lung cancer may be explained by effects of the nAChRs pathway through CHRNA proteins on tumor cell proliferation^[15], apoptosis, epithelial-mesenchymal transition, and proinvasive and angiogenic phenotypes^[19]. This review will provide an overview for better understanding the genetic risk factors for lung cancer in the nAChR gene cluster, the roles of nicotine receptors, and the molecular mechanisms of the cholinergic pathways to lead to more opportunities for intervention and prevention of lung cancer.

GENETIC RISK FACTORS IN THE NACHR GENE CLUSTER FOR LUNG CANCER

GWA studies for lung cancer

Lung cancer is a heterogeneous and challenging disease to treat. With the arrival of genotyping and genomic profil-

ing, management of non-small-cell lung cancer (NSCLC) has made several advances with the understanding of activating mutations in epidermal growth factor receptor (EGFR), fusion genes involving anaplastic lymphoma kinase (ALK), rearrangements in ROS-1. The next era of personalized treatment will involve a comprehensive genomic characterization of lung cancer. One important task in personalized medicine is to predict disease risk based on a person's genome, *e.g.*, on a large number of SNPs. GWA studies make SNP and phenotype data available to researchers.

Notably, the 15q24-15q25.1 region (CHRNA5/CHRNA3/CHRNA4) has been identified as a lung cancer risk spot by several GWA studies^[2-11]. Of one GWA study in the International Agency for Research on Cancer (IARC), there is 14% increased lung cancer susceptibility associated with nAChR cluster variation irrespective of smoking status^[3]. The same GWA result was identified by the other studies from the MD Anderson Cancer Center, United States^[2], and deCODE Genetics, Iceland^[4], respectively. Importantly, a non-synonymous variant rs16969968 of CHRNA5, inducing an amino acid substitution (D398N) in the second intracellular loop of the protein, can increase lung cancer risk by 30%^[3]. The SNP rs8023462 in CHRNA3/CHRNA4 intergenic region, interfering CHRNA3/CHRNA4 gene expression by interacting with GATA transcription factors, was the first functional evidence for association with lung cancer risk^[20].

To better understand the GWA studies for the nAChR gene cluster and lung cancer risk, we further investigate a catalog of all existing published GWA study data updated to December 20, 2013 from National Human Genome Research Institute (NHGRI) (<http://www.genome.gov/>), using the subject terms (CHRNA OR nAChR OR 15q25) AND (lung cancer OR lung adenocarcinoma OR lung adenocarcinoma (clinical stage) OR lung cancer (DNA repair capacity) OR lung cancer-asbestos exposure interaction). Twenty potentially relevant articles^[2,3,6,8-11,21-33] were produced according to the first primary screening strategy, of which 7 GWA studies met the subject terms for further evaluating the relationship of the nAChR cluster with lung cancer susceptibility^[2,3,6,8-11]. A flow diagram summarized the process of selecting and excluding articles with detailed reasons (Figure 1). All GWA studies for lung cancer at the 15q25.1 region are summarized in Table 1.

Considering that the statistical power of individual GWA study has been limited by the modest effect sizes of genetic variants and the financial constraints on the numbers of variants, it is stringent to perform meta-analyses of existing genetic data on the nAChR gene cluster to better understand disease loci harboring common variants associated with lung cancer risk. A total of 12 qualified articles between 2008 and 2011 screened from 40 potentially relevant articles were selected, including 16 studies with 9 in Caucasians, 4 in East Asians, 2 in African-Americans, and 1 in mixed (Caucasian, African-American and Hispanic) populations^[34]. CHRNA3 gene

Table 1 Qualified genome-wide association studies at the 15q25.1 region on the risk of lung cancer

Date	PubMed ID	Ref.	Initial sample size	Replication sample size	Chromosome position	Mapped gene	SNP	Context	P value	OR (95%CI)	Platform
8/4/2009	19654303	Broderick <i>et al</i> ^[9]	1952 European ancestry cases, 1438 European ancestry controls	5608 European ancestry cases, 6767 European ancestry controls	78806023	AGPHD 1	rs8034191	intron	3.00E-26	1.29 (1.23-1.35)	Illumina
11/2/2008	18978790	McKay <i>et al</i> ^[6]	2971 cases, 3746 controls	2899 cases, 5573 controls	78894339	CHRNA 3	rs1051730	cds-synon	1.00E-15	1.35 (1.25-1.45)	Illumina
11/2/2008	18978787	Wang <i>et al</i> ^[8]	1952 cases, 1438 controls	7579 cases, 8236 controls	78908032	CHRNA 3	rs8042374	intron	8.00E-12	NR	Illumina
9/17/2008	18780872	Liu <i>et al</i> ^[10]	194 cases, 219 controls	3878 cases, 4831 controls	78806023	AGPHD 1	rs8034191	intron	1.00E-8	1.38 (1.17-1.64)	Affymetrix
4/3/2008	18385676	Amos <i>et al</i> ^[2]	1154 cases, 1137 controls	2724 cases, 3694 controls	78806023	AGPHD 1	rs8034191	intron	3.00E-18	1.30 (1.15-1.47)	Illumina
4/3/2008	18385738	Hung <i>et al</i> ^[3]	1926 cases, 2522 controls	2513 cases, 4752 controls	78806023	AGPHD 1	rs8034191	intron	5.00E-20	1.30 (1.23-1.37)	Illumina
10/15/2009	19836008	Landi <i>et al</i> ^[11]	5739 European descent cases, 5848 European descent controls	7561 European descent cases, 13818 European descent controls	78894339	CHRNA 3	rs1051730	cds-synon	2.00E-51	1.31 (1.27-1.36)	Illumina

rs1051730-A allele, compared with G allele, is associated with a 36% higher risk for lung cancer (95%CI, 1.27-1.46; $P < 0.0005$)^[34]. Surprisingly subgroup analyses suggested that rs1051730-A allele might be the factor for lung cancer susceptibility in Caucasians (OR = 1.32; 95%CI, 1.25-1.44; $P < 0.0005$), but not in East-Asians (OR = 1.51; 95%CI, 0.76-3.00; $P = 0.237$)^[34]. Allele frequency of rs1051730 in Asians was lower than that in Caucasians. For rs1051730, the minor allele frequency (MAF) was reported to be 0.39, 0.15, and 0.04 in Whites, African Americans, and Chinese, respectively, based on the HapMap data (<http://hapmap.ncbi.nlm.nih.gov/>), which was reported in our previous study^[35]. The Japanese finding^[36] consistent with our reported Chinese study^[35] also suggests that, like EGFR story, different genetic backgrounds may cause the discrepancy in lung cancer risk in different populations.

Owing to SNPs with weaker effects in influencing lung cancer risk could be missed given the stringent requirements for adjustment for multiple comparisons, nAChR pathway analysis has been proposed as a complementary approach to single SNP analyses in GWA studies. Using two lung cancer GWA studies data sets based on four studies: one a combined data set from Central Europe and Toronto (CETO)^[3]; the other a combined data set from Germany (HGF study)^[11,37] and MD Anderson Cancer Center (GRMD)^[2], comparison of pathway analysis approaches demonstrated that the nAChR pathway was identified as associated with lung cancer in CETO and GRMD^[38].

DNA methylation in the nAChR cluster with lung cancer risk

Although CHRNA5, CHRNA3 and CHRNB4 are clus-

tered together at the same locus, they exhibit differential susceptibility to aberrant methylation. Treatment of cancer cells exhibiting CHRNA3 hypermethylation with DNA methylation inhibitors caused demethylation of the CHRNA3 promoter and gene reactivation, inducing apoptotic cell death^[39]. Small hairpin RNA-mediated depletion of CHRNA3 in CHRNA3-expressing lung cancer cells led to resistance to apoptosis-inducing agents, underscoring the importance of epigenetic silencing of the CHRNA3 gene in human cancer^[39]. Silencing of the CHRNA3-encoding gene may result in over-representation of other nAChR subunits, notably CHRNA7 and CHRNA5^[39], which may stimulate cell survival and provide a proliferation advantage to tumor cells^[40,41]. In contrast to CHRNA3 hypermethylation, elevated CHRNB4 methylation, insufficient to induce significant silencing of the gene, failed to inhibit gene expression. Conclusively, the 15q25.1 locus may be under epigenetic regulation and that its deregulation may lead to lung cancer.

Interplay of smoking behaviors, nAChR cluster and lung cancer risk

Evidence that the nAChR cluster on 15q25 locus is associated with smoking status, nicotine dependence and the risk of lung cancer is inconsistent in different populations. The region of the nAChR cluster has been confirmed to be associated with a number of smoking-related traits, including nicotine dependence, cigarettes smoked per day, and heavy smoking, in some lung cancer GWA studies^[4,42], and in some genome-wide meta-analyses in Caucasian populations^[42-44]. For example, the Caucasian population with variant rs1051730 SNP in the nAChR cluster was related with lung cancer risk and

nicotine dependence, approximately smoking one and two more cigarette per day than those without variant rs1051730 SNP^[4]. Another study demonstrates further that association signals in the nAChR cluster affecting early smoking behaviors may have disparity with those contributing the mature nicotine dependence^[45].

Surprisingly, a Japanese case-control study^[36] reported associations between the selected SNPs in the nAChR cluster and risk of lung cancer and found that associations among never and ever smokers were similar. The association was consistent among non-smokers and smokers in our study^[35]. These studies might argue for a role of the nAChR cluster in lung cancer that is independent of smoking behavior in Asians.

These findings in different populations suggest a role for racial differences in the association between smoking behaviors, nAChR cluster, and risk of lung cancer. Reasons underlying the racial difference in the genotype with smoking associations are unclear. This discrepancy may be due to differences in genetic and environmental backgrounds. Alternatively, other factors that have not been taken into account, such as food intake and passive smoking, differentiate the mode of contribution of the nAChR cluster in non-smokers.

STRUCTURE AND FUNCTION OF NACHRS

The regulation of acetylcholine receptor pathways has been established from the primitive organisms, irrespective of neurons. There exist two types of AchRs, nAChRs and Muscarinic Ach receptors (mAChRs) of cholinergic signaling. nAChRs comprise five subunits, including ten α subunits ($\alpha 1$ - $\alpha 10$), four β subunits ($\beta 1$ - $\beta 4$), one δ , and one ϵ or γ subunit, which form hetero- or homo-pentamers enclosing a central ion channel. The nAChR subunit composition in turn further regulates the function and pharmacology of nAChRs^[16,17].

An autocrine or a paracrine acetylcholine receptor pathway for nAChRs

Acetylcholine receptor signaling in non-neuronal cells is comparable to acetylcholine receptor neurotransmission^[46]. Both nAChR families are expressed in cancer cells^[46], and both NSCLC and small-cell-lung cancer (SCLC) cell lines can synthesize and release Ach^[47]. The widespread synthesis of Ach beyond the nervous system has changed the paradigm of Ach acting merely as a neurotransmitter, and it may also act as an autocrine or a paracrine messenger able to interact with nAChRs. For example, non-neuronal Ach is released from the living NSCLC or SCLC cells and binds to nAChRs of its source and neighbouring cells to mediate autocrine and paracrine regulatory loops, prolonging cell survival with subsequent cell proliferation through mitogen-activated protein kinase (MAPK) pathway^[48-50] or with increase of vascular endothelial growth factor (VEGF) stimulating neoangiogenesis^[19]. All these effects can be blocked at

the level of the nAChRs^[48,51,52]. This novel paradigm necessitates the opportunity of marker-guided lung cancer intervention and prevention strategies, making balance between nAChR-mediated stimulation and inhibition^[16,53].

Metabolism of Ach

Ordinarily Ach is regarded as a classical neurotransmitter. Ach is synthesized intracellularly by the enzyme choline acetyltransferase (ChAT) from choline and acetyl-coenzyme A (AcCoA) before being released into the extracellular space to act on synaptic-adjacent cells. In contrast, the enzyme acetylcholinesterase (AChE) can rapidly clear the extracellular Ach pool into its inactive metabolites choline and acetate. This signaling system is targeted by various biological modulators which can inhibit Ach release or AChE activity^[54]. ChAT is strongly up-regulated, whereas AChE is down-regulated in squamous cell carcinoma (SqCC)^[54], which increases levels of Ach, providing endogenous proliferative stimuli to nAChRs.

Classical ionic channel activity of nAChRs

All nAChRs allow the influx of different cations (Na^+ , K^+ , Ca^{2+}), and the $\alpha 7$ nAChR is selective for Ca^{2+} . Binding of nicotine to $\alpha 7$ nAChR can cause Ca^{2+} influx into lung cancer cells and the subsequent membrane depolarization activates voltage-gated Ca^{2+} channels, which activates the MAPK pathway^[48]. Subsequently, MAPK activates complexes of the transcription factor NF- κ B that induce entry into S phase to promote cancer cell proliferation^[48]. Moreover, tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) has been found to promote Ca^{2+} influx thought binding to nAChRs, activating both protein kinase C (PKC) and the MAPK pathway to stimulate the proliferation of SCLC cells^[49].

Cell proliferation mediated by nAChRs

The role of nAChRs in the growth regulation of cancer was first identified in 1989^[15], and reinforced further by the ability of generalized nAChR antagonists (*e.g.*, hexamethonium and mecamylamine) to reverse the proliferative effects of nicotine. Nowadays, the mechanism of nicotine-induced tumor cell proliferation and associated angiogenesis is an active area of research. In particular, $\alpha 7$ nAChR has been implicated in mediating the proliferative effects of nicotine, and $\alpha 7$ nAChR antagonist a bungarotoxin (α -BTx) or methyllycaconitine can attenuate the proliferative effects of nicotine in NSCLC and SCLC cells^[48,52]. These findings have been confirmed by the transfection of small interfering RNA targeting $\alpha 7$ nAChR in lung cells^[52]. This point implies that $\alpha 7$ nAChR is being considered a target for cancer therapy^[48], marking a new chapter in lung cancer research and the feasibility of using nAChRs as a viable molecular target for cancer therapy.

Cross-talk of nAChRs with the other signaling pathways

Beyond the above channel activity, nAChRs can be involved in other intracellular events involving various

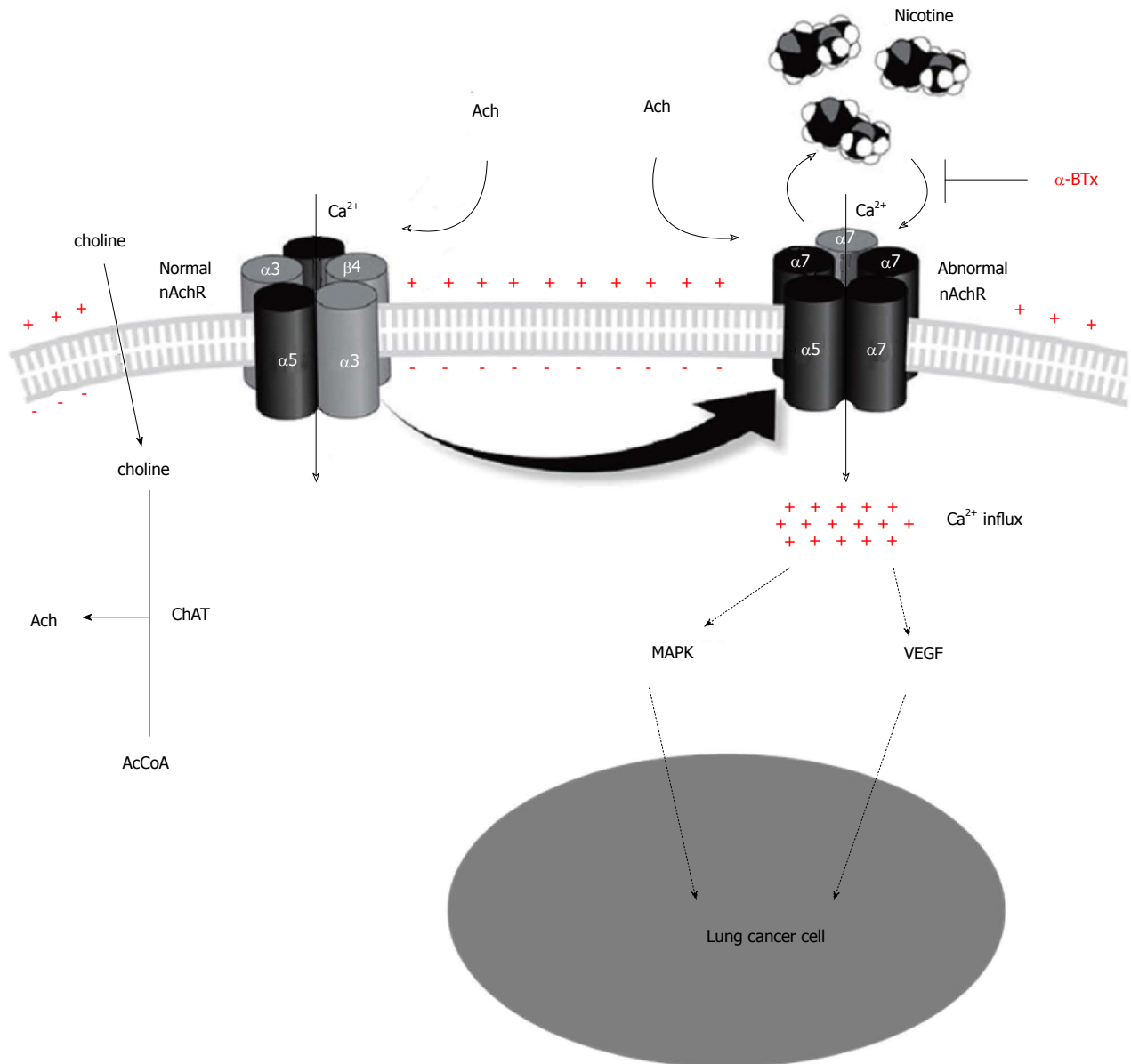


Figure 2 nAChRs pathway in lung cancer. (1) ACh is synthesized intracellularly by the enzyme choline acetyltransferase (ChAT) from choline and acetyl-coenzyme A (AcCoA) before being released into the extracellular space. This signaling system is targeted by various biological modulators which can inhibit ACh release or ChAT activity. ChAT is strongly up-regulated in squamous cell carcinoma (SqCC), which increases levels of ACh, providing endogenous proliferative stimuli to nAChRs; (2) All nAChRs allow the influx of different cations (Na^+ , K^+ , Ca^{2+}), and the $\alpha 7$ nAChR is selective for Ca^{2+} . Binding of nicotine to $\alpha 7$ nAChR can cause Ca^{2+} influx into lung cancer cells and the subsequent membrane depolarization activates voltage-gated Ca^{2+} channels, which activates the MAPK pathway to promote cancer cell proliferation; (3) Hypermethylation of 15q25.1 locus and epigenetic silencing of the CHRNA3 gene may result in under-representation of $\alpha 3$ containing nAChR-subtypes, which in turn leads to over-representation of $\alpha 7$ and $\alpha 5$ containing nAChRs on the cell surface, further increasing Ca^{2+} influx mediated activation of the MAPK signaling cascade. As a result, cells with the complete or partial absence of the CHRNA3 subunit containing nAChRs may display defective cell death response; (4) $\alpha 7$ nAChR antagonist abungarotoxin (α -BTx) can attenuate the proliferative effects of nicotine in NSCLC and SCLC cells; and (5) The other findings suggest a strong connection between growth factor mediated VEGF pathway and the cholinergic angiogenic pathway. The endothelial cell (EC) migration induced by basic fibroblast growth factor (bFGF) and VEGF can be inhibited by blocking the nAChRs with α -BTx.

downstream nAChR-mediated signaling pathways, including Ca^{2+} /calmodulin, $\text{PKC}^{[49]}$, $\text{MAPK}^{[48-50]}$, and $\text{VEGF}^{[19]}$. It has been reported that EGFR was found to be on the list of high $\alpha 6\beta 3$ tumors, and nAChR may trigger the MAPK pathway in which EGFR was involved, leading to promotion of cell growth and proliferation. Thus, cross-talk between signaling downstream of EGFR and nAChR activation *via* the MAPK pathway may together promote

carcinogenesis^[50]. The other findings suggest a strong connection between growth factor mediated angiogenic pathway and the cholinergic angiogenic pathway^[19,55]. For instance, the endothelial cell (EC) migration induced by basic fibroblast growth factor (bFGF) and VEGF can be inhibited by blocking the nAChRs with α -BTx^[19]. Figure 2 simply summarizes the above mentioned nAChRs pathway in lung cancer.

NACHRS RESEARCH IN SOME SPECIAL LUNG CANCERS

Lung cancer in non-smokers

The carcinogenic nitrosamine N-nitrosodiethylamine (DeN) was identified recently to have the similar structure with ACh, acting as a high-affinity ligand for homo- and hetero-meric nAChRs in lung cancer cells^[56]. Considering the fact that DeN is contained in numerous foods and drinks^[57], such agents may lead to non-tobacco-related modulations of nAChRs. In addition, different nAChR subunit gene expression patterns were found between NSCLCs in smokers and non-smokers, and higher nAChR $\alpha 6\beta 3$ expression was associated with NSCLC tumors from non-smokers, as compared with those from smokers^[5].

Lung cancer in female patients

Neurotransmitter γ -aminobutyric acid (GABA) is recognized as the most important inhibitory neurotransmitter in the brain, but it also acts as a tumor suppressor for pulmonary adenocarcinoma (PAC)^[58] and carcinomas of the pancreas^[59], breast^[60] and colon^[61]. As $\alpha 4\beta 2$ nAChR regulates the release of GABA, desensitization of this receptor may therefore lead to GABA deficiency^[62]. Moreover, it has been shown that mRNA levels of the $\alpha 4$ nAChRs were significantly lower in PAC tissues than in normal lung tissue or other types of NSCLCs^[5]. Considering that oestrogen and phyto-oestrogens were reported to desensitize $\alpha 4\beta 2$ nAChR^[63], the predominance of PAC in women may therefore at least in part be the result of impaired $\alpha 4\beta 2$ nAChR function. Recent reports of clinical trials of the nAChR $\alpha 4\beta 2$ antagonist, which targets $\alpha 4\beta 2$ receptors in the brain, have shown its clinical efficacy in smoking cessation^[64]. It is possible that similar nAChR antagonists could block the effect on lung tumors, including lung cancer in women.

Lung cancer in pulmonary neuroendocrine cells

Pulmonary neuroendocrine cells (PNeCs) and SCLC can express high levels of $\alpha 7$ nAChR^[51]. $\alpha 7$ nAChR is the central regulator of proliferation, apoptosis and migration in SCLC cells through stimulating the release of some autocrine growth factors, including serotonin and neuropeptides^[51]. $\alpha 7$ nAChR antagonist α -BTx can attenuate the proliferative effects of nicotine in SCLC cells^[48,52].

SURVIVAL STUDIES FOR NACHRS

nAChRs are associated with resistance to gemcitabine, cisplatin and paclitaxel in NSCLC cell lines. Our research is the first study to investigate whether or not a genetic variant in the 15q25 region has a prognostic effect on the survival outcome of patients with lung cancer^[35]. The patients with CHRNA3 gene rs3743073G > T allele showed a higher risk of lung cancer and worst survival in Chinese Han patients with advanced stage NSCLC^[35]. A Caucasian study showed that CHRNA3 (rs1051730)

genotyping can improve customized chemotherapy based on tumor assessment of excision repair cross-complementing 1 (ERCC1) mRNA in stage IV NSCLC patients with a performance status of 0 (clinicaltrials.gov. identifier: NCT00174629)^[65]. A further Korean survival study demonstrated that a functional SNP, rs6495309C > T, in the promoter of the CHRNA3 gene, was the prognostic factor for resected early stage NSCLC^[66]. Compared with rs6495309 CC genotype, the patients in the studied Korean cohort with rs6495309 CT/TT genotype had a better 5-yr OS by 5% and better 5-yr DFS by 7%^[66].

NACHRS AS BIOMARKERS FOR LUNG CANCER - THE FUTURE PERSPECTIVE?

The use of nAChR antagonists that block the receptors still has some issues, because these receptors regulate many vital cell and organ functions, and deficiency or impairment of nAChR signaling will lead to overproduction of cytokines in some tissues and enhance tissue damage. Carefully designed animal studies are essential to investigate the potential side effects of nAChR antagonists on the brain, central nervous system, immune cells and muscle cells, which express high levels of nicotinic receptors.

In summary, strategies for marker-guided lung cancer intervention that targets nAChRs seem promising. nAChR antagonists could be potentially used in combination with established chemotherapeutic drugs to enhance the therapeutic response to chemotherapy. Future studies involving the design of nAChR antagonists with improved selectivity might trigger novel strategies for the intervention and prevention of lung cancer.

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WJCO 5th Anniversary Special Issues (2): Breast cancer**Cyclooxygenase-2 and the inflammogenesis of breast cancer**

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Abstract

Cohesive scientific evidence from molecular, animal, and human investigations supports the hypothesis that constitutive overexpression of cyclooxygenase-2 (COX-2) is a ubiquitous driver of mammary carcinogenesis, and reciprocally, that COX-2 blockade has strong potential for breast cancer prevention and therapy. Key findings include the following: (1) COX-2 is constitutively expressed throughout breast cancer development and expression intensifies with stage at detection, cancer progression and metastasis; (2) essential features of mammary carcinogenesis (mutagenesis, mitogenesis, angiogenesis, reduced apoptosis, metastasis and immunosuppression) are linked to COX-2-driven prostaglandin E₂ (PGE-2) biosynthesis; (3) upregulation of COX-2 and PGE-2 expression induces transcription of CYP-19 and aromatase-catalyzed estrogen biosynthesis which stimulates unbridled mitogenesis; (4) extrahe-

patic CYP-1B1 in mammary adipose tissue converts paracrine estrogen to carcinogenic quinones with mutagenic impact; and (5) agents that inhibit COX-2 reduce the risk of breast cancer in women without disease and reduce recurrence risk and mortality in women with breast cancer. Recent sharp increases in global breast cancer incidence and mortality are likely driven by chronic inflammation of mammary adipose and upregulation of COX-2 associated with the obesity pandemic. The totality of evidence clearly supports the supposition that mammary carcinogenesis often evolves as a progressive series of highly specific cellular and molecular changes in response to induction of constitutive overexpression of COX-2 and the prostaglandin cascade in the "inflammogenesis of breast cancer".

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Key words: Breast Cancer; Cyclooxygenase-2; Nonsteroidal anti-inflammatory drugs; Inflammogenesis; Estrogen; Aromatase

Core tip: Mammary carcinogenesis often evolves as a series of highly specific cellular and molecular changes in response to induction of constitutive over-expression of cyclooxygenase-2 (COX-2) and the prostaglandin cascade; reciprocally, agents that block COX-2 have significant value in the chemoprevention and therapy of breast cancer.

Harris RE, Casto BC, Harris ZM. Cyclooxygenase-2 and the inflammogenesis of breast cancer. *World J Clin Oncol* 2014; 5(4): 677-692 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i4/677.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i4.677>

INTRODUCTION

More than a century ago, Virchow *et al*^[1,2] suggested that

chronic inflammation leads to cancer development by increasing cellular proliferation^[3]. Various models of carcinogenesis have been proposed involving inflammatory stimuli and mediators of wound healing^[4-6]. The recent discovery of the inducible cyclooxygenase-2 (COX-2) gene has rekindled interest in the causal link between inflammation and cancer, and various models of carcinogenesis have been proposed involving inflammatory stimuli and COX-2 expression^[7-10].

The current review synthesizes and interprets the accumulating body of evidence supporting COX-2 driven inflammogenesis as a general model of breast cancer development and the use of anti-inflammatory compounds that block COX-2 for breast cancer prevention and therapy. Evidence from molecular studies and meta-analyses of COX-2-inhibiting agents and breast cancer are discussed and based upon results, a general model of inflammogenesis of breast cancer is proposed involving induction of constitutive COX-2 over-expression and up-regulation of the prostaglandin cascade.

COX, PROSTAGLANDINS AND INFLAMMATION

Vane *et al.*^[11] discovered that the anti-inflammatory effects of aspirin [and all other nonsteroidal anti-inflammatory drugs (NSAIDs)] are primarily due to their inhibition of cyclooxygenase, the rate-limiting enzyme of the prostaglandin cascade. Metabolism of the essential fatty acid, arachidonic acid, *via* the cyclooxygenase pathway produces various prostaglandins that have a diverse array of physiologic activities throughout the human system. Indeed, these short lived molecules appear to control not only the inflammatory response, but they also help regulate constriction of blood vessels, contraction of smooth muscle, aggregation of platelets, sensitization of neurons to pain, flux of intracellular calcium, cell division, apoptosis, and many other molecular events that are critical for homeostatic physiology.

Two primary genes encode cyclooxygenase, a constitutive gene (*COX-1*) and its inducible isoform (*COX-2*)^[12-14]. The inducible *COX-2* gene is the master switch that activates the inflammatory response. Induction of COX-2 by any inflammatory stimulus (*e.g.*, tobacco, alcohol, ischemia, trauma, pressure, foreign bodies, toxins, bacteria, viruses, lipopolysaccharides, *etc.*) quickly results in the biosynthesis of prostaglandins of the E-series, particularly prostaglandin E₂ (PGE-2), and these prostaglandins in turn orchestrate the inflammatory response.

The cyclooxygenase pathway produces various prostaglandins, prostacyclins and thromboxanes from arachidonic acid and other fatty acids. In the initial step, COX catalyzes the oxidation of arachidonic acid to prostaglandin H-2 (PGH-2) which is rapidly converted to biologically active prostaglandins by specific enzymes. For example, PGH-2 is converted to the chief inflammatory prostaglandin, PGE-2, by PGE-2 synthetase.

Prostaglandin structure and function depend upon

the cell of origin and the level and type of catalytic COX enzyme. COX-1 is constitutively expressed at basal levels in many cells throughout the body, *e.g.*, gastrointestinal epithelium, renal tubules, vascular smooth muscle and blood platelets. Ordinarily, COX-1 expression is constitutive and sustains low levels of prostaglandins that are cytoprotective and maintain homeostasis. Conversely, the *COX-2* gene is silent (not transcribed) unless induced by inflammatory stimuli. Induced COX-2 transcription and expression markedly amplify the biosynthesis of PGE-2 which is the chief effector molecule of inflammation^[15].

Under normal conditions, acute inflammation is a tightly controlled self-limiting response to the offending stimulus. The process involves the integration of multiple cell types of the vascular and immune systems for the purpose of targeting, capturing, degrading, and removing the offending agent from the tissue under attack. Concurrent with acute inflammation, COX-2 expression and PGE-2 production by endothelial cells, epithelial cells, stromal cells, monocytes and lymphocytes increases up to 100 fold of basal levels. Amplification of the COX-2 inflammatory cascade is triggered by recognition of pro-inflammatory stimuli by toll-like receptors on the cell membranes of exposed cells and activation of nuclear factor kappa β (NF- $\kappa\beta$) which is often touted as a universal transcription factor^[16]. In addition, a variety of cytokines are secreted by infiltrating macrophages and other cells of the innate immune system. In particular, tissue necrosis factor α , γ -interferon and interleukins 1 and 6 (IL-1 and IL-6), stimulate the production of acute phase proteins such as C-Reactive protein, Amyloid A and complement, which assist in the inflammatory response^[17].

With abatement of the inflammatory stimulus, specific cytokines, particularly IL-1 and IL-6, exert feedback inhibition causing COX-2 expression and PGE-2 production to cease and the inflammatory process to subside. However, with sustained exposure to pro-inflammatory stimuli, continued overexpression of the COX-2 inflammatory cascade promotes the transition from acute to chronic inflammation. Molecular studies suggest that specific cytokines such as IL-6 and IL-1 β are responsible for recruiting monocytes to chronically inflamed tissues which may in turn disrupt the inhibitory feedback loop by secreting a variety of other pro-inflammatory cytokines^[18,19].

Constitutive expression of the *COX-2* gene and sustained biosynthesis of PGE-2 appear to be irrevocably linked to the initiation and promotion of mammary carcinogenesis. This review builds upon the evidence from molecular studies of COX-2, the rate-limiting enzyme of the prostaglandin cascade, reflecting its virtually ubiquitous role in mammary carcinogenesis, and reciprocally, epidemiologic studies documenting the beneficial impact of COX-2 blockade in breast cancer prevention and therapy.

Molecular studies are reviewed and updated data compiled to elucidate the role of COX-2 in the progression of breast cancer^[10]. Epidemiologic studies are reviewed and composite estimates derived by meta-analysis to

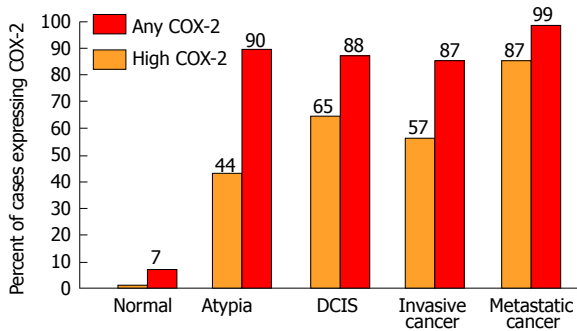


Figure 1 Cyclooxygenase-2 expression in the progression of breast cancer. COX-2: Cyclooxygenase-2.

quantify the impact of selective and non-selective agents that reduce breast cancer risk by inhibition of COX-2^[20]. Convincing evidence is presented showing that mammary carcinogenesis often evolves as a progressive series of highly specific cellular and molecular changes in response to induction of constitutive over-expression of COX-2 and the prostaglandin cascade in the “inflammogenesis of breast cancer”.

MOLECULAR EVIDENCE: COX-2 IN MALIGNANT AND PREMALIGNANT MAMMARY NEOPLASMS

Molecular studies using immunohistochemistry and reverse transcriptase polymerase chain reactions (RT-PCR) reveal that over-expression of COX-2 is a prominent feature of all stages of breast cancer. Furthermore, COX-2 is commonly found in premalignant lesions (dysplasia and atypia), carcinoma *in situ*, invasive cancer, and in particular, metastatic disease. In stark contrast to mammary cell populations that are in various stages of carcinogenesis, COX-2 is ordinarily not detectable in normal (non-inflamed) mammary tissues^[21,22].

The first investigation of COX-2 in human breast cancer specimens was conducted using immunohistochemistry and a human COX-2 primer^[23]. The study revealed the presence of COX-2 protein in 13 of 13 invasive human breast tumors, but not in samples of normal breast tissue. There was a statistically significant linear association between COX-2 and high (> 50%) tumor cell density ($P < 0.01$) with COX-2 protein localized to tumor cells.

Subsequently, molecular biologists from multiple independent laboratories have consistently observed COX-2 over-expression in all stages of breast cancer^[23-42]. Figure 1 shows the mean frequency of specimens over-expressing COX-2 in the progression of mammary carcinogenesis. Among studies of invasive breast cancer, 87% of specimens were positive for COX-2 and 57% had high levels of COX-2 expression. Significantly elevated frequencies of specimens with high COX-2 expression were also observed in premalignant lesions such as atypical hyperplasia (44%) and ductal carcinoma *in situ* (65%). Furthermore, several of the studies suggest that COX-2 expression is

correlated with the metastatic spread of breast cancer and has strong potential as a prognostic indicator of disease severity and progression^[30,31,35,37-39]. By comparison, all studies have found negligible or very weak focal COX-2 expression in normal tissues. It is indeed remarkable that high levels of COX-2 expression are evident throughout mammary carcinogenesis.

In an important prospective study conducted by Hartmann *et al.*^[43] at the Mayo Clinic, COX-2 expression was measured by immunohistochemistry in biopsy specimens from 235 women with atypical hyperplasia of the breast. Forty-one (17%) of the 235 women subsequently developed breast cancer during a median follow-up of 15 years. Notably, COX-2 expression at baseline was a significant predictor of risk. Compared to women without atypia, the cumulative incidence of breast cancer increased with increasing COX-2 expression, relative risk (RR) = 2.6 for weak or negligible expression, RR = 3.6 for moderate expression and RR = 5.7 for strong expression. The authors concluded that “COX-2 appears to be a biomarker that further stratifies breast cancer risk among women with atypia and may be a relevant target for chemoprevention strategies”^[43,44].

The molecular evidence clearly demonstrates that COX-2 over-expression is not only an early event in the genesis of breast cancer, but is present throughout the entire evolutionary process of breast cancer development and progression. Thus, COX-2 may be a useful biomarker of impending cancer and a prime target for molecular intervention in breast cancer prevention and therapy^[45].

COX-2 BLOCKADE IN BREAST CANCER PREVENTION AND THERAPY

The molecular evidence suggests that induction and constitutive upregulation of COX-2 and the prostaglandin cascade play a significant role in mammary carcinogenesis. But if inflammogenesis of breast cancer is to be upheld as a viable model, then the reciprocal relationship must also be true, *vis a vis.*, blockade of COX-2 should have significant inhibitory impact against mammary carcinogenesis. Critical evidence from animal and human investigations is discussed next.

Animal studies of breast cancer and COX-2 blockade

In the past quarter century, several independent investigations employing animal models of mammary carcinogenesis have generated compelling evidence that NSAIDs have significant and consistent chemopreventive effects against breast cancer development.

Karmali *et al.*^[46,47] first observed chemopreventive effects of NSAIDs against breast cancer and also elucidated differential effects of essential dietary fatty acids in prostaglandin (PG) biosynthesis and tumor promotion. Their studies showed that dietary supplementation with the n-6 fatty acid, linoleic acid, promoted tumor growth and development *via* enhanced arachidonic acid metabolism and elevated levels of PG activity, whereas the n-3

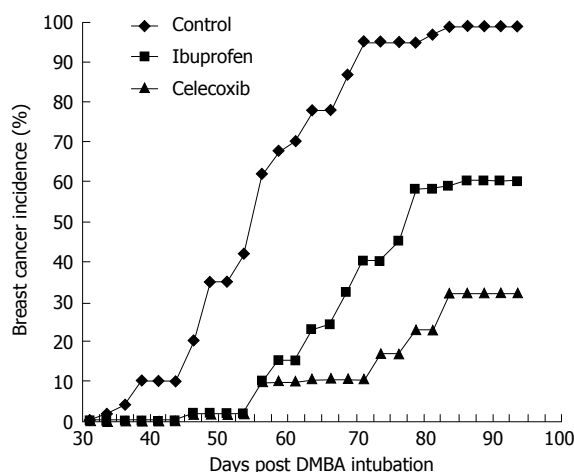


Figure 2 Effects of selective cyclooxygenase-2 blockade on DMBA-induced breast cancer in Sprague-Dawley rats^[51].

essential fatty acid, linolenic acid, had the opposite effect.

In several subsequent preclinical investigations of chemically induced breast cancer, supplemental administration of general NSAIDs such as aspirin, ibuprofen, piroxicam, sulindac, and others, in the diet or drinking water consistently reduced the growth and progression of breast tumors^[48-50]. The molecular basis for the antineoplastic effects of these general NSAIDs is linked to their inhibition of cyclooxygenase gene expression and enzyme activity. However, general NSAIDs have nonselective activity against both COX-1 and COX-2.

It is therefore important to note that recent preclinical studies have demonstrated even stronger antineoplastic effects of selective COX-2 inhibitors such as celecoxib, rofecoxib, valdecoxib, and nimesulide against breast cancer. Harris and colleagues initially reported that celecoxib markedly reduced the incidence of DMBA-induced breast cancer in Sprague-Dawley female rats^[51]. In their study, celecoxib reduced the incidence of breast cancer by 70% compared to controls. In the same trial, ibuprofen reduced the incidence of breast cancer by 40% (Figure 2). Further evidence for the primary role of COX-2 in mammary carcinogenesis comes from transgenic mouse models in which the overexpression of COX-2 is sufficient to induce malignant transformation of normal epithelial cells of the mammary gland^[52].

In summary, animal models of carcinogenesis provide compelling evidence that NSAIDs inhibit growth and development of breast tumors. While preclinical investigations provide consistent evidence that both selective and nonselective NSAIDs inhibit chemically induced carcinogenesis of mammary epithelial tumors, the strongest antineoplastic effects are clearly the result of intervention by administration of COX-2 blocking agents.

Human studies of non-selective COX-2 inhibitors and breast cancer

Meta-analysis of NSAIDs and breast cancer: Independent estimates from 37 studies were used in an updated meta-analysis of over-the-counter NSAIDs (primarily

aspirin or ibuprofen) and breast cancer^[53-91]. These reports were ascertained by a search of MEDLINE in the period 1970-2013 using combinations of key words: breast cancer with NSAIDs, aspirin and ibuprofen. Methods developed by Schlesselman and Greenland were adapted for combined analysis of the data from these studies^[92,93]. Estimates of RR and 95% confidence intervals were converted to $\ln(RR)$ with corresponding variance estimates (v). The combined estimate of risk in logarithmic form, $\ln(RR^*) = \sum \ln(RR)w / \sum w$, was obtained by weighting individual estimates by $w = 1/v$. A χ^2 test of heterogeneity was utilized to test for differences among studies.

RR with 95% CIs from these reports are shown in Figure 3. Among the 37 estimates, 25 were significantly less than 1.0 and only one was significantly greater than 1.0. The test for heterogeneity was not significant and the composite estimate shows a 25% reduction in the relative risk of breast cancer with regular use of aspirin or other OTC NSAIDs (Combined RR = 0.75, 95%CI: 0.67-0.84, $P < 0.001$).

Our review and meta-analysis of data from the epidemiologic literature therefore provides compelling evidence that regular intake of NSAIDs that nonselectively block COX-2 protects against the development of breast cancer. When data are combined by meta-analysis, it is estimated that regular NSAID intake is associated with a 25% reduction in overall breast cancer risk. This estimate is similar to the results of earlier meta-analysis by González-Pérez *et al.*^[94] who reported a 23% reduction in breast cancer risk with NSAID use^[93]. The available data suggest that significant reductions in breast cancer risk occur with 5 or more years of using low dosages of aspirin or other NSAIDs on a regular basis and long term studies suggest that the risk declines to maximal levels with regular intake for 10-20 years^[94]. It is also notable that some studies have found that NSAIDs may have a greater effect against estrogen receptor positive breast cancer^[72,77,80,86], and in one such study, a genetic polymorphism of the COX-2 gene was associated with a significant reduction in the risk of estrogen positive breast cancer^[80].

Study of selective COX-2 inhibitors and breast cancer

Based on the epidemiologic evidence that nonselective NSAIDs reduce human breast cancer risk, we initiated a case control study of selective COX-2 inhibitors to assess their effects on the relative risk of breast cancer. The study was conducted for women diagnosed with breast cancer during the window of time (1998-2004) in which two selective COX-2 inhibitors, celecoxib and rofecoxib, were available by prescription in the United States. In the study, 323 cases with pathologically confirmed invasive breast cancer were compared to 649 controls without cancer who were frequency-matched at a 2:1 rate to the cases by age and county of residence^[79].

Results of the investigation are shown in Table 1. Coxib use reduced the risk of breast cancer development by 71% (OR = 0.29, $P < 0.01$). Significant reductions in

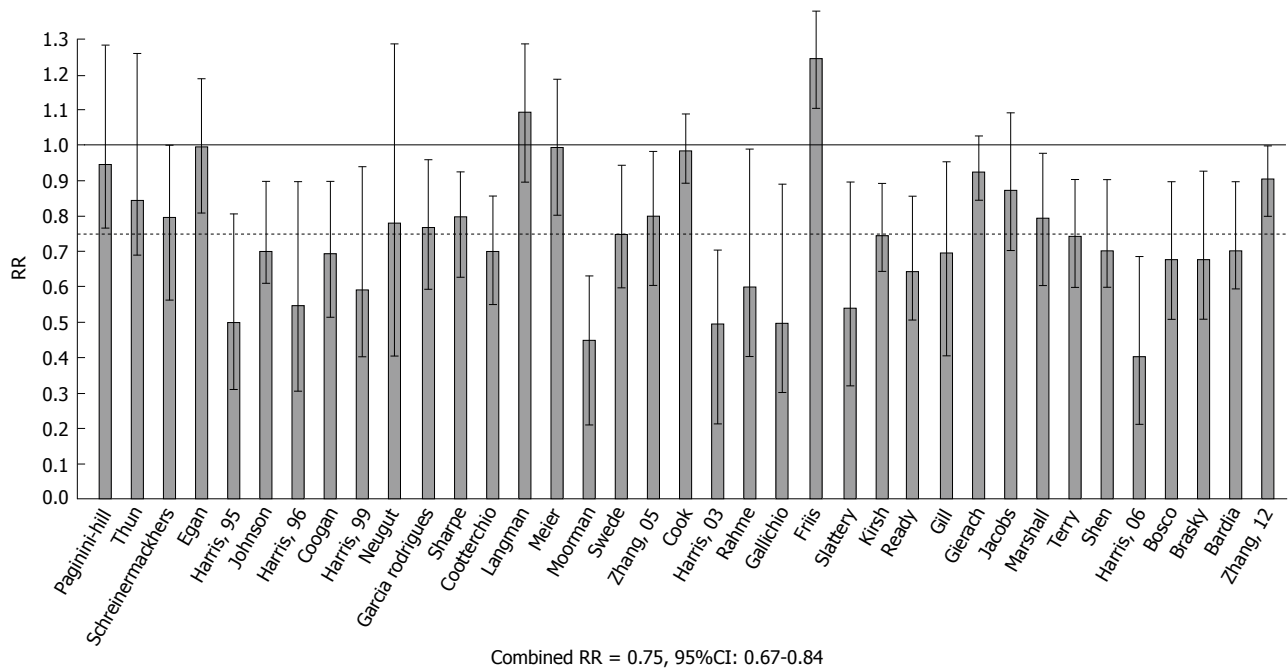


Figure 3 Meta-analysis of breast cancer and nonsteroidal anti-inflammatory drugs. Estimates of relative risk (RR) and 95%CI are shown for individual studies. The horizontal dotted line reflects the combined estimate of RR = 0.75, 95%CI: 0.67-0.84, $P < 0.001$.

Table 1 Breast cancer and cyclooxygenase-2 blocking agents: Results of a case control study^[79]

Agent	OR (95%CI)
COX-2 inhibitor	0.29 (0.14-0.59)
Ibuprofen	0.37 (0.18-0.72)
Regular aspirin (325 mg)	0.51 (0.27-0.98)
Low dose aspirin (81 mg)	0.77 (0.41-1.41)
Acetaminophen	1.02 (0.39-2.20)
Selective COX-2 inhibitors: Celecoxib or Rofecoxib	

COX-2: Cyclooxygenase-2.

breast cancer risk were also noted for ibuprofen (63%) and regular 325 mg aspirin (49%) but not for low dose (81 mg) aspirin (23%). There was no effect of acetaminophen, an analgesic without COX-2 inhibiting properties (OR = 1.02). The inverse pattern of risk for acetaminophen, low dose aspirin, regular aspirin, ibuprofen and coxibs was significant by a linear trend test ($P < 0.05$) suggesting that chemopreventive effects become progressively stronger with greater selective COX-2 inhibition.

Comparative studies of breast cancer and other neoplasms

During the time period 1987-2008, we conducted a series of epidemiologic studies of NSAIDs and cancers of the breast, prostate, colon and lung^[57,59,61,69,79,95-99]. Five of these studies focused on cancer of the breast. In each investigation, information was obtained about the entire profile of NSAID use for each participant including both over-the-counter and prescription drugs. All studies were designed to specifically evaluate and compare the two major over-the-counter compounds, aspirin and ibuprofen. Following their FDA approval, selective COX-2

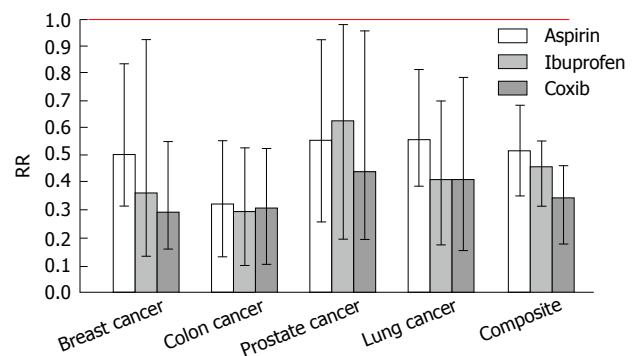


Figure 4 Comparison of selective and non-selective cyclooxygenase-2 inhibitors in cancer prevention. Bars represent 95%CI of each estimate^[100].

inhibiting agents were also evaluated. Effects of specific agents were quantified by estimating relative risks (or odds ratios) adjusted for cancer risk factors with standard errors and 95%CI. In each study, estimates for specific compounds were derived by comparison with a reference group that did not report using any type of NSAID. Furthermore, acetaminophen, a commonly used analgesic with little or no activity against either COX-1 or COX-2 was always evaluated as a comparator drug. Meta-analysis as described above was applied to examine effects of individual compounds for each individual cancer and across all malignancies^[100,101].

Figure 4 presents the individual and composite risk estimates for the four cancer sites with exposure to regular aspirin, ibuprofen or selective COX-2 inhibitors (celecoxib or rofecoxib). Daily intake of a selective COX-2 inhibitor (either celecoxib or rofecoxib) produced a significant reduction in the risk for each type of cancer (71% for breast cancer, 55% for prostate cancer, 70% for

colon cancer, and 79% for lung cancer). The observed chemopreventive effects of coxibs were associated with recommended daily doses of celecoxib (median dose = 200 mg) or rofecoxib (median dose = 25 mg). Significant risk reductions of slightly lesser magnitude were observed for over-the-counter NSAIDs with nonselective COX-2 activity, such as regular (325 mg) aspirin and (200 mg) ibuprofen. Daily intake of baby (81 mg) aspirin produced marginally significant risk reductions for colon cancer and lung cancer, but did not significantly reduce the risk of breast cancer or prostate cancer whereas daily acetaminophen, an analgesic without COX-2 activity, did not produce a significant change in the risk of any of the cancers studied. Composite risk reductions of 64%, 53% and 46% were observed for the selective COX-2 inhibitors (either celecoxib or rofecoxib), ibuprofen and aspirin, respectively; a significant dose response pattern that is consistent with the degree of selective COX-2 blockade (celecoxib > ibuprofen > aspirin).

Notably, selective COX-2 inhibitors (celecoxib and rofecoxib) were only recently approved for use in 1999. In 2004, rofecoxib (Vioxx) was withdrawn from the marketplace due to concerns about cardiovascular risk. Nevertheless, even in the short window of exposure to these compounds, the selective COX-2 inhibitors produced significant reductions in the risk of the four major human cancers (breast, prostate, colon, and lung). It is also important to note that ibuprofen produced effects similar in magnitude to the coxibs which is consistent with its high activity against COX-2. These results tend to substantiate the important role of COX-2 in carcinogenesis, and reciprocally, the strong potential for selective COX-2 blockade in cancer chemoprevention.

Therapeutic studies of NSAIDs in human breast cancer

Randomized clinical trials of nonselective COX-2 inhibitors such as aspirin and ibuprofen for human cancer therapy are lacking. Nevertheless, since these drugs are frequently regularly taken for pain relief in randomized clinical trials of cancer, some investigators have examined their therapeutic impact among patients.

Remarkably, the treatment-adjusted hazard ratios for NSAID users show significant reductions of recurrence risk or death in three cohorts of breast cancer patients. It is emphasized that effects of ibuprofen and aspirin estimated from these studies are adjusted for stage at cancer detection, surgical treatment, chemotherapy, radiation therapy and other prognostic indicators such as age, race and gender.

Kwan *et al.*^[102] examined the association between NSAID use and breast cancer recurrence among 2292 women diagnosed with breast cancer in the Life After Cancer Epidemiology Study. They observed that regular ibuprofen users experienced 44% less recurrence than non-users after five years of follow-up.

Blair *et al.*^[103] examined effects of NSAID intake on survival after invasive breast cancer diagnosis among 591 postmenopausal women ascertained through the Iowa Women's Health Study. Compared to nonusers, women

who regularly took an NSAID experienced a 36% reduction in breast cancer mortality and a 43% reduction in all-cause mortality after approximately 10 years of followup.

Holmes *et al.*^[104] examined effects of taking aspirin or non-aspirin NSAIDs such as ibuprofen among 4164 women presenting with invasive breast cancers in the Nurses Health Study. They found that aspirin intake after breast cancer diagnosis was associated with a decreased risk of breast cancer recurrence, death from breast cancer and death from any cause. Significant decreases in breast cancer mortality of 71% and 64% were noted for aspirin intake 2-5 times per week and 6-7 times per week, respectively. More limited results from the study suggested that daily intake of non-aspirin NSAIDs also reduced breast cancer mortality whereas acetaminophen use showed no evidence of survival benefit.

MODEL OF INFLAMMOGENESIS OF BREAST CANCER

Interaction of mammary epithelium and adipose tissue

White adipocytes are intimately and inseparably connected to the parenchyma of the human female breast throughout life^[105]. These cells provide the nutrients essential for the morphogenesis, maturation and function of the mammary epithelium. Homeostasis of the breast epithelium therefore depends vitally upon the integrity of the adipocyte population of the mammary gland. Far from being an inert fat storage depot and energy resource for parenchymal cells (*e.g.*, the mammary epithelium), white adipose tissue is an active endocrine organ that secretes a variety of bioactive proteins collectively called adipokines^[106].

Obesity, inflammation and breast cancer

Recent data from the World Health Organization and the International Agency for Research on Cancer reflects a 20% increase in the global incidence of breast cancer and a 14% increase in breast cancer mortality during the past five years^[107]. These increases are most likely largely attributable to the global pandemic of obesity that influences breast cancer development and progression.

In molecular studies of tissues from humans and animals, obesity leads to inflammation and infiltration of mammary and visceral adipose tissue by macrophages with activation of NF- κ B, overexpression of COX-2 and hypersecretion of PGE-2 and pro-inflammatory mediators and adipokines such as leptin, resistin, IL-6, IL-1 β and tumor necrosis factors (TNF)- α ^[108-111]. Furthermore, COX-2 driven PGE-2 biosynthesis induces transcription of CYP-19 and aromatase-catalyzed production of estrogen in a paracrine mechanism. Local estrogen biosynthesis in the breast parenchyma has been hypothesized to be a key feature of breast cancer development, particularly in postmenopausal women^[112,113].

Inflammogenesis of breast cancer by COX-2: Molecular mechanisms

Various molecular mechanisms may be responsible for

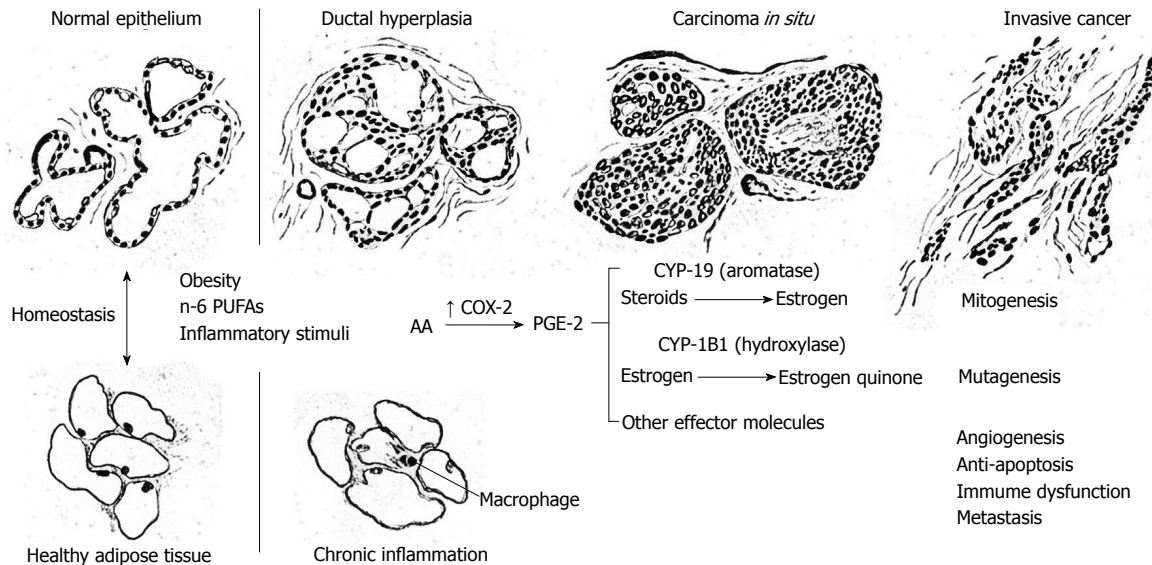


Figure 5 Model of COX-2-driven inflammogenesis of breast cancer. Key steps in the transition of normal mammary ductal epithelium to invasive cancer are as follows: (1) Healthy mammary adipose maintains homeostasis of the mammary epithelium; (2) Pro-inflammatory stimuli (e.g., adipokines, cytokines and n-6 PUFA) induce macrophage infiltration and chronic inflammation of the mammary stroma; (3) COX-2 is upregulated and constitutively expressed by adipocytes, immune cells and epithelial cells; (4) COX-2-catalyzed PGE-2 induces transcription of the *CYP-19* gene and aromatase-catalyzed estrogen biosynthesis by adipocytes; (5) Estrogen stimulates estrogen receptors of epithelial cells to promote cell proliferation; (6) PGE-2 induces CYP-1B1 in epithelial cells resulting in hydroxylation of estrogen that is converted to estrogen quinone that has mutagenic impact; and (7) PGE-2 induces a profile of genes in epithelial cells that promote angiogenesis, loss of apoptosis, immunosuppression and metastasis (see text for discussion). COX-2: Cyclooxygenase-2; PGE-2: Prostaglandin E2; n-6 PUFA: n-6 polyunsaturated fatty acids.

the initiation and promotion of mammary carcinogenesis by COX-2. It is indeed remarkable that the induction of constitutive COX-2 expression and PGE2 biosynthesis are sufficient to stimulate all of the key features of mammary carcinogenesis including mutagenesis, mitogenesis, angiogenesis, metastasis, inhibition of apoptosis and immunosuppression with reduced antineoplastic activity of T and B lymphocytes. These mechanisms are thoroughly reviewed and discussed elsewhere, e.g., Shiff *et al.*^[114] and Subbaramaiah *et al.*^[115].

As depicted in Figure 5, continuous over-expression of COX-2 can initiate and promote carcinogenesis by (1) increasing production of PGE-2 and other prostaglandins that strongly promote cell proliferation, e.g., correlative up-regulation of the gene for aromatase (CYP19) and estrogen biosynthesis in stromal cells, or activation of epidermal growth factor receptor (EGFR) that stimulate an intracellular cascade of mitogenic signaling (mitogenesis); (2) increasing production of estrogen quinones and other reactive oxygen species, e.g., malondialdehyde, that are carcinogenic (mutagenesis); (3) stimulation of vascular endothelial growth factor (VEGF) and platelet derived growth factor by PGE-2 resulting in de novo formation of blood vessels (angiogenesis); (4) increasing production of matrix metalloproteinases (MMP) *via* co-expression of COX-2 and the *Her-2/Neu* gene, thus enhancing invasive potential (metastasis); (5) stimulating telomerase expression, decreasing bioavailable arachidonic acid pools necessary for conversion of sphingomyelin to ceramide, and stimulation of the *Bcl-2* gene and inhibition of the *BAX* gene thereby reducing cell differentiation and apoptosis (anti-apoptosis); and (6) inhibiting proliferation of B and T lymphocytes, particularly natural killer T cells, thus lim-

iting antineoplastic activity (immunosuppression). All of these processes are discussed in some detail below.

Induction of COX-2

A key event in the carcinogenic process is induction of constitutive expression of the *COX-2* gene. Molecular studies from multiple laboratories reveal that adenocarcinoma of the breast is characterized by aberrant over-expression of COX-2 by breast cancer epithelial cells^[24,33-38]. As shown by Karmali *et al.*^[116] Rose *et al.*^[117], and others^[33-38], arachidonic acid production, COX-2 expression, and prostaglandin biosynthesis are increased *in vivo* by dietary n-6 polyunsaturated fatty acids (n-6-PUFAs) such as unconjugated linoleic acid, and decreased by n-3-PUFAs such as linolenic acid. High dietary intake of n-6-PUFAs may therefore be an important factor in the induction of constitutive COX-2 expression. This mechanism is compatible with the high rates of cancers of the breast, colon and prostate (neoplasms that characteristically over-express COX-2) in populations where n-6-PUFAs, particularly unconjugated linoleic acid, are abundant in the diet. The COX-2 enzyme efficiently catalyzes the conversion of essential dietary fats (principally arachidonic acid and unconjugated linoleic acid) into prostaglandins^[100,113,118].

Obesity has reached epidemic proportions in most industrialized nations in association with increased rates of a variety of chronic conditions such as type 2 diabetes, coronary heart disease and certain malignant neoplasms including breast cancer. The obese phenotype is characterized by the presence of fat-laden adipocytes that secrete pro-inflammatory adipokines (e.g., leptin and resistin) and stimulate infiltration of the mammary fat by

macrophages which secrete pro-inflammatory cytokines (*e.g.*, IL-6 and TNF- α). Since the COX-2 gene contains multiple promoter binding sites, these effector molecules may also participate in signal transduction cascades to induce constitutive overexpression of COX-2 and PGE-2 biosynthesis^[45,108-111,114,115]. Furthermore, *in vitro* studies of breast cancer tissues suggest that mutations or methylation of CpG islands at binding sites for these transcription factors in the promoter region of the COX-2 gene regulate the induction of COX-2 transcription^[119]. Thus, induction of constitutive COX-2 genetic expression may involve synergistic interactions between a number of micro-environmental epigenetic and genetic cofactors.

Mitogenesis

The COX-2 enzyme efficiently catalyzes the conversion of essential dietary fats (principally arachidonic acid and unconjugated linoleic acid) into prostaglandins. Induction of constitutive over-expression of COX-2 in a cell predominantly increases the biosynthesis of PGE-2, which is the chief prostaglandin of the inflammatory cascade. This short-lived intercellular hormone is capable of inducing the transcription of specific genes in the nucleus of nearby cells. In particular, PGE-2 has been found to stimulate the transcription of genes that have powerful mitogenic effects.

Importantly, it has recently been discovered that there is a strong link between prostaglandins and a paracrine mechanism of estrogen biosynthesis. This occurs when the chief prostaglandin, PGE-2, activates the promoter II region of the aromatase gene (*CYP-19*), which is responsible for local estrogen biosynthesis catalyzed by aromatase^[112]. Furthermore, several molecular studies have revealed a significant correlation between up-regulation of cyclooxygenase expression and CYP-19 transcription and aromatase-catalyzed estrogen biosynthesis in breast cancer tissues^[108,109,120-122]. Notably, this mechanism has been demonstrated in other malignant neoplasms including cancers of the lung, colon, and prostate and may in fact be a ubiquitous feature in cancer promotion and development^[123-130]. Clearly, the established molecular link between heightened levels of n-6 polyunsaturated fatty acids, COX-2, PGE-2, aromatase and estrogens, provides a basis for mammary carcinogenesis through unbridled mitogenesis.

An additional mitogenic mechanism is PGE-2 activation of EGFR that in turn triggers cell division through the mitogen-activated protein kinase (MAPK) cascade^[131-133]. Polakis *et al.*^[134] discovered that PGE-2 rapidly phosphorylates EGFR and triggers the extracellular kinase, ERK-2, thereby activating the mitogenic signaling cascade in normal gastric epithelium and colon cancer. Their studies indicate that PGE-2-induced EGFR trans-activation involves signal transduction *via* TGF- α and activated MMP. Other investigators have confirmed that co-expression of COX-2, PGE-2, and EGFR results in mitogenic activation in precancerous and cancerous tissues of multiple anatomic sites^[135-139]. In a recent molecular study of COX-2 and EGFR in human breast cancer

tissues from 55 patients, COX-2 expression was detected in cancer cells of more than 95% of specimens and EGFR expression was found to be dependent on COX-2 upregulation^[140].

Other COX-2 driven mechanisms may also be involved in delimiting cell proliferation of the ductal epithelium of mammary tissues. For example, PGE-2 expression is associated with disruption of contact inhibition in malignant cells from specimens of cancerous tissues from multiple anatomic sites.

Molecular examination of colon cancer specimens first revealed accumulation of the cell adhesion molecule, beta-catenin, in the nucleus of malignant cells^[133-136]. Cell adhesion is under the control of the gene for adenomatous polyposis coli (APC) and involves maintenance of the integrity of a molecular cell adhesion complex comprised of beta-catenin, APC protein, T-cell factor and actin. Familial adenomatous polyposis is caused by a mutation of the APC gene that causes dissociation of these cell adhesion complexes and the migration of beta catenin to the cell nucleus where it activates one of the peroxisome proliferator activated receptors (PPAR gamma) on the nuclear membrane. Castellone *et al.*^[137] conducted a series of experiments demonstrating that inhibition of PGE-2 biosynthesis by NSAIDs effectively reduces the accumulation of beta-catenin and the progression of colon cancer. Based on their results, over-expression of COX-2 with increased PGE-2 biosynthesis and binding to its receptor in turn activates a cytoplasmic G-protein receptor that binds axin thereby reducing phosphorylation of beta-catenin. This chain of molecular events leads to dissociation of the adhesion complex, accumulation of unphosphorylated beta-catenin in the cell nucleus, activation of the nuclear receptor, PPAR-gamma, and stimulation of cell proliferation through transcription of cell cyclin genes. Recent molecular studies suggest that this mechanism is not limited to the colon; that is, induction of cyclooxygenase and increased PGE-2 can result in cellular beta-catenin accumulation, nuclear PPAR-gamma activation, and subsequent cell proliferation and carcinogenesis in a variety of tissues including the mammary epithelium^[138,139].

Mutagenesis

Accumulated mutagenic damage to DNA is believed to contribute substantially to the etiology of breast cancer. Notably, there is strong experimental evidence to support the role of estrogen metabolites as carcinogenic agents. Specifically, the metabolism of estrogens by certain enzymes of the cytochrome P450 system produces catechol estrogens that can be further oxidized to form quinones that react directly with DNA or undergo redox cycling to generate reactive oxygen species that cause oxidative damage to DNA. This mechanism is of major significance in chronically inflamed breast tissue (*e.g.*, in obese women) wherein PGE-2 activates aromatase-catalyzed estrogen biosynthesis.

The quinone metabolites of estrogen are formed by the action of the same cytochrome P450 enzymes

responsible for the metabolism of polycyclic aromatic hydrocarbons (cytochrome P450 isoforms CYP-1A1, CYP-1B1, and CYP-3A). While most P450 enzymes are produced in the liver, CYP-1B1 is constitutively expressed in the mammary gland and other extrahepatic tissues. In the mammary gland, CYP-1B1 preferentially metabolizes estrogen to 4-hydroxyestrogen which is oxidized to form carcinogenic 3,4 estrogen quinone which in turn forms unstable adducts with adenine and guanine in DNA, leading to depurination and mutation *in vitro* and *in vivo*. Reduction of estrogen quinones to hydroquinones and catechols can also form reactive oxygen species by redox recycling^[140-147].

Another established mechanism of mutagenesis involves constitutive COX-2 expression and intermediate compounds formed by activation of the prostaglandin cascade. It is well known that lipid peroxidation in the human system generates reactive electrophilic compounds that have mutagenic potential. COX catalyze the two-step oxidation and peroxidation of arachidonic acid to form the intermediate prostaglandin endoperoxides, PGG-2 and PGH-2^[13,14,45,114,115,148]. Spontaneous breakdown of PGH-2 yields the mutagen, malondialdehyde (MDA) plus hydroxyheptadecatreonic acid, and specific enzymes of the cytochrome P450 system as well as thromboxane synthetase can also catalyze the breakdown of PGH-2 to MDA^[149]. Malondialdehyde reacts with DNA under physiological conditions to form DNA adducts, predominantly pyrimidopurine adducts of deoxyguanosine^[150]. Sharma *et al.*^[151] demonstrated that induction of COX-2 in human non-malignant colon epithelial cells produced increases in PGE-2, MDA, and characteristic DNA adducts that were similar to the levels observed in malignant colon epithelial cells. These findings underscore the potential for carcinogenesis due to oxidative damage and mutagenesis attributable to constitutive over-expression of COX-2.

Angiogenesis

VEGF is a potent stimulant of de novo blood vessel formation (angiogenesis) in a variety of tissues. Once believed present only in the endothelial lining of blood vessels, VEGF has now been discovered in virtually all types of cancers^[152,153]. Molecular investigations of breast cancer tissues provide strong evidence that COX-2-derived PGE-2 stimulates the synthesis and release of VEGF resulting in angiogenesis and ingrowth of new blood vessels that are immature and highly permeable thereby facilitating metastatic spread of tumor cells^[7,28,154]. Tumor secretion of VEGF (and other growth factors) may further amplify COX-2 expression in a positive feedback loop to produce lymphangiogenesis^[155,156]. Notably, inhibition of this vicious cycle by COX-2 inhibiting agents such as celecoxib has been found to limit angiogenesis and halt the progression and metastatic spread of tumors in animals^[157].

Suppression of apoptosis

Apoptosis or controlled cell death is an important regula-

tory mechanism for the maintenance of homeostasis in cell populations. Dysfunctional apoptosis results in immortalization of cells, a key feature of cancer cells. Inflammation, COX-2 over-expression, and increased PGE-2 are clearly anti-apoptotic, whereas, anti-inflammatory compounds that inhibit COX-2 are pro-apoptotic^[21,22,114,115,158].

Apoptosis is regulated by an intrinsic pathway that originates inside the cell and an extrinsic pathway that originates outside the cell and in molecular studies of breast cancer tissues, both pathways are inhibited by COX-2 over-expression^[159-161]. The intrinsic pathway involves mitochondrial release of cytochrome c and activation of caspase 9 and other enzymes that destroy the cell. Intrinsic apoptosis is triggered when the expression of two nuclear genes, Bcl-2 and BAX, favors BAX. Notably, COX-2 over-expression and prostaglandin biosynthesis promotes Bcl-2 and inhibits BAX, thereby blocking intrinsic apoptosis^[159,160].

The extrinsic pathway involves activation of death receptors on the cell membrane by TNF- α , - β and other epigenetic factors. This results in activation of caspase 8 and other enzymes that destroy the cell. Over-expression of COX-2 attenuates activation of this mechanism thereby blocking extrinsic apoptosis^[161].

Compounds that inhibit COX-2 and PGE-2 appear to enhance both intrinsic and extrinsic apoptosis and as a consequence, COX-2 inhibitors used in combination with radiation show beneficial synergism in the elimination of cancer cells in inoperable solid tumors^[162,163]. Nonsteroidal anti-inflammatory drugs have also been found to increase apoptosis by other mechanisms, *e.g.*, by increasing bio-available arachidonic acid pools necessary for conversion of sphingomyelin to ceramide since ceramide accumulation in the cell triggers apoptosis^[164]. In an interesting study of a breast cell line immortalized by introduction of the human telomerase gene, a selective COX-2 inhibitor, celecoxib, induced apoptosis and inhibited growth in association with upregulation of insulin-like growth factor^[165].

Metastasis

The Her-2/Neu oncogene is a member of the EGFR family. It is an important mediator of cancer cell growth and metastasis. Koki *et al.*^[7] and Subbaramaiah *et al.*^[166] demonstrated that COX-2 and Her-2/Neu are co-expressed in breast cancer tissues. Co-expression of COX-2 and Her-2/Neu stimulate the MAPK/AP-1 signaling cascade. When the Her-2/Neu receptor protein is activated, multiple other factors are activated that promote tumor development and metastatic spread of cancer cells^[7]. Overexpression of Her-2/Neu is now widely used by clinicians as a biomarker of poor prognosis and metastasis for patients with invasive breast cancer^[167].

Molecular studies of breast cancer tissues have demonstrated that high levels of COX-2 and PGE-2 are correlated with amplified Her-2/Neu expression and increased activity of MMP^[168,169]. The MMP are proteolytic enzymes that degrade basement membranes and are thus associated with tumor invasiveness, metastasis, and poor survival. Reciprocally, in animal models of breast cancer,

agents that inhibit COX-2 or block membrane receptors of PGE-2 have been found to reduce Her-2/Neu and MMP levels thereby decreasing the metastatic potential of cancer cells^[170,171].

Immunosuppression

Immunosuppression is a characteristic feature of cancer patients that correlates with disease promotion and progression. It is an interesting paradox that COX-2 over-expression and prostaglandin biosynthesis empowers cancer cell proliferation, immortalization, and metastasis on the one hand, while suppressing the function of important cells of the immune system on the other, thereby creating an immunosuppressed host with little ability to mount an immune defense against a developing tumor. Indeed, the induction of T cell anergy is an early event in the course of tumor progression^[172].

Prostaglandins, particularly PGE-2, are important modulators of immunosuppression. Pockaj *et al.*^[173] found that increased levels of PGE-2 suppress the immunocompetence of helper T-cells and dendritic cells in newly diagnosed breast cancer patients. Specifically, elevated levels of PGE-2 were associated with reduced secretion of antitumor factors by T-cells (interferon-gamma, TNF-alpha, and interleukins IL-2 and IL-12) and loss of immunocompetence in dendritic cells (reduced secretion of stimulatory molecules, loss of antigen-sensitizing function, reduced phagocytic activity, and lack of maturation potential). Defective T-cell and dendritic cell function due to COX-2 driven PGE-2 biosynthesis is therefore an important mechanism by which tumors evade immunosurveillance.

Web of causation of mammary carcinogenesis

It should be emphasized that the proposed “inflammogenesis model of breast cancer” is not mutually exclusive and may in fact be synergistic with other mechanisms of mammary carcinogenesis. For example, polycyclic aromatic hydrocarbons and other carcinogens present in tobacco smoke are mutagenic in mammary tissues^[174] and acetaldehyde, the primary metabolite of alcohol metabolism has powerful mutagenic impact in all tissues studied^[175]. The web of breast cancer causation may thus be particularly strong in obese women who are chronically addicted to both tobacco and alcohol and regularly consume diets with a high content of n-6 PUFA.

CONCLUSION

Cohesive scientific evidence from molecular, animal, and human investigations supports the hypothesis that induction of constitutive COX-2 over-expression and upregulation of the prostaglandin cascade play a significant role in mammary carcinogenesis, and reciprocally, blockade of the process has strong potential for breast cancer prevention and therapy. A summary of the evidence supporting the “inflammogenesis of breast cancer” is given below: (1) Epidemiologic investigations have consistently demonstrated that nonselective COX-2 inhibitors, such

as aspirin and ibuprofen, used on a regular basis, significantly reduce the risk of human breast cancer; (2) Selective COX-2 inhibitors, such as celecoxib, used on a regular basis have been shown to reduce the risk of human breast cancer; (3) Follow-up studies of women with breast cancer have consistently demonstrated that nonselective COX-2 inhibitors significantly reduce recurrence risk and breast cancer mortality; (4) Molecular investigations show that COX-2 expression is a characteristic feature of premalignant mammary neoplasms and ductal carcinoma *in situ*; (5) Molecular investigations show that COX-2 expression is a characteristic feature of invasive breast cancer and expression tends to intensify with stage at detection and cancer progression and metastasis; (6) All essential features of carcinogenesis (mitogenesis, mutagenesis, angiogenesis, reduced apoptosis, metastasis, and immunosuppression) are linked to COX-2-driven PGE-2 biosynthesis; and (7) Most notably, upregulation of COX-2 and PGE-2 expression induces transcription of CYP-19 and aromatase-catalyzed estrogen biosynthesis by the mammary adipose tissue which stimulates unbridled mitogenesis of ductal epithelium, and the extrahepatic mammary enzyme, CYP-1B1, converts paracrine estrogen to carcinogenic quinones that have potent mutagenic impact.

This review documents compelling evidence that mammary carcinogenesis often evolves as a progressive series of highly specific cellular and molecular changes in response to induction of constitutive over-expression of COX-2 and the prostaglandin cascade in the “inflammogenesis of cancer”. Based upon results, a general model of inflammogenesis of cancer is proposed involving induction of constitutive COX-2 expression and upregulation of the prostaglandin cascade.

It is emphasized that encouraging results regarding the chemopreventive and therapeutic effects of both selective and non-selective COX-2 inhibiting agents against cancer of the breast as well as other malignant neoplasms have been tempered by concerns about cardiovascular risk associated with taking compounds that inhibit COX-2^[10]. For example, data from randomized trials suggests that high dosages of some NSAIDs or combinations of such drugs may increase the risk of cardiovascular disease^[176]. Before recommendations can be made, more studies are needed to determine if certain COX-2 inhibiting drugs can be taken at dosages that prevent cancer without increasing the risk of heart conditions.

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WJCO 5th Anniversary Special Issues (2): Breast cancer

Caring for the breast cancer survivor's health and well-being

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Abstract

The breast cancer care continuum entails detection, diagnosis, treatment, and survivorship. During this time, focus on the whole woman and medical concerns beyond the breast cancer diagnosis itself is essential. In this comprehensive review, we critically review and evaluate recent evidence regarding several topics pertinent to and specific for the woman living with a prior history of breast cancer. More specifically, we discuss the most recent recommendations for contraceptive options including long-acting reversible contraception and emergency contraception, fertility and pregnancy considerations during and after breast cancer treatment, management of menopausal vasomotor symptoms

and vulvovaginal atrophy which often occurs even in young women during treatment for breast cancer. The need to directly query the patient about these concerns is emphasized. Our focus is on non-systemic hormones and non-hormonal options. Our holistic approach to the care of the breast cancer survivor includes such preventive health issues as sexual and bone health, which are important in optimizing quality of life. We also discuss strategies for breast cancer recurrence surveillance in the setting of a prior breast cancer diagnosis. This review is intended for primary care practitioners as well as specialists caring for female breast cancer survivors and includes key points for evidence-based best practice recommendations.

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Key words: Breast cancer; Contraception; Vasomotor symptoms; Vulvovaginal atrophy; Sexual health; Osteopenia; Osteoporosis; Breast cancer recurrence; Breast cancer surveillance

Core tip: Caring for women with breast cancer at the time of diagnosis, during, and after treatment goes well beyond addressing the breast cancer alone. Holistic care includes safe, effective and convenient contraceptive options; local progestin intrauterine contraception may present an option for some breast cancer survivors. Discussion regarding the effects of chemotherapy on future fertility and pregnancy is an important part of survivorship care for women in child-bearing years. Addressing the effects of breast cancer treatment including vasomotor symptoms, vulvovaginal atrophy and sexual well-being is vital for all breast cancer survivors, but especially important in the setting of adjuvant therapy with aromatase inhibitors.

Casey PM, Faubion SS, MacLaughlin KL, Long ME, Pruthi S. Caring for the breast cancer survivor's health and well-being. *World J Clin Oncol* 2014; 5(4): 693-704 Available from: URL:

INTRODUCTION

Breast cancer is the most common cancer diagnosis in women, and one in eight women will be diagnosed with breast cancer during her lifetime^[1]. Treatment, both surgical and chemotherapeutic, has evolved from the Halsted radical mastectomy to less invasive surgical techniques, combination treatment with focused radiation, and more effective adjuvant therapeutic options including tamoxifen and aromatase inhibitors (AIs)^[2]. These advances in early detection and effective therapies have led to a growing number of cancer survivors worldwide. As vital as these interventions are for a woman's ultimate survival, the overall quality of life of a woman with breast cancer encompasses additional issues to be addressed by the health care provider caring for her during and after the diagnosis and treatment. These issues are often only elicited with compassionate, yet direct inquiry. Adequately addressing these concerns may ultimately make a significant difference in the general health, adherence to recommended therapy, and overall well-being of the woman and her loved ones. In this review, we focus on issues of contraceptive options, fertility and pregnancy after breast cancer treatment, management of bothersome vasomotor symptoms (VMS), vulvovaginal atrophy (VVA) and other sexual health issues, prevention of bone loss, and evidence-supported surveillance for breast cancer recurrence. Ideally, coordination of care with specialists in oncology, reproductive endocrinology, women's health, breast health, gynecology, and primary care to address psychological and medical needs can provide a sense of a "medical home" extending beyond a woman's cancer diagnosis, and can contribute to her overall quality of life.

SURVEILLANCE AFTER BREAST CANCER

Breast cancer survival has been attributed to advances in screening mammography and adjuvant therapy. In the United States, this survival benefit has led to a growing population of women who are living with a history of breast cancer^[3]. With the new focus on patient centeredness, a multidisciplinary approach is the expectation for survivorship care in order to best address the woman's needs. A survey study demonstrated that breast cancer survivors reported a high rate of distress, neuropathy, chest wall, and arm pain. The majority of survivors stated that their medical needs were met, but only 49% reported that their psychological and spiritual needs were met following completion of cancer treatment^[4]. Surveillance to monitor for recurrence, management of treatment related adverse effects, and promotion of preventive and general health care are all integral components of improving quality of care of breast cancer survivors. The Institute of Medicine published a report in 2005, "From

Cancer Patient to Cancer Survivor: Lost in Transition," which was instrumental in promoting awareness of the importance of standardization of survivorship care, development of cancer treatment plans, and improvement in the quality of care of breast cancer survivors^[5].

The American Society of Clinical Oncology (ASCO) has outlined evidence-based recommendations for survivorship care^[6]. A study comparing intensive *vs* standard surveillance for early-stage breast cancer demonstrated no difference in the disease-free or overall survival^[7]. Recommendations for follow-up care after breast cancer include taking a history of symptoms and performing a physical examination every 3-6 mo for 3 years, then every 6-12 mo for 2 years, and then annually. Women are encouraged to perform monthly breast awareness and promptly report new findings to their health care provider. Breast imaging includes an annual mammogram for women with remaining breast tissue. Routine laboratory testing and radiologic studies are not recommended. Preventive health and screening guidelines for other cancers should follow average-risk recommendations. Women are advised to maintain a healthy lifestyle with regular exercise, avoidance of alcohol, and maintenance of a healthy weight^[8]. Those with a hereditary predisposition for breast cancer and those with a known breast cancer mutation are advised to have an annual breast MRI in conjunction with mammography^[9].

Adjuvant hormonal therapy has been shown to decrease breast cancer recurrence for hormone-dependent breast cancer^[10]. Both AIs and tamoxifen are typically prescribed for 5 years for estrogen receptor-positive breast cancer. There is further evidence that longer therapy is beneficial for estrogen receptor-positive disease. A recent large study, the Adjuvant Tamoxifen: Longer Against Shorter (ATLAS) trial, comparing 5 years *vs* 10 years of tamoxifen demonstrated a further reduction in recurrence and mortality after 10 years of tamoxifen in women with early-stage estrogen receptor-positive breast cancer^[11].

However, common side effects of antiestrogen therapies such as exacerbation of VMS, vaginal dryness, vaginal bleeding or spotting, and arthralgias can negatively impact quality of life for many women. These adverse effects can result in early discontinuation and nonadherence to adjuvant hormonal therapy^[12]. Clinicians can be proactive in assessing and counseling patients experiencing medication-related side effects. Various management options are available to provide relief of bothersome symptoms and evaluation of worrisome findings, such as postmenopausal bleeding in the setting of tamoxifen therapy, and can improve therapy adherence and survival.

MANAGEMENT OF VASOMOTOR SYMPTOMS

Vasomotor symptoms are among the most common bothersome symptoms associated with the menopausal transition, occurring in up to 80% of women^[13]. Though the experience of VMS varies, recent evidence suggests

that VMS begin before the final menstrual period and may last for over a decade^[14]. Further, up to 10% of women in a Scandinavian study continued to experience VMS well into their 70s^[15]. Women undergoing treatment for breast cancer may also experience VMS as a consequence of therapy, specifically tamoxifen or AIs^[16].

Hormone therapy (HT) whether estrogen alone, estrogen plus progestin, or progestin alone effectively treats VMS^[17,18]. However, systemic hormone therapy has been associated with an increased recurrence risk in breast cancer survivors in some but not all studies^[19,20]. In a review of 15 studies between 1967 and 2001 by Batur *et al.*^[21], menopausal HT (estrogen plus progestin in 14 of the 15) was not associated with increased cancer recurrence, cancer-related mortality, or total mortality. Nonetheless, synthetic progestins demonstrate proliferative effects in the breast and may augment carcinogenesis by stimulating conversion of differentiated cancer cells to cancer stem cells^[22]. In addition, the 13-year follow-up of the Women's Health Initiative showed increased risk of breast cancer after approximately 5 years of therapy in the estrogen plus progestin group and not in the estrogen alone group, adding to the concern that certain progestins may increase breast cancer risk^[22,23]. There is no current data to support differential management of VMS for women with different receptor-positive tumor types (*i.e.*, ER, PR, and HER-2neu).

In short, women on HT diagnosed with breast cancer need to discontinue therapy. There is no evidence-based guidance as to whether a taper is preferable to abrupt discontinuation, though a slow taper may be preferable based on expert opinion^[24]. Approximately 50% of women who discontinue HT will experience recurrence of VMS^[25].

NONHORMONAL TREATMENT OPTIONS FOR VASOMOTOR SYMPTOMS

Given the safety concerns with HT use in breast cancer survivors, nonhormonal treatments are often considered^[26]. Lifestyle modifications for management of VMS are recommended as first-line interventions. They include avoidance of triggers (caffeine, alcohol, tobacco, warm beverages, spicy foods), dressing in layers, and the use of cooling or wicking clothing and bed linen. The results of studies regarding soy, exercise, and acupuncture have been mixed^[27-29], whereas paced respirations may be of some benefit for VMS management^[30,31]. Caution should be exercised with nonprescription products claiming efficacy for VMS, as robust scientific evidence is lacking. Further, these products are not regulated by the Food and Drug Administration (FDA) raising questions about safety.

A 2010 Cochrane review of 16 randomized controlled trials of nonhormonal interventions for VMS management in breast cancer survivors showed a mild-to-moderate effect with selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors

(SNRIs), gabapentin, clonidine, and relaxation therapy^[32]. Limitations of existing studies include significant placebo effect, differing measures of efficacy, and in many, short duration of treatment. Areas of uncertainty are potential long-term drug effects, optimal duration of therapy, and symptom recurrence upon discontinuation^[31].

Gabapentin has been shown to reduce VMS in breast cancer survivors^[33,34]. However, extended-release gabapentin did not meet FDA approval for this indication in 2013, due to concerns regarding marginal effectiveness and adverse effects such as dizziness.

Low-dose mesylate salt of paroxetine (Brisdelle 7.5 mg) modestly reduces VMS frequency in clinical trials and is the first FDA-approved nonhormonal option for VMS management^[35]. In a recent year-long randomized, double-blind, placebo-controlled trial, desvenlafaxine (Pristiq) was found to be associated with a statistically significant and clinically meaningful VMS reduction^[36]. However, desvenlafaxine did not receive FDA approval for this indication in 2011 due to concerns regarding the risk-benefit profile.

Sexual side effects of antidepressants, including difficulty with arousal and orgasm, are associated with many antidepressants with some exceptions (bupropion and mirtazapine)^[37]. In fact, bupropion has been used in the setting of sexual side effects associated with other antidepressants, though it has not been studied specifically in women with breast cancer^[38].

In breast cancer patients, an interaction between tamoxifen and SSRIs that strongly inhibit cytochrome P450 2D6 (CYP2D6) may exist, potentially reducing endoxifen, the bioactive form of tamoxifen. Newer evidence is concerning for a potential correlation between tamoxifen efficacy or risk of breast cancer recurrence with an inherited variant of the CYP2D6 allele or with the use of medications which may inhibit the CYP2D6 enzyme^[39]. Therefore, caution is currently advised when using strong inhibitors of CYP2D6 in breast cancer patients on tamoxifen.

Though some data suggest short-term improvement in VMS with stellate ganglion block in breast cancer survivors, results have been mixed and further investigation is needed^[40,41].

Providers may choose a VMS management option based on potential side effects, medical comorbidities, and patient preference. For example, if a patient suffers from migraine, gabapentin may be reasonable. If a mood disorder is present, an antidepressant may be most appropriate.

SEXUAL HEALTH CONCERNS IN THE BREAST CANCER SURVIVOR

While sexual health is increasingly identified as an important issue in cancer survivors, providers don't always query patients about sexual function, and patients are often reluctant to bring up concerns, fearing nothing can be done or dissuaded by provider discomfort^[42]. Most

patients are, however, interested in discussing sexual function and want their providers to broach the topic. Survivors of breast cancer face well-described sexual health challenges, including changes in body image, loss of fertility, impairment of relationships, lack of libido, VVA, and dyspareunia. Both the experience of cancer and its treatment can have a significant impact on sexual functioning. Chemotherapy-induced ovarian insufficiency (or risk-reducing oophorectomy) as well as medications used to treat or control disease may result in hormonal loss or blockade, potentially leading to changes in libido, VVA, dyspareunia, and decreased quality of life^[43].

MANAGEMENT OF VULVOVAGINAL ATROPHY IN THE BREAST CANCER SURVIVOR

In contrast with VMS, which tend to improve with time, symptoms associated with VVA progress over time. In women with VVA, meticulous vulvar care is important, and products with perfumes or dyes should be avoided (toilet tissue, soaps, fabric softeners, stimulating lubricants, vaginal hygiene products)^[44]. Vaginal moisturizers can be used on a regular basis to replace vaginal moisture, whereas lubricants are needed with sexual activity to reduce friction^[45]. First-line therapies for VVA include not only vaginal moisturizers and lubricants, but also regular sexual activity (with partner, device, or solo) to stimulate blood flow to the area. Though data is lacking, expert opinion favors proactively educating postmenopausal women about vulvovaginal health in order to preserve sexual function^[46]. This concept is perhaps even more pertinent for the breast cancer survivor. Pelvic floor physical therapy may be utilized to facilitate pelvic floor relaxation, and may include education, relaxation techniques of the pelvic floor muscles, deep breathing, biofeedback, and instruction on the use of lubricated vaginal dilators in graduated sizes to gently stretch vaginal tissues^[47]. Psychosocial interventions, including individual or couple counseling, cognitive behavioral therapy, and mindfulness-based interventions are utilized in breast cancer survivors to improve coping strategies. These also serve to address a number of concerns that impact sexuality including altered body image, anxiety, fear of recurrent disease, changes in relationships, and dyspareunia, though supportive evidence is sparse^[43].

In particular, breast cancer survivors on AI therapy experience profound estrogen deprivation, vaginal dryness, and dyspareunia^[48]. A few small studies have demonstrated significant increases in plasma estradiol concentrations in postmenopausal breast cancer patients on AIs treated with local vaginal estrogen therapy (LVET). However, no studies to date have revealed an increased risk of breast cancer recurrence with LVET use^[49]. It is becoming increasingly evident that severe VVA can have a negative impact on quality of life and may result in medication noncompliance. LVET for severe symptoms

is available in ring, cream, or tablet form, but the long-term safety and systemic absorption from these various preparations are still largely unknown. Newer evidence has demonstrated that serum pharmacokinetics can be used to determine the maximum annual dose delivered and serum estradiol levels of various LVETs. Vaginal estrogen tablets (10 µg) prescribed twice a week demonstrated the lowest annual delivered systemic dose as compared to other LVET^[44]. Providers should be aware of barriers to breast cancer treatment adherence and take into account individual patient's symptom severity, preference, and potential cancer recurrence risk when considering LVET.

A trial utilizing topical testosterone vaginal cream in 20 breast cancer patients on AIs revealed improvements in vaginal dryness, dyspareunia, and vaginal maturation index (VMI) without a change in estradiol levels. The significance of increased reported testosterone levels is unclear^[50]. Likewise, intravaginal dehydroepiandrosterone (DHEA) has been associated with improvements in bothersome vaginal symptoms, VMI, and vaginal pH without significant changes in serum estrogen or androgen levels^[51]. A trial of vaginal DHEA in 465 breast cancer survivors with VVA is underway, with results expected in 2014^[52].

Ospemifene is a selective estrogen receptor modulator (SERM) FDA-approved for treatment of moderate-to-severe dyspareunia. While it has demonstrated antiestrogenic effects in preclinical models of breast cancer, it has not been studied in breast cancer survivors and is not approved for use in this population or in those at high risk of breast cancer^[53].

FERTILITY AFTER BREAST CANCER

While many women are diagnosed with breast cancer after menopause, a 20-year-old woman in the United States has about a 0.5% chance of developing breast cancer in her reproductive years, bringing up the impact of cancer therapy on future fertility, and of pregnancy on cancer recurrence^[54]. In general, the impact of chemotherapy on fertility is related to age-related baseline fertility as well as the type and dose of chemotherapy used^[55,56]. In women who desire pregnancy after breast cancer, fertility preservation should be discussed at the time of diagnosis. While a full discussion is beyond the scope of this review, both embryo and oocyte cryopreservation are considered standard options. With cycle-day-independent ovarian stimulation protocols, the timing of cancer therapy initiation can be minimally affected. In order to reduce potential risks associated with increased estrogen levels at ovarian stimulation, many regimens now utilize letrozole. Gonadotropin-releasing hormone analogs should not be used for fertility preservation due to unproven efficacy^[57].

While women were previously counseled to wait two years prior to conceiving after breast cancer diagnosis, newer published data does not demonstrate an alteration in survival with breast cancer to pregnancy intervals

longer than 10 mo^[58]. Estrogen-receptor status does not impact breast cancer recurrence with pregnancy^[59]. Initial studies noting improved outcome in women who become pregnant after breast cancer were attributed to better baseline status in women who chose pregnancy, the so-called “healthy mother effect”^[60]. A meta-analysis designed to correct for the “healthy mother effect” included over 1000 women with pregnancy after breast cancer diagnosis and over 13000 health-matched controls. This study noted improved survival in women who became pregnant after breast cancer treatment as compared to those who did not, hazard ratio 0.51 [95% confidence interval (CI), 0.42-0.62] suggesting that pregnancy at least 10 mo post breast cancer treatment in women < 45 years does not adversely affect prognosis and may in fact significantly improve survival^[60].

About 50% of women can lactate after breast cancer therapy, but breast milk volume tends to be reduced^[61].

ASSOCIATION BETWEEN HORMONAL CONTRACEPTION AND BREAST CANCER

Though data is difficult to interpret given varying hormonal doses and lengths of follow-up, there is no clear evidence that hormonal contraceptive use (oral contraceptive have been most thoroughly studied), past or present, is associated with a significantly increased breast cancer risk^[62]. Importantly, older studies reporting a weak association contain data pertaining to older, higher dose contraceptive formulations not in use today^[63,64]. Therefore, patients can be reassured of the unlikely contribution of prior hormonal contraception to the breast cancer diagnosis.

CONTRACEPTION OPTIONS DURING BREAST CANCER TREATMENT AND BEYOND

Safe, effective, and convenient contraception should be discussed and made available to all women undergoing diagnosis, treatment, and surveillance for breast cancer. Women not wishing further fertility may consider male or female sterilization with inherent failure rates < 1%^[65,66]. A number of minimally invasive options for sterilization now exist including laparoscopic tubal ligation and hysteroscopic sterilization (Essure). It is important to note that the latter does not have immediate efficacy, and tubal occlusion must be confirmed by hysterosalpingogram 12 wk following the procedure^[67]. Therefore, as with male sterilization, another form of contraception should be used until efficacy can be assured^[65,67].

During the evaluation for breast cancer, a woman on hormonal contraception, including combined hormonal contraception, should continue using this method until she receives appropriate counseling regarding future reproductive goals, an assessment of medical needs beyond the breast cancer, and, most importantly, until a new

method is initiated. The United States Medical Eligibility Criteria for Contraceptive Use (US MEC) considers all hormonal contraception as “advantages generally outweigh theoretical or proven risks” in the setting of an undiagnosed breast mass^[68]. Nonhormonal methods including the copper T380 (CuIUD or ParaGard) and barrier methods have “no restriction in use” in this setting. However, even with perfect use, barrier method alone is unlikely to provide sufficient efficacy and convenience to most women, given its 15% failure rate^[69]. An unplanned pregnancy at a time when breast cancer treatment should be initiated can lead to needlessly difficult choices about pregnancy termination or treatment delay.

The Cu-IUD is the only hormone-free, long-acting reversible contraceptive (LARC) currently available in the United States and has “no restrictions for use” in the setting of current breast cancer^[68]. Pregnancy rates are < 1% per year with this method indicated for up to 10 years^[70]. In addition, a Cu-IUD can be placed at any time that pregnancy can be reasonably ruled out, as well as within 5 d of unprotected intercourse if emergency contraception is desired. Additional back-up contraception is not required after insertion. Further, due to its non-systemic mechanism of action, chemotherapy-associated nausea and vomiting does not alter efficacy.

While the Cu-IUD represents the first-line reversible contraception in women with breast cancer, concurrent medical issues such as endometrial proliferation on tamoxifen, anemia, menorrhagia, and dysmenorrhea may warrant consideration of local progestin therapy, especially in women with hormone receptor-negative tumor types. These considerations need to be balanced with US MEC rating of “unacceptable health risks, method not to be used” for all hormone-containing contraceptives. The newer 13.5 mg levonorgestrel releasing system (low dose LNG-IUS) (Skyla) has lower circulating levels than the 52 mg LNG-IUS (Mirena) used in prior studies of LNG-IUS and breast cancer^[71-74]. In addition to being an extremely effective reversible contraception that is effective for up to 3 years, low dose LNG-IUS may improve anemia, menorrhagia, and dysmenorrhea that can be associated with Cu-IUD, while exposing a woman to lower circulating levels of levonorgestrel than LNG-IUS (Mirena). However, there is currently no evidence regarding low dose LNG-IUS efficacy for menorrhagia treatment or its safety in the setting of breast cancer.

A small case-control study of women using LNG-IUS compared 79 women, who started or continued using LNG-IUS after diagnosis of breast cancer, and 120 controls. While there was no increased risk of recurrence overall, there was concern about the 3.39 adjusted hazard ratio of breast cancer recurrence (95%CI, 1.01-11.35) in a subgroup who developed breast cancer while using LNG-IUS and continued its use^[74]. This finding contrasts with population data that has not found an increased risk of breast cancer in LNG-IUS users as compared to Cu-IUD users in 5100 breast cancer patients and 20000 controls^[73].

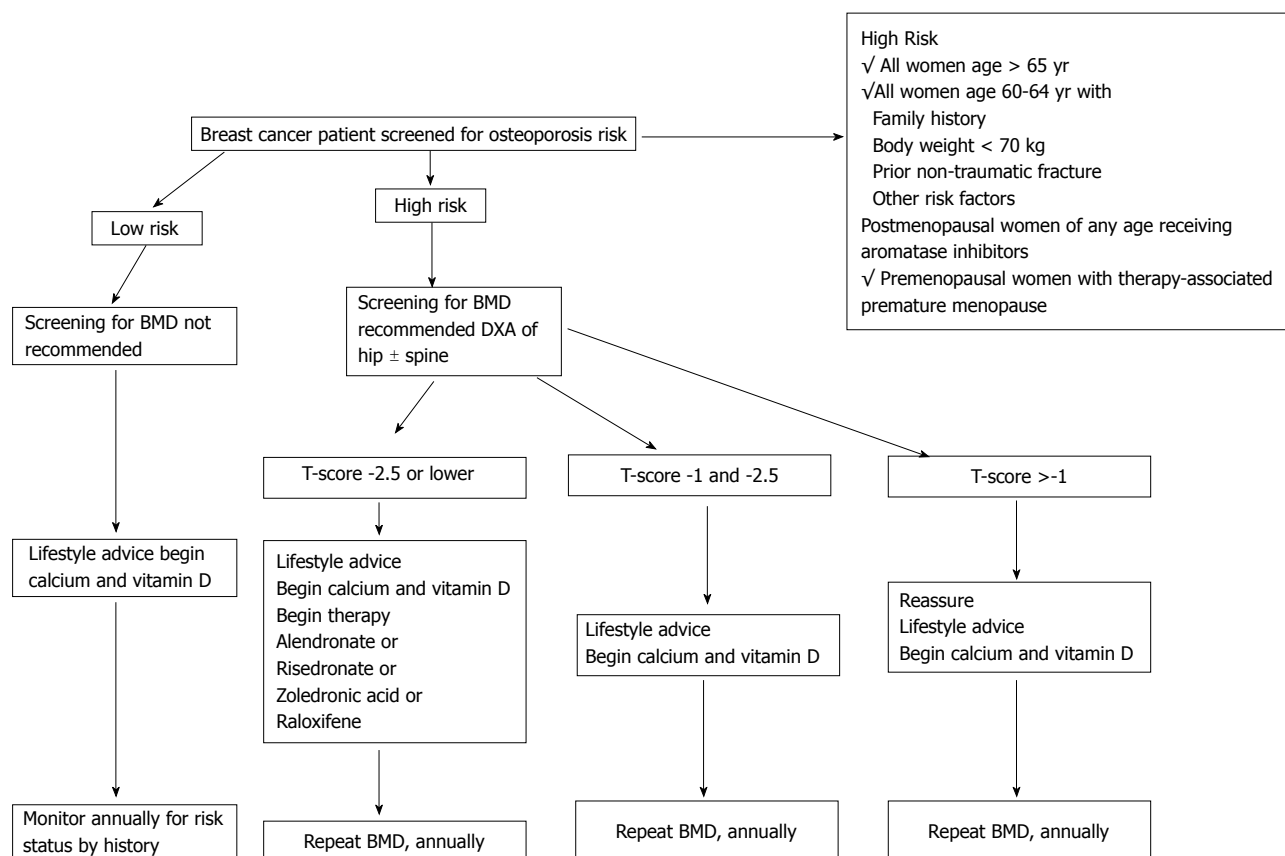


Figure 1 Recommended management strategy for patients with diagnosed nonmetastatic breast cancer^[97]. This management strategy is largely based on influence from results in non-breast cancer populations. BMD: Bone mass density; DXA: Dual energy X-ray absorptiometry bone scan. Reprinted with permission. © (2003) American Society of Clinical Oncology. All rights reserved.

LNG-IUS is indicated for the treatment of menorrhagia and can decrease menstrual blood loss by up to 90%^[75]. This is compared with a 40% reduction in menstrual blood loss with antifibrinolytics and about 25% with NSAIDs^[76,77]. Unfortunately, there is virtually no data on the contraceptive implant (Implanon, Nexplanon) in the setting of breast cancer^[78]. As with all other hormonal LARC methods, the US MEC rates the implant as “unacceptable health risk, method should not be used” with current breast cancer and as “theoretical or proven risks usually outweigh the advantages” after 5 years without breast cancer recurrence^[68].

When determining the need for contraception after breast cancer diagnosis, amenorrhea and elevated gonadotropins such as follicular stimulating hormone (FSH) are unreliable markers of infertility in women who have received chemotherapy^[55,79]. The incidence of chemotherapy-induced amenorrhea is reported to be 53%-89%, and is more likely to be reversible in women under 40 years than older women^[80].

EMERGENCY CONTRACEPTION

The World Health Organization (WHO) has determined that there is no medical condition wherein the risks of emergency contraception outweigh its benefits^[81]. There-

fore emergency contraception should be available to women diagnosed with and undergoing treatment for breast cancer.

The Cu-IUD can serve as 96% effective emergency contraception if inserted within 5 d post-unprotected intercourse, and can remain as primary hormone-free, yet reversible contraception for up to 10 years. In one study, over 80% of women who received Cu-IUD for emergency contraception continued using it for primary contraception thereafter^[82].

Additional emergency contraception methods include levonorgestrel (LNG or PlanB one step) and Ulipristal (UPA or Ella)^[83,84]. Plan B is now available in the United States without a prescription regardless of patient age, and is indicated for use within 72 h of unprotected intercourse^[84]. An efficacy rate of 85% has been reported. LNG alone is more effective than the combined Yuzpe regimen of oral contraceptives and is associated with fewer side-effects^[85]. UPA is indicated in a single dose for emergency contraception up to 5 d post-unprotected intercourse^[83]. Lower failure rates than LNG have been reported with UPA, (OR, 0.35, 0.58, 0.55 at 24, 72, and 120 h respectively)^[86]. Differences in efficacy of emergency contraception have been reported in women with normal vs elevated body mass index (BMI). As compared to women with a BMI < 25 kg/m², women with a BMI of

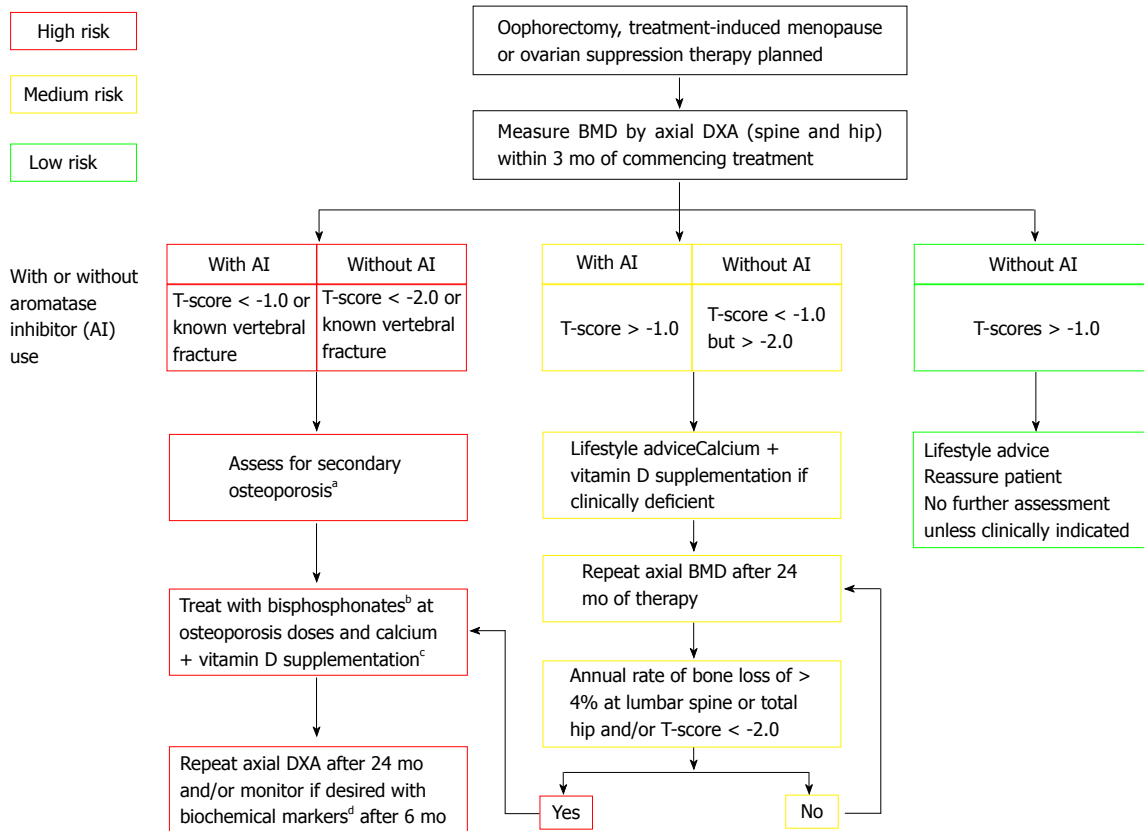


Figure 2 Adjuvant treatment associated with ovarian suppression/failure with or without concomitant aromatase inhibitor use in women who experience premature menopause^[92]. ^aErythrocyte sedimentation rate, full blood count, bone and liver function (calcium phosphate, alkaline phosphatase, albumin, AST/ALT), serum creatinine, endomysial antibodies, serum thyroid stimulating hormone; ^bAlendronate 70 mg per week, risedronate 35 mg per week, ibandronate (150 mg po monthly or 3 mg iv 3-monthly), zoledronic acid 4 mg iv 6-monthly; ^cTo be given as ≥ 1 g of calcium + ≥ 800 IU of vitamin D; ^dBiochemical markers such as serum C-terminal telopeptide of type I collagen or urinary N-telopeptide of type I collagen. Reprinted with permission. © (2008) Elsevier.

25–29 kg/m² had a 1.5-fold increased risk of pregnancy whereas those with a BMI > 30 kg/m² had a 3.6-fold increased risk with LNG EC *vs* UPA^[86]. Therefore, UPA or Cu-IUD, which do not have BMI-related efficacy differences, should be considered over LNG in women with elevated BMI.

BONE HEALTH

Bone health has been increasingly recognized as a significant issue for breast cancer survivors from the standpoint of osteoporosis prevention as well as its diagnosis and treatment^[87–89]. A recently published survey study found that women aged 65 and older with a breast cancer diagnosis had a higher prevalence of osteoporosis and falls. However, their risk was not more likely to have been identified by their health care provider, and bone health or fall prevention discussed^[90].

There are multiple mechanisms by which breast cancer treatment impacts bone health. Primary ovarian insufficiency or premature menopause often results from treatment with gonadotropin-releasing hormone agonists or chemotherapeutic agents in previously premenopausal women and increases risk of osteoporosis^[88]. The use of antiestrogen therapies can cause estrogen deficiency resulting in bone loss and reduced bone integ-

egrity^[91]. Tamoxifen has different effects on bone in pre-*vs* postmenopausal women. In premenopausal women, tamoxifen has been shown to cause a 1%–2% bone loss over 1–2 years, but experts note that this is not a clinically significant change, and that monitoring or treatment solely based on tamoxifen use is not indicated. In contrast, tamoxifen is associated with increased bone density in postmenopausal women^[92]. Of greater concern for a negative impact on bone health and fracture risk is the use of AIs. AIs are used in postmenopausal women with hormone receptor-positive breast cancer to reduce recurrence risk with a demonstrated survival benefit^[93,94]. However, AIs result in substantial reduction in estrogen production and estradiol levels, and are associated with decreased bone mineral density (BMD) and higher rate of fracture^[95].

Strategies for prevention of bone loss in all women, including those receiving antiestrogen therapies, include counseling on the importance of adequate calcium (1200 mg per day) and vitamin D (800–1000 IU per day) through diet or supplement, regular exercise including both weight-bearing and muscle strengthening, advice on fall prevention, smoking cessation, and avoidance of excess alcohol^[96].

In 2003 the ASCO published an algorithm (Figure 1) for screening and treatment specifically for breast cancer

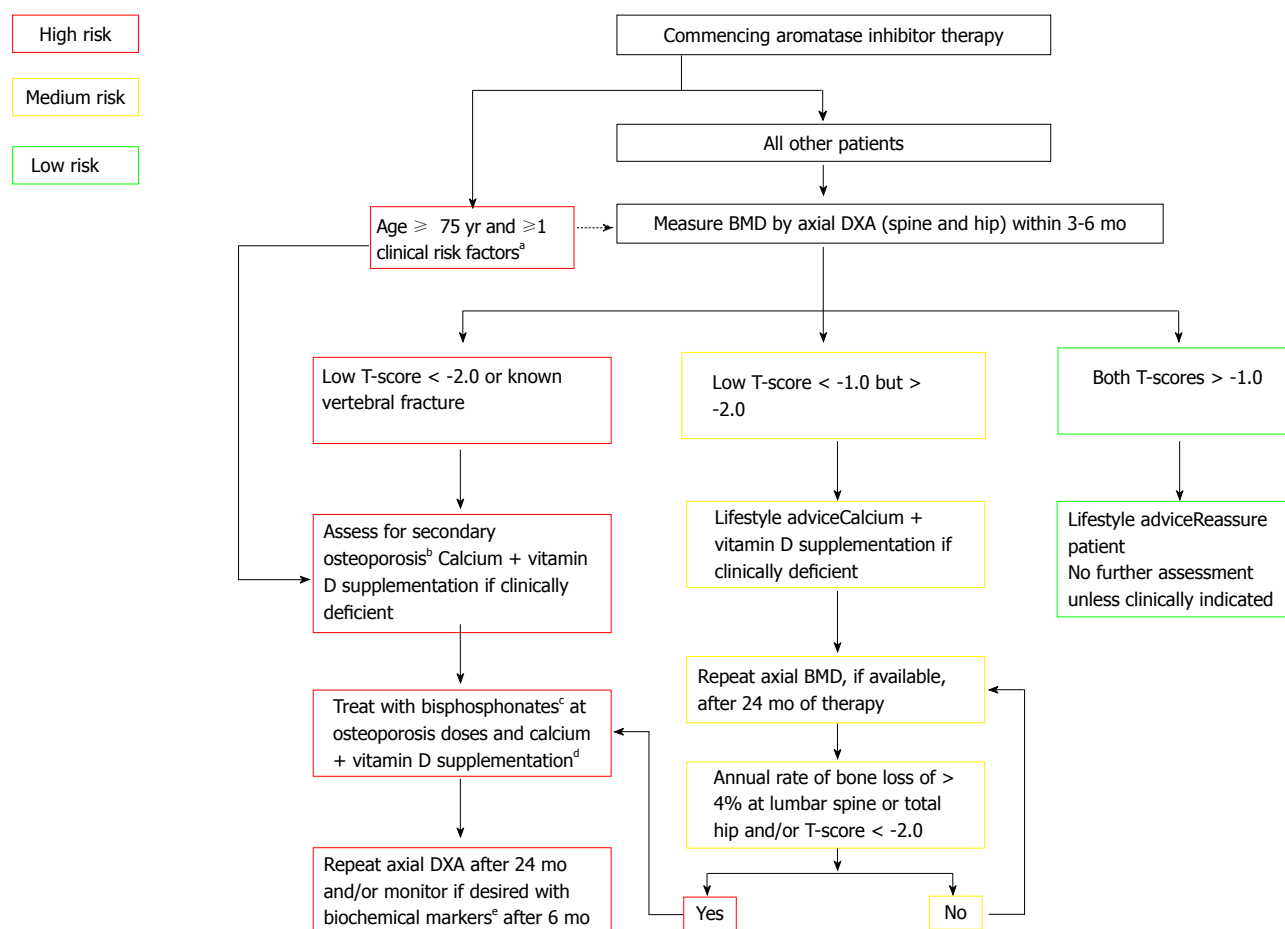


Figure 3 Postmenopausal adjuvant treatment with aromatase inhibitors^[92]. ^aPrevious low-trauma fracture after age 50, parental history of hip fracture, alcohol intake of ≥ 4 units/day, diseases associated with secondary osteoporosis, prior corticosteroids for > 6 mo, low BMI (< 22); ^bErythrocyte sedimentation rate, full blood count, bone and liver function (calcium phosphate, alkaline phosphatase, albumin, AST/ALT), serum creatinine, endomysial antibodies, serum thyroid stimulating hormone; ^cAlendronate 70 mg per week, risedronate 35 mg per week, ibandronate (150 mg po monthly or 3 mg iv 3-monthly), zoledronic acid 4 mg iv 6-monthly; ^dTo be given as ≥ 1 g of calcium + ≥ 800 IU of vitamin D; ^eBiochemical markers such as serum C-terminal telopeptide of type I collagen or urinary N-telopeptide of type I collagen. Reprinted with permission. © (2008) Elsevier.

patients that recommended BMD screening annually for all postmenopausal women on AIs and for premenopausal women with treatment-induced premature menopause, as well as for all breast cancer patients aged 65 years and older or those aged 60-64 years with risk factors. The treatment guideline at that time only called for bisphosphonate or raloxifene for women with a T-score of -2.5 or lower^[97]. A consensus statement from a UK Expert Group in 2008 divided that algorithm into specific guidelines for formerly premenopausal women with treatment-induced premature menopause (defined in the publication as < 45 years old) and for postmenopausal women treated with AIs (Figures 2 and 3). For women with continued menstruation or postmenopausal women older than 45 years old and either on tamoxifen or not on AIs, no specific recommendation is given for screening. Any woman with a T-score < -2.0 or with a history of vertebral fracture is advised to have evaluation for secondary causes of osteoporosis^[92].

For breast cancer patients with treatment-induced early menopause < 45 years old, a BMD measurement by spine and hip dual energy X-ray absorptiometry (DXA)

is recommended. If a woman not on an AI had a T-score < -2.0 , bisphosphonate treatment is recommended with a follow up DXA in 24 mo. With a score of -1.0 or higher, no further testing is indicated. If the T-score is between -1.0 and -2.5 , a repeat DXA in 24 mo is advised. For the woman on AI, the threshold for treatment drops to a T-score of < -1.0 with repeat DXA recommended at 24 mo post bisphosphonate initiation. For a woman on AI and in premature menopause at < 45 years old, a T-score > -1.0 results in a recommendation for DXA in 24 mo^[92].

For postmenopausal women starting AI therapy, DXA of the spine and hip is recommended. If the T-score is < -2.0 or if the woman is 75 years or older with any osteoporosis risk factors, a bisphosphonate is recommended with follow-up DXA in 24 mo. Similar to the algorithm for breast cancer patients with early menopause, a T-score of -1.0 or higher requires no further screening unless indicated by a change in the clinical situation. For a T-score between -1.0 and -2.5 , a follow-up DXA in 24 mo is recommended^[92].

All breast cancer patients with known vertebral fractures should be considered for treatment per the UK

Expert Group guidelines. Another indication for treatment is an annual bone loss rate of > 4% in both the premature menopause and postmenopausal AI-treated groups^[92].

CONCLUSION

In focusing on the woman undergoing treatment and surveillance for breast cancer, attention to a number of concurrent issues beyond the breast cancer itself will impact her satisfaction with treatment and overall care. Appropriate counseling and evidence-supported surveillance strategy along with age-appropriate testing will promote overall health and perhaps a sense of some control over her care. Careful attention to medication-related side effects including chemotherapy-induced VVA with resultant dyspareunia can certainly affect her well-being and relationship with her partner. The availability of nonhormonal treatment options for VVA and VMS can help her focus on her recovery. If future fertility is a concern, she should ideally be evaluated by a reproductive endocrinologist prior to initiation of chemotherapy and counseled regarding fertility preservation options. Pregnancy is generally not advised for at least 10 mo after breast cancer therapy, but advancing maternal age and other factors need to be considered in individual counseling. If contraception is desired, women should be counseled about both reversible and permanent hormone-free options, but they should not discontinue current contraception until the initiation of an alternate method. Chemotherapy-induced amenorrhea should not be considered permanent ovarian insufficiency, especially in women younger than 40 years. Likewise, elevated FSH in this setting is not an indicator of permanent ovarian insufficiency. Attention to bone health is important in breast cancer survivors, particularly in the context of chemotherapy-induced premature menopause or AI use. A multidisciplinary, comprehensive, and holistic approach to the woman with breast cancer can facilitate her transition from breast cancer patient to breast cancer survivor.

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WJCO 5th Anniversary Special Issues (2): Breast cancer

Phytoestrogens and prevention of breast cancer: The contentious debate

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Abstract

Phytoestrogens have multiple actions within target cells, including the epigenome, which could be beneficial to the development and progression of breast cancer. In this brief review the action of phytoestrogens on oestrogen receptors, cell signalling pathways, regulation of the cell cycle, apoptosis, steroid synthesis and epigenetic events in relation to breast cancer are discussed. Phytoestrogens can bind weakly to oestrogen receptors (ERs) and some have a preferential affinity for ER β which can inhibit the transcriptional growth-promoting activity of ER α . However only saturating doses of phytoestrogens, stimulating both ER α and β , exert growth inhibitory effects. Such effects on growth may be through phytoestrogens inhibiting cell signalling pathways. Phytoestrogens have also been shown to inhibit cyclin D1 expression but increase the expression of cyclin-dependent kinase inhibitors (p21 and p27) and the tumour suppressor gene p53. Again these effects are only observed at high (> 10) μ mol/L doses of phytoestrogens. Finally the effects of phytoestrogens on breast cancer may be mediated by their ability to

inhibit local oestrogen synthesis and induce epigenetic changes. There are, though, difficulties in reconciling epidemiological and experimental data due to the fact experimental doses, both *in vivo* and *in vitro*, far exceed the circulating concentrations of "free" unbound phytoestrogens measured in women on a high phytoestrogen diet or those taking phytoestrogen supplements.

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Key words: Phytoestrogens; Breast cancer; Cell signalling; Cell cycle; Epigenomics

Core tip: Phytoestrogens have multiple actions within target cells, including the epigenome, which could be beneficial to the development and progression of breast cancer. In this brief review the action of phytoestrogens on oestrogen receptors, cell signalling pathways, regulation of the cell cycle, apoptosis, steroid synthesis and epigenetic events in relation to breast cancer are discussed. The difficulties in interpreting experimental evidence relating to the beneficial effects of phytoestrogens in light of dietary/supplementary intake and bioavailability of ingested phytoestrogens is also addressed.

Bilal I, Chowdhury A, Davidson J, Whitehead S. Phytoestrogens and prevention of breast cancer: The contentious debate. *World J Clin Oncol* 2014; 5(4): 705-712 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i4/705.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i4.705>

INTRODUCTION

Phytoestrogens are naturally occurring plant compounds which are structurally similar to oestrogens and can have weak oestrogenic actions. There are six major classes

Table 1 Major classes of phytoestrogens and their common dietary sources

Flavonoids	Isoflavonoids	Lignans	Coumestans	Stilbenes
Apigenin Quercetin Narigenin Catechins Red/yellow fruits and vegetables, tea	Genistein Biochanin A Daidzein → equol Soy beans, soy foods, vegetables	Enterodiol Enterolactone Flaxseed, whole grains, fruit, vegetables	Coumesterol Peas, beans, alfafa, sunflower seeds	Resveratrol Red wine

of phytoestrogens, all of which have distinct common dietary sources (Table 1) but the most intensively investigated phytoestrogens are the isoflavones and the stilbene, resveratrol. The link between breast cancer and phytoestrogens arose from the early epidemiological evidence showing that the incidence of breast cancer is lower in Asian populations who consume high dietary concentrations of soy products which have a high isoflavonone content^[1,2]. This fuelled the widespread belief that consumption of soy foods reduces the risk of breast cancer and other hormone dependent cancers and led to further research on the protective effects of many other phytoestrogens^[3,4].

However reconciling experimental evidence, mainly based on high supraphysiological doses of single phytoestrogens, coupled with the limited bioavailability of orally consumed phytoestrogens has raised questions and concerns about the validity of promoting the health benefits of diets rich in phytoestrogens and/or taking dietary supplements^[5-7]. Furthermore phytoestrogens exert a plethora of actions beyond weak oestrogenic effects (Figure 1) and these include antagonist effects oestrogen receptors (ERs), modulation of cell signalling pathways, regulation of the cell cycle, enzyme inhibition, anti-oxidant properties, angiogenesis and epigenetic alterations^[8]. This review will focus on the action of phytoestrogens on oestrogen receptors and cell signalling pathways, their regulation of the cell cycle and apoptosis, inhibition of steroidogenic enzymes and induced epigenetic changes.

DIETARY INTAKE, METABOLISM AND BIOAVAILABILITY OF PHYTOESTROGENS

In foods phytoestrogens are present as mixtures and are usually found as biologically inactive glycoside conjugates containing glucose or carbohydrate moieties. Blood levels can vary widely between individuals depending both on dietary preferences as well the phytoestrogen content of a particular food product resulting from local and/or seasonal variations^[9]. For example Asian diets can result in isoflavone consumption as high as 50 mg/d compared with 1-3 mg/d for individuals eating a typical Western diet although a vegetarian diet or use of supplements can increase dietary intake to levels of an Asian diet^[10-12] and references therein.

In the gut phytoestrogens are broken down by glucosidases to their respective aglycones allowing more effi-

cient absorption, although intestinal bacteria may further metabolise these products. For example the phytoestrogens genistein and daidzein, can be further metabolised to *p*-ethyl phenol and to equol and/or *O*-desmethyldanglensin (*O*-DMA) respectively though it should be noted that only 30%-50% of the population can produce equol and approximately 80%-90% *O*-DMA^[10,13]. Thus not only will dietary factors contribute to phytoestrogen intake but also individual variations in metabolism.

Once absorbed the aglycone phytoestrogens are rapidly conjugated to glucuronic acid and to a lesser extent sulphuric acid in the hepatic circulation. They are then de-conjugated prior to excretion with urinary concentrations increasing in parallel to consumption^[14]. There is generally very low levels of biologically active 'free' unconjugated phytoestrogens in the circulation (< 3% of the total) and blood levels are in the ng/mL range or lower^[10,15]. One may then argue what is the relevance of *in vitro* studies showing that only high micromolar doses of unconjugated phytoestrogens can inhibit the growth the breast cancer cells, inhibit oestrogen-dependent gene transcription or inhibit cell signalling pathways?^[16,17]. Similarly *in vivo* studies have only shown that dietary supplements far in excess of those consumed with an Asian diet had any effect on inhibiting experimentally-induced tumour growth and even this data is conflicting^[16,18,19] and references therein.

PHYTOESTROGENS, OESTROGEN RECEPTORS AND CELL SIGNALLING PATHWAYS

Two major oestrogen receptors (ERs) have been identified, ER α and ER β , which are encoded by separate genes and have different tissue distributions and roles in gene regulation^[20]. They also have differential effects in oestrogen-sensitive tissue and in breast tissue ER α activation can stimulate proliferation whilst ER β activation can counteract this proliferative effect. This is thought to be mediated by dimerization of ER β with ER α ^[20,21]. In breast tumours the ratio of ER α to ER β is raised and tumour aggressiveness is increased in those that are ER β negative^[21].

The relative binding affinity (RBA) of phytoestrogens to ERs is weak and are in the order of 1000-10000 times less than that of oestradiol although some phytoestrogens

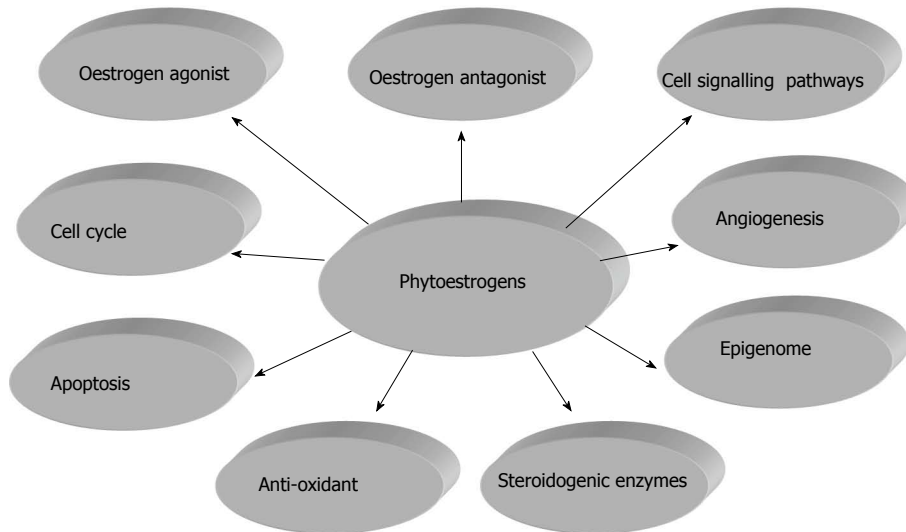


Figure 1 Multiple targets for the action of phytoestrogens.

such as genistein, coumestrol and apigenin have a higher affinity for ER's and their RBAs are in the order of only 10-100 times that of oestradiol^[22]. Interestingly several phytoestrogens such as genistein, daidzein and apigenin have a 9-10 fold increased affinity for ER β than ER α ^[22] and a more recent study showed that after dietary supplementation total genistein and daidzein concentrations were 20-40 fold higher than oestradiol equivalents in breast adipose/glandular tissue^[23]. Thus their ability to preferentially activate ER β and their ability to accumulate in breast tissue may have some clinical significance. That said, the concentrations required to induce apoptosis or at least inhibit cell growth arrest are induced only by over-saturating doses ($\geq 10 \mu\text{mol/L}$) doses of phytoestrogens^[16].

However, phytoestrogens can also act on cell surface oestrogen receptors or interact with growth factor and cytokine signalling pathways (Figure 2). Thus phytoestrogens can modulate the responses to growth factors or activate/inhibit kinases which may alter ligand-independent transcriptional activity of oestrogen receptors or other transcription factors such as AP-1 and NF- κ B^[24]. For example genistein is a tyrosine kinase inhibitor and has been shown to alter the activity/expression of both extracellular regulated kinase (ERK) and the PI-3/Akt pathway as have other phytoestrogens including resveratrol^[25,26]. Long-term treatment of breast cancer cells with dietary levels of genistein (10^{-8} mol/L) have also been shown to down-regulate the expression of Akt^[27]. In context of the growth-repressing effect of ER β and cell signalling, a recent study showed that activation of the MEK 1/2 and PI-3K/Akt pathways inhibited the ER β growth-mediated repression in breast cancer cells^[28]. Thus down regulation of these pathways by long-term dietary phytoestrogens could promote the effectiveness of ER β activation and inhibit proliferation.

PHYTOESTROGENS AS REGULATORS OF THE CELL CYCLE AND APOPTOSIS

Recently much attention has focussed on the action of

phytoestrogens in regulating the expression of proteins regulating the cell cycle and apoptosis (Figure 2). Cyclins are a family of proteins which regulate transition of the cell cycle through the G₁, S, G₂ and metaphase (M) phases and, through coalescing with cyclin-dependent kinases (CDKs), they initiate gene transcription controlling regulation of the cell cycle. Cyclin D1, which regulates the G₁ to S phase of the cell cycle, is the most widely investigated being established as an oncogene and over-expressed in more than 50% breast cancers^[29]. The majority of studies have shown that high concentrations of phytoestrogens ($\geq 10 \mu\text{mol/L}$ range) inhibit the expression of cyclin D1^[30-32] although another study reported a transient increase mimicking the effects of oestradiol^[33] and other studies reported no effects^[34,35].

The activity of cyclin/CDK complexes is regulated by CDK inhibitors (CDIs) and thus these proteins can inhibit the cell cycle^[36,37]. The two most widely investigated CDIs are p21^(CIP1/WAF1) and p27^(Kip1) and the expression of p21 is controlled by the tumour suppressor gene p53^[38] which has many other actions as a tumour suppressor including inducing apoptosis^[39]. High doses of phytoestrogens have been shown to increase the expression/activity of p21, p27 and p53^[33,35,40-43] which parallel changes in the reduction of cyclin D1. Such effects have been seen with high doses of phytoestrogens but a microarray analysis showed that low levels of genistein (1 and 5 $\mu\text{mol/L}$) that stimulated growth of MCF-7 cells had no effect on the expression of p53 target genes such as p21^(CIP1/WAF1) and only at a higher pharmacological dose of genistein (25 $\mu\text{mol/L}$) cell growth was inhibited and increased the expression of p53 target genes. This would result in increased apoptosis and decreased proliferation^[44].

The pro-apoptotic protein, Bax, is regulated positively by p53 whilst the anti-apoptotic protein, Bcl-2, is negatively regulated by p53^[45]. Both *in vivo*^[46-48] and *in vitro* studies^[33,49-52] have shown that phytoestrogens can stimulate apoptosis and increase the Bax/Bcl-2 ratio but evidence as to whether this effect is due to increased or decreased activity of ERK1/2 is controversial^[49-52].

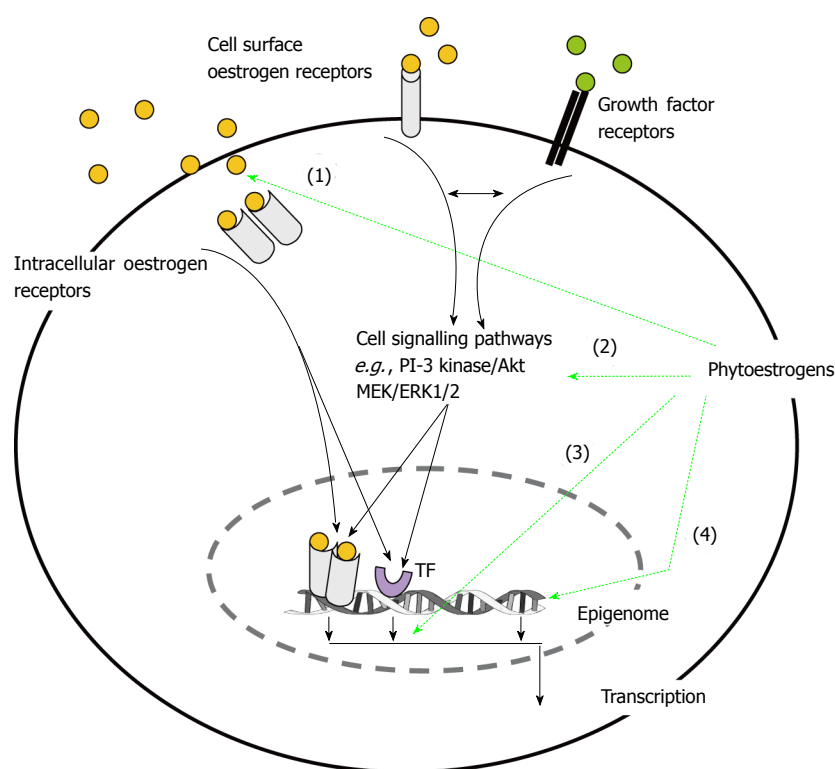


Figure 2 Different ways in which phytoestrogens may alter gene transcription. (1) Acting as an oestrogen agonist/antagonist and the transcriptional activity of oestrogen receptors; (2) Modulating cell signalling pathways which can be activated by cell surface oestrogen receptors or growth factor receptors and ultimately activate gene transcription by activating oestrogen receptors or other transcription factors (TF); (3) Inhibiting/stimulating transcription of genes regulating apoptosis and the cell cycle; and (4) Epigenetic alterations in DNA, histone proteins and RNA to alter transcription/translation of proteins.

PHYTOESTROGENS AS INHIBITORS OF OESTROGEN SYNTHESIS

Low doses of phytoestrogens are generally found to stimulate growth of breast cancer cells with only high supraphysiological doses inhibiting growth and with evidence that they regulate the expression of proteins involved in controlling the cell cycle and apoptosis as opposed to their action as weak oestrogen agonists/antagonists at ER α and ER β . There is, however, evidence that phytoestrogens can also inhibit steroid synthesis and this may be particularly significant in relation to the local production of oestrogens in breast tissue^[53] and references therein. Fatty tissues are a major storage site for phytoestrogens^[13] and the most abundant cells surrounding breast cancer cells are mature adipocytes and progenitors which may be a key component of breast cancer progression by locally affecting breast cancer cell behaviour^[54].

Approximately 60%-70% breast cancers express oestrogen receptors and these are considered to promote tumour growth. Hence initial treatments are directed towards reducing oestrogenic effects by inhibitors of oestrogen receptors and inhibitors of aromatase which converts androgens to active oestrogens^[55]. The incidence of breast cancer increases with age despite the loss of ovarian hormones in post-menopausal women. Peripheral tissue can convert circulating androgens (dehydroepiandrosterone/DHEA) and oestrone sulphate into 17 β -oestradiol (E₂) and in post-menopausal women with breast cancer concentrations of E₂ in the tumour is at least 20-fold higher than in the circulation^[56].

Flavones and isoflavones are the most potent phytoestrogens that inhibit aromatase and the IC₅₀ values are in

the order of 0.1-10 μ mol/L which is more than 100 times higher than the IC₅₀ value for the steroidal inhibitor, 4-hydroxyandrostendione^[53,57]. Isoflavones are generally weak inhibitors of aromatase but like other phytoestrogens can inhibit 17 β -hydroxysteroid dehydrogenase (HSD) type 1 which reduces oestrone (E₁) to E₂ and 17 β -HSD type 5 that converts androstenedione to testosterone, which can subsequently be converted to E₂ by aromatase^[53] and references therein. More recently certain phytoestrogens have been shown to alter the activation of breast cancer-associated aromatase promoters^[58]. Overall, however, the inhibition of these enzymes by unconjugated phytoestrogens are in the order of 1-10 μ mol/L whilst total circulating phytoestrogens are in the low nanomolar range except in vegetarians or those eating a high soy diet where concentrations of 100 nM to 1 μ mol/L may be achieved^[59]. Thus there is another discrepancy between experimental results and levels of phytoestrogens achieved by dietary means. However our study on human granulosa cells showed that low dose mixtures of three isoflavones in the nM range inhibited expression and activity of aromatase though a similar inhibition was only achieved with a single phytoestrogen at 100 times the dosage^[60]. Further studies are required to investigate mixtures of phytoestrogens as occurs through dietary means.

EPIGENETIC MODULATION BY PHYTOESTROGENS

Over the last decade there has been an explosion in the number of studies concerning epigenetic changes and the development and progression of breast cancer^[61] and not surprisingly these have included studies on the ability

of phytoestrogens to alter the epigenome which could be useful in the prevention of cancer^[61-63]. In fact studies have indicated that early childhood exposure to phytoestrogens could protect against breast cancer in later life^[62] and references therein and this could involve epigenetic events (Figure 2). Epigenetic changes are defined as heritable changes in gene expression which do not involve mutations of DNA nucleotide sequences. They include DNA methylation, histone acetylation and microRNAs (miRNAs).

DNA methylation occurs on cytosine in the cytosine-phosphate-guanine (CpG) dinucleotide sequence of genomic DNA, a reaction catalysed by DNA methyl transferases (DNMTs). CpG dinucleotide rich regions (known as CpG islands) are found in the promoter region of approximately 60% of all human genes and, whilst most CpG islands are unmethylated in normal cells, they become hypermethylated in cancerous cells leading to gene suppression, including the tumour-suppressing genes^[61]. Along with DNMTs are the methyl-CpG-binding domain family of proteins which bind to a methylated gene and can inhibit transcriptional activity by altering chromatin structure. Chromatin structure can also be modified by histone acetylation which is catalysed by histone acetylase (HAT) and results in a more open structure of chromatin allowing access for transcription factors to DNA. The reverse occurs when histone proteins become deacetylated and this reaction is catalysed by histone deacetylases (HDACs). Histones may also be methylated by histone methyl transferases (HMT's) and generally methylation causes gene transcription to be switched off. The most recent participant of the epigenetic field are the miRNAs, small non-coding RNAs that inhibit protein expression of target genes by binding to the 3'-untranslated region of mRNA causing degradation or inhibition of mRNA of the target gene^[61,62] and references therein.

The most widely studied dietary components in relation to epigenetic changes are the tea polyphenols, epicatechins and epigallocatechins (EGCCs), the isoflavones, genistein and diadzein, resveratrol and curcumin and all have been well reviewed recently^[63-66]. Relatively few studies have been directed towards epigenetic changes in breast cancer models and results have been inconclusive^[61,62].

Recent studies have shown that 20-40 µmol/L genistein stimulated expression of the tumour suppressing genes, *p21^{WAF1}* and *p16^{INK4a}*, in breast cancer cells and that this was associated with a small reduction in the activity of HDACs but a large increase in the activity of HMTs^[67]. The same group also showed that genistein can reactivate ERα expression in ER^{ve} breast cancer cells and that this effect was associated with increased markers of histone acetylation in the ERα promoter region and decreased activity of HDAC and DNMT^[68]. Another study showed that µmol/L doses of genistein and diadzein "might reverse" DNA hypermethylation in breast cancer cells thus restoring expression of the oncosuppressor genes BRCA1 and BRCA2^[69]. In biopsies of human

breast tissue specific DNMT transcripts were increased in cells taken from the tumourous tissue compared to adjacent normal breast tissue and parallel studies showed that treatment of breast cancer cells lines with genistein, resveratrol, curcumin and EGCC also reduced the mRNA of the same DNMTs^[70]. Whilst all these studies have been performed acutely with high doses of single phytoestrogens, we showed that long-term treatment with 10 nmol/L genistein down-regulated the expression of acetylated histone3, cyclin D1 and procaspase 9 and reduced the growth promoting effects of E2 and epidermal growth factor^[71].

It is clear that both nutrition and exposure to phytoestrogens and other phytochemicals can have dramatic effects on epigenetic events and that these may become heritable through transgenerational mechanisms. Thus their impact on both disease and the health of future generations needs to be carefully considered.

CONCLUSION

Phytoestrogens have multiple targets within cells and whilst acute studies with supraphysiological doses of these compounds indicate that they may inhibit the development and progression of breast cancer, lower doses have been shown to promote the growth of breast cancer cells *in vitro* and experimentally induced tumours *in vivo*. More studies utilizing long-term exposure to lower doses and mixtures of phytoestrogens are required to demonstrate unequivocally that dietary supplements do have beneficial rather than detrimental effects on breast cancer. However their multiple targets in breast cancer cells and their ability to modulate epigenetic events associated with breast cancer and prevention may lead to new, non-toxic therapeutic approaches through development of highly specific and long-acting analogues of phytoestrogens.

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WJCO 5th Anniversary Special Issues (2): Breast cancer**Alcohol drinking and mammary cancer: Pathogenesis and potential dietary preventive alternatives**

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Abstract

Alcohol consumption is associated with an increased risk of breast cancer, increasing linearly even with a moderate consumption and irrespectively of the type of alcoholic beverage. It shows no dependency from other risk factors like menopausal status, oral contraceptives, hormone replacement therapy, or genetic history of breast cancer. The precise mechanism for the effect of drinking alcohol in mammary cancer promotion is still far from being established. Studies by our laboratory suggest that acetaldehyde produced *in situ* and accumulated in mammary tissue because of poor detoxicating mechanisms might play a role in mutational and promotional events. Additional studies indicated the production of reactive oxygen species accompanied of decreases in vitamin E and GSH contents and of glutathione transferase activity. The resulting oxidative stress might also play a relevant role in several stages of the carcinogenic process. There are reported in literature studies showing that plasmatic levels of

estrogens significantly increased after alcohol drinking and that the breast cancer risk is higher in receptor ER-positive individuals. Estrogens are known that they may produce breast cancer by actions on ER and also as chemical carcinogens, as a consequence of their oxidation leading to reactive metabolites. In this review we introduce our working hypothesis integrating the acetaldehyde and the oxidative stress effects with those involving increased estrogen levels. We also analyze potential preventive actions that might be accessible. There remains the fact that alcohol drinking is just one of the avoidable causes of breast cancer and that, at present, the suggested acceptable dose for prevention of this risk is of one drink per day.

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Key words: Alcohol; Ethanol; Acetaldehyde; Free radicals; Mammary cancer; Oxidative stress; Estrogens; Polyphenols

Core tip: Excessive alcohol drinking of any type of alcoholic beverage is known to linearly increase the risk of breast cancer. The precise mechanism involved to produce this effect is still far from being established. In this review we introduce our present working hypothesis integrating the participation in cancer initiation and promotion of: *in situ* accumulation of the acetaldehyde metabolite, the local promotion of oxidative stress and the effects of the increased levels of estrogen occurring during alcohol drinking. Some potential preventive alternatives were also analyzed.

Castro GD, Castro JA. Alcohol drinking and mammary cancer: Pathogenesis and potential dietary preventive alternatives. *World J Clin Oncol* 2014; 5(4): 713-729 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i4/713.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i4.713>

EPIDEMIOLOGY OF ALCOHOL DRINKING INDUCED BREAST CANCER

Alcohol drinking is causally related to an increased risk of cancer of the upper aero-digestive tract, liver, colorectum, and female breast^[1-4]. Of particular concern is the case of breast cancer promotion by chronic alcohol consumption. In effect, breast cancer is an extremely relevant cause of disease and death in women and alcohol intake is one of the few modifiable risk factors for breast cancer. The International Agency for Research on Cancer recently reported that by 2010 more than 100 epidemiological studies have evaluated the association between the consumption of alcoholic beverages and the risk of breast cancer^[4]. Further, combined analysis of data from 53 studies around the world showed a clear dose-response relationship between alcohol consumption and increased risk of breast cancer^[2]. This study showed 9% increase in risk per 10 g intake of alcohol per day. In fact, other recently epidemiological studies in a total of 1280296 middle-aged women in the United Kingdom reported that even drinking women consuming an average in only 10 g of alcohol (one drink) per day showed 12% increased risk of breast cancer^[1]. In addition, a detailed prospective study in 254870 women, made in eight European countries, reported that 5% of the female breast cancer was attributable to alcohol consumption^[5].

Unfortunately, there is limited information regarding a possible mechanism for this effect and about positive modulatory effects of dietary factors if alcohol drinking is not avoided. That issue was of particular interest in the studies made by our laboratory and by other workers^[6-9]. In this review we are going to describe our working hypothesis on the pathogenesis of alcohol drinking induced mammary cancer and its potential prevention.

ACETALDEHYDE FORMATION, ACCUMULATION AND EFFECTS

Despite the relevance of the problem, the mechanism for ethanol-increased risk of breast cancer remains unknown.

Several lines of evidence indicate that acetaldehyde, a product of alcohol metabolism, might play a role in alcohol-related carcinogenic effects in different target tissues^[10,11]. Animal experiments have clearly shown that acetaldehyde is an established mutagenic and carcinogenic chemical^[12-14]. In addition, other factors such as oxidative stress, altered methyl transfer, abnormal metabolism of vitamin A and retinoic acid and perturbed levels of hormones might be of particular relevance according to the target tissue involved^[3,8].

Concerning the specific case of mammary tissue, the knowledge of the acetaldehyde concentrations was of particular interest to our laboratory. In general terms, the concentration of acetaldehyde in any tissue, and also in the mammary, depends of its ability to produce it *in situ*, plus the additional arriving to the organ *via* blood supply

and in the capacity of the given organ to degrade it^[15].

The ability of mammary tissue to produce acetaldehyde *in situ* was studied in our laboratory. The available information was scarce to null at that time. Two different pathways of bioactivation of ethanol to acetaldehyde were reported by our laboratory to be present in the rat mammary tissue. One is in the cytosolic fraction and the other is in microsomes^[16,17]. Both were preliminary characterized and showed to be susceptible to inhibitory effects by chemicals present in food (this last subject will be analyzed ahead when discussing the preventive potential of findings).

The enzyme involved in the cytosolic pathway was evidenced to be xanthine oxidoreductase (XOR) because of its susceptibility to inhibitory effects of allopurinol and by the ability of the process to occur only when the presence of NAD⁺ was accompanied by substrates of the XOR form of the enzyme such as hypoxanthine, xanthine, caffeine, theobromine, theophylline or 1,7-dimethylxanthine^[16].

Moreover, it is also known that during acute alcohol intoxication, there is an increased purine degradation and hyperuricemia^[18,19]. The enhanced supply of purines resulting from this process could also provide an extra amount of cofactors for the XOR-mediated pathway of metabolism of ethanol to acetaldehyde (and also free radicals) in the mammary tissue.

The presence of XOR in mammary tissue is well known^[20,21], and past studies from our laboratory evidenced their presence in high amounts in the rat mammary tissue epithelial cells^[22]. Interestingly, the activity of this cytosolic pathway significantly increased after repetitive alcohol drinking through a Lieber and De Carli diet for 28 d^[22].

The contribution of enzymes present in cytosolic fraction of mammary tissue, other than XOR, to the activation of ethanol to acetaldehyde, for example, alcohol dehydrogenase (ADH) may be more limited. On one hand, previous studies^[23] showed that no ADH activity was found in homogenates of rat mammary tissue. More recently, our laboratory reported traces of ADH activity in the cytosolic fraction of mammary tissue that was about 16 times smaller than in the liver^[15]. By the other hand, Triano *et al.*^[24] reported that human mammary tissue contains a class of ADH having a limited potential to transform alcohol to acetaldehyde.

In addition to the mammary tissue cytosolic pathway of ethanol oxidation to acetaldehyde described above, our laboratory reported the presence of another one occurring in the microsomal fraction of that tissue^[17].

In our earlier studies of that pathway it was established that the enzymatic transformation involved was oxygen and NADPH-dependent, but that the cytochrome P450 was not involved because it was not inhibited by CO:O₂ (80:20 v/v) or by SFK525A^[17].

Interestingly, this microsomal transformation of alcohol to acetaldehyde was strongly inhibited by diphenyleneiodonium (DPI), sodium diethyldithiocarbamate, sodi-

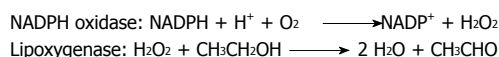


Figure 1 Cooperative mechanism between NADPH oxidase and lipoxygenase in the oxidation of ethanol to acetaldehyde in microsomes.

um azide, nordihydroguaiaretic acid but not by dapsone, aminotriazole or indomethacin. Those results suggested us the potential participation in this biotransformation of an oxidase or a peroxidase but not of lactoperoxidase or cyclooxygenase^[17]. We were unable to detect the formation of either hydroxyl or 1-hydroxyethyl radicals in those studies. In the course of following studies performed at the opportunity in rats exposed to a standard Lieber and De Carli diet for 28 d, we observed the induction not only of XOR cytosolic activation pathways but also of the microsomal one^[22].

That was of particular significance, since we showed in the course of additional recent work that the enhancing effect is not due to the participation of CYP2E1 after chronic alcohol drinking as it is known for the liver microsomal fraction^[25].

Further, acetone, another inducer of microsomal CYP2E1-mediated alcohol metabolism in liver microsomes, failed to enhance ethanol bioactivation and CYP2E1 enzymatic activity in the microsomal rat mammary tissue^[25]. To ensure that CYP2E1 enzymatic activity in the microsomal rat mammary tissue was not present or was very low, we also included in those studies determination of chlorzoxazone hydroxylase activity. This activity was considered in literature as having a significant response to the presence of CYP2E1 in a given tissue^[25]. We were not able to detect CYP2E1-mediated metabolism of chlorzoxazone in the mammary tissue microsomal fraction despite the fact we employed a particularly sensitive procedure developed in our laboratory, where the formation of 6-hydroxychlorzoxazone metabolite could be determined by HPLC with coulometric detection^[25]. That further excluded the participation of CYP2E1 in this microsomal pathway of alcohol metabolism in the mammary tissue and encouraged us to challenge the possibility that a peroxidase or a lipoxygenase was involved in that process instead. That hypothesis was originally coined because of the potent inhibitory effect of nordihydroguaiaretic acid. This polyphenol is a known inhibitor of lipoxygenases^[26,27]. We also envisaged the possibility that the potent inhibitory effect of DPI could be suggesting the additional participation of a NADPH oxidase enzyme as a supplier of hydrogen peroxide. Under this view, the role of NADPH oxidase would be the generation of the necessary co-substrate required by lipoxygenase to exert its activity against xenobiotics^[8]. On behalf of this hypothesis is the fact that the specific inhibitory effect of DPI on NADPH oxidase is well established^[28]. That hypothesis visualizes the overall process of microsomal ethanol oxidation to acetaldehyde in rat mammary tissue as a cooperative mechanism between NADPH oxidase and lipoxygenase (Figure 1).

The concentration of acetaldehyde in different tissues

depends on the production and degradation of acetaldehyde and on the amount of it arriving to the given tissue *via* blood supply. In a detailed study from our laboratory, we attempted to evaluate how much acetaldehyde accumulates in mammary tissue after single doses of ethanol and also the levels of it arriving *via* blood at different periods of time. The studies were performed at three different dose levels (high, medium and low). Values were compared with the equivalent occurring in liver^[15].

The levels of acetaldehyde in mammary tissue were higher than in plasma and lower than in liver for at least 15 h for the higher dose tested or six hours for the medium dose or two hours for the case of the lower one. The shape of the curve concentration of acetaldehyde in mammary tissue *vs* time after p.o. administration always mimicked that observed in liver. But, more important, the levels of acetaldehyde in plasma were similar for the three ethanol doses given^[15]. These results suggest that acetaldehyde present in the mammary tissue (and in the liver) reflects the balance between the ability of these tissues to generate acetaldehyde and the one to metabolize and excretes it. The liver is able to get rid of the acetaldehyde formed with the participation of aldehyde dehydrogenase (ALDH) and glutathione transferase (GST)^[19]. In the case of mammary tissue the situation appears to be different. On one hand, ADH activity is about 16 times smaller than in the liver, but perhaps more important, ALDH activity present in three subcellular fractions tested in our study were in all of them at least ten times smaller than in the liver^[15].

The overall conclusion of those results was that acetaldehyde is able to accumulate during significantly relevant periods of time in mammary tissue mainly as result of its ability to oxidize ethanol to acetaldehyde *in situ* and to a less important extent to the arrival of it *via* blood and produced elsewhere (for example, in the liver)^[15].

Which might be the expected consequences of acetaldehyde accumulation in a given tissue for long lasting periods of time?

It is important to point out that acetaldehyde is a reactive chemical that proved to be toxic, mutagenic and carcinogenic and able to interact with many cellular constituents, including DNA, proteins (nuclear proteins), lipids (including nuclear lipids), glutathione and others^[19,29,30].

The reactions of acetaldehyde with DNA are of particular concern because they clearly suggest that it acts as a tumor initiator and mutagenic compound. Structures of the identified DNA adducts of acetaldehyde were recently reviewed in literature^[31-33]. Representative structures are shown in Figure 2.

DNA adducts may cause polymerase errors and induce mutations in critical genes. Furthermore, they may lead to mutations that activate proto-oncogenes and inactivate tumor suppressor genes in replicating cells^[34,35].

Notwithstanding, there are known DNA repair enzymes that can modify DNA damage caused by acetaldehyde, removing its adducts from DNA. The relation between adducts formation and their repair would be relevant to molecular epidemiology of cancer, particularly

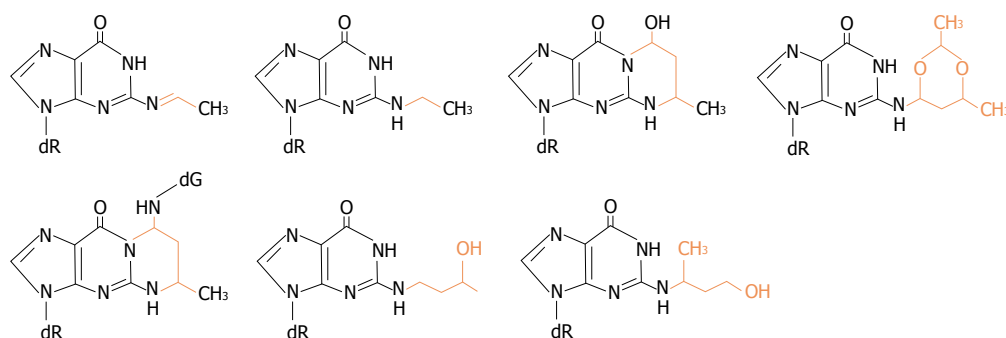


Figure 2 Structures of some of acetaldehyde-guanine adducts.

in replicating cells^[35].

It is of particular interest in this respect that studies by Freudenheim *et al.*^[36] reported results that were consistent with an increased likelihood of tumors with p53 mutations for pre-menopausal breast cancer with increased alcohol intake 10 or 20 years previous. For intakes of 16 or more drinks per month in the period of 20 years before the interview compared with non drinkers, the OR was 5.25 (95%, 1.48-18.58)^[36].

Acetaldehyde is also known to bind to proteins and amino acids and the subject might also be relevant to potential carcinogenic effects of alcohol drinking in different organs.

However, non specific studies related to breast cancer and alcohol drinking and the potential participation of acetaldehyde protein binding are available at present in literature. Notwithstanding, it is well known that acetaldehyde binds to reactive lysine residues, to some aromatic amino acids, cysteine, or to free alpha-amino groups, such as the terminal valine of hemoglobin. The subject was thoroughly reviewed by Niemela^[37], who also pointed the relevance of those interactions in different pathologies occurring in several tissues other than the breast.

In this respect, it might be relevant to take in consideration that acetaldehyde was shown to bind covalently to liver nuclear proteins^[30]. In the case of liver, the biotransformation of ethanol to acetaldehyde may occur not only in the cytoplasm or in the endoplasmic reticulum but also in the nearby outer nuclear membrane^[30,38]. Acetaldehyde, despite being a reactive molecule, does not have the equivalent reactivity to the one of a low molecular weight free radical (for example, trichloromethyl or 1-hydroxyethyl). This allows it to travel from cellular distant sites (cytoplasm or endoplasmic reticulum) to the nucleus and interact with DNA and nuclear proteins or lipids when acetaldehyde accumulates for long periods of time as those observed to occur in mammary tissue after alcohol drinking.

In the case of other liver carcinogenic chemicals, like carbon tetrachloride, a good correlation was observed between the covalent binding of its reactive metabolites to nuclear proteins and carcinogenicity. The interaction involved histone and non-histone proteins. Acidic and residual nuclear proteins were the favorite targets of that interaction and showed a good correlation between those

covalent interactions and carcinogenicity^[39].

In addition, is important to take in mind that ethanol is a well known inducer of CYP2E1-mediated metabolisms^[19] and that this enhancing effect was observed in the outer nuclear membrane of the liver^[40,41].

Far reaching consequences may also be expected from alterations in nuclear proteins resulting from an attack by acetaldehyde. In effect, it is known that nuclear proteins are critical for cell division, growth, differentiation and apoptosis^[42-44]. A clear example of relevant nuclear proteins is the one of those involved in the cell cycle clock. They include cyclins, cyclin-dependent kinases; protein coded by proto-oncogenes and tumor suppressor genes (such as the myc and Bcl proteins or the pRB or p53 and others); enzymes required for DNA synthesis and for DNA repair^[45,46].

Critical evidence that some of these activities may be affected by acetaldehyde produced during ethanol consumption was provided by several authors, who showed that chronic ethanol consumption results in inhibition of the DNA alkylation repair by O⁶-methylguanine transferase (O⁶-MeGT), which removes alkyl groups from the O⁶-position of guanine. Acetaldehyde has been shown to inhibit O⁶-MeGT^[10,47-49]. Further, exposure to ethanol was reported in recent studies to interfere with the cell division machinery by perturbing the key G2 cycling protein^[50].

Concerning the covalent binding to nuclear lipids, it should be noted that all lipid fractions were involved in our studies performed in the case of liver. Part of the covalent binding was acid labile, but a significant part was not. The labile portion might be attributable to the presence of Schiff base adducts of acetaldehyde with amino groups from phospholipids^[30]. In effect, previous studies from other laboratories reported that amino-containing phospholipids like phosphatidylserine and phosphatidylethanolamine formed Schiff base adducts^[51,52].

The fraction of covalent binding resistant to acid hydrolysis might be attributed in part to addition reactions of 1-hydroxyethyl on unsaturated fatty acid double bonds or on cholesterol moieties and on nitrogen-containing phospholipids. That behavior was previously reported for the case of other carbon-centered free radicals, for example the trichloromethyl radicals^[53,54].

Another part of the covalent binding to lipids resistant to mild acid media might also be attributed to the

formation of ethyl esters of fatty acids. It is known that liver microsomes and cytosol contain enzyme systems able to esterify free fatty acids or transesterify other esters to ethyl esters^[55-57]. Whether nuclear preparations from mammary tissue have similar ability remains to be established. Irrespectively of the present lack of knowledge about the structure of the reaction products formed, it is not unexpected to envisage that the alteration of nuclear membranes by their reaction with either acetaldehyde or 1-hydroxyethyl or resulting from ester formation with ethanol, might lead to profound alterations in liver nuclear functions. It is well established that ethanol also strikingly alters other liver membranes and their fluidity^[58]. It is known that nuclear lipids may be part of an intracellular signaling system modulating protein kinase C, an enzyme involved in phosphorylation of nuclear proteins^[59]. The covalent binding to nuclear phospholipids deserves special interest since they have a known ability to regulate gene function, nucleosome structure, RNA synthesis and activation of DNA polymerase alpha^[59-65]. Past studies from the laboratory reported correlations between alterations in nuclear lipids resulting from a free radical attack and the different response of the rat strains employed to the carcinogenic effects of CCl₄^[54].

FREE RADICALS, OXIDATIVE STRESS AND EFFECTS

Oxidative stress was also considered to be a potential factor involved in the alcohol drinking promoted carcinogenic effect on mammary tissue. Notwithstanding this, no evidence of its occurrence in mammary tissue were reported until our recent studies where we showed that oxidative stress can be observed in this tissue after an experimental protocol of 28 d of alcohol drinking through the Lieber and De Carli diet^[15,22,25]. It is relevant at this point to have present what "oxidative stress" is all about. Oxidative stress has been defined as an imbalance between oxidants and antioxidants in favor of the former, resulting in an overall increase in cellular levels of reactive oxygen species (ROS). Many pathways play a role in how ethanol induces oxidative stress in the liver (the issue has exercised the interest of numerous researches for years) and is reviewed in Cederbaum *et al.*^[66]. Ethanol-induced oxidative stress in the liver included the ability to generate free radicals able to initiate the process (for example, 1-hydroxyethyl and hydroxyl radicals) and formation of hydroperoxides, peroxides, superoxide, H₂O₂ and other. In the case of liver exposed to alcohol, the evidence included the formation of products derived from the attack of ROS to relevant target molecules (for example, DNA, proteins or lipids) like 8-oxodeoxyguanosine, protein carbonyls, decreases in protein sulfhydryls, lipid hydroperoxides and other products such as malondialdehyde or 4-hydroxy-2-nonenal (4HNE)^[66].

The evidence that cellular antioxidant defenses were overwhelmed might include determinations of the variety of enzymatic and non-enzymatic mechanisms that have

evolved to protect cells against ROS such as superoxide dismutase, catalase, glutathione peroxidase, GST and other enzymes. Also are relevant low molecular weight antioxidants, such as glutathione itself, vitamin E, ascorbate, vitamin A and others. In summary, toxicity induced by ROS reflects the balance between the rates of production of ROS compared to the rates of removal of ROS plus repair of damage to cellular macromolecules^[66].

After these introductory remarks learnt from free radical cell injury and from the effects of alcohol on liver, let us to introduce what kind of evidence we obtained for the case of mammary tissue.

Initially we began to consider the possibility that during alcohol drinking an oxidative stress process occurred in mammary tissue because in our hands repetitive alcohol drinking exposure led to increased XOR and lipoxygenase activities in the rat mammary tissue^[22]. In effect, increased XOR and lipoxygenase activities by themselves would lead to not only higher generation of ROS, but also when occurring in the presence of ethanol, to increased generation of free radicals. The formation of hydroxyl radicals was detected during XOR mediated cytosolic alcohol metabolism^[16].

Other reason leading to a similar potential effect was derived from our observation that acetaldehyde accumulates in mammary tissue during repetitive alcohol drinking^[15]. It is well known that the generation of increased level of acetaldehyde may provoke decreases in GSH as it was respectively observed in liver^[66]. Further, GSH and GSH-dependent enzymes such as glutathione peroxidase, glutathione reductase and glutathione transferase are a vital first line defense against oxidative stressful conditions^[66].

The initial observation indicating that alcohol drinking could provoke oxidative stress that we obtained was derived from our studies in the *t*-butylhydroperoxide-induced chemiluminescence in mammary tissue homogenates from rats exposed to repetitive alcohol drinking when compared to those from control animals^[15].

The samples from alcohol treated animals had a completely different response to the *t*-butylhydroperoxide challenge and the shape of their response curve compared to that observed in the control samples clearly suggested that animals exposed to alcohol have diminished defenses against *t*-butylhydroperoxide challenge^[15]. However, that experiment did not give indication about the nature of the defensive process that was compromised. Further, it did not show which target molecule involved the potential oxidative process that was occurring.

To answer those questions, other studies were performed and we found that after repetitive alcohol drinking, the levels of lipid hydroperoxides in mammary tissue were significantly increased. Further, the protein sulfhydryl and vitamin E content in the alcohol-treated animals decreased. However, in those experiments we did not observe protein carbonyl enhancement or increased formation of 8-hydroxyguanine^[22]. One reason to explain that different response to alcohol drinking-provoked oxidative stress might be that much longer periods of

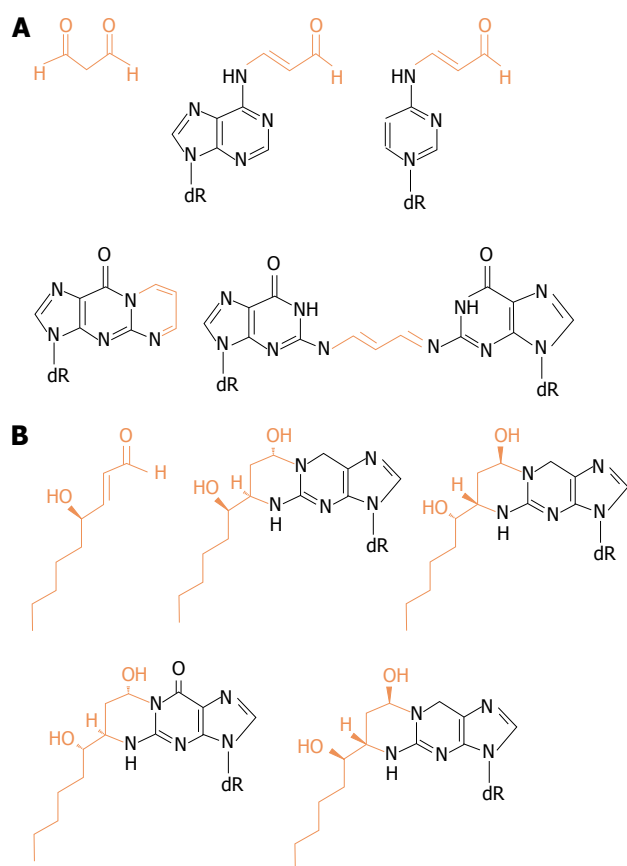


Figure 3 Structures of adducts formed between DNA bases and (A) malondialdehyde or (B) 4-hydroxy-2-nonenal.

expose to alcohol could be necessary. That is in our work we were detecting early manifestations of alcohol drinking induced oxidative stress in mammary tissue.

If that was the case, after more prolonged periods of alcohol drinking, other relevant effects might occur derived from generation of harmful reactive aldehydes produced from lipid peroxidation such as malondialdehyde and 4HNE. They are able to react with proteins, lipids and with sulfhydryl-containing molecules of low molecular weight^[33,66]. See Figure 3 for their structures.

The reaction with sulfhydryl groups that we observed might be of significance considering that many sulfhydryl-containing enzymes play a key role in cell functioning and also in cell signaling processes^[67-70].

Free radicals not only are relevant in relation to the carcinogenesis initiation step. The progress of human breast cancer to the metastasis stage was linked to hydroxyl radical-induced DNA damage^[71].

These findings should be of some significance in light of the well established correlation between oxidative stress and tumor promotion and cancer^[72-74].

INCREASED LEVELS OF ESTROGENS AND CONSEQUENCES

Increased plasmatic levels of estrogen by alcohol drinking have been clearly demonstrated in controlled feed-

ing studies in human female volunteers^[75-84]. Further, the hypothesis about the role of estrogens in alcohol drinking-induced breast cancer is strongly supported by the fact that their higher risk was related to estrogen receptor ER-positive rather than to ER-negative mammary tumors^[85-88]. However, estrogens may exert their carcinogenic effects in mammary tissue not only *via* ER, but also by direct damage to DNA^[89]. Metabolic activation of estrogens to catechol-3,4-quinones is an example. These reactive metabolites are able to interact with DNA to give mutational events^[90-93]. Those increased levels of estrogen after alcohol drinking would arise because alcohol increases aromatase activity and that leads to enhanced conversion of testosterone to estrogen, resulting in decreased testosterone and increased estrogen level^[94].

These considerations might be of particular relevance, since there are available in literature clear evidences that high levels of estrogen in blood are associated with an increased risk of breast cancer^[94].

One major mechanism of action of estrogens is that by which estrogen stimulates cell proliferation through nuclear ER-mediated signaling pathways, thus resulting in an increased risk of genomic mutations during DNA replications^[95-97].

Another pathway involves estrogen metabolism that is mediated by cytochrome P450 1B1, which generates 2- and 4-catechol estrogens that easily autoxidize to the respective quinones, even without the need for enzymatic or metal ion catalysis^[98]. At this point it is particularly relevant to do mention that alcohol consumption was reported to significantly increase the rate of NADPH-dependent oxidative metabolism of estrogens to quinones in females. CYP1B1 had a distinct, selective activity for the 4-hydroxylation of estradiol and estrone^[99]. This inductive effect of alcohol drinking might increase chances of participation of cytochrome-mediated pathway in relation to other related to direct interactions of the estrogens with ER.

It is our idea that, under conditions where oxidative stress or peroxidizing conditions occur (like those reported by our laboratory to occur during exposure of mammary tissue to alcohol), the transformation of one to the other might be even further facilitated.

In addition, o-quinones are also potent redox-active compounds^[98,100]. For example, these quinones can be reductively detoxified by a quinone reductase in a two-electron transfer process^[101]. However, they can also undergo redox cycling with the semiquinone form of P450 reductase (a one-electron process) and generate superoxide radicals, that in the presence of iron or other transition metal give hydroxyl radicals^[100] (Figure 4).

By the other hand, the estrogen quinones can directly damage DNA leading to genotoxic effects^[102,103]. DNA adducts of estrogen quinones have been detected in the mammary glands of ACI rats treated with 4-hydroxyestradiol or its quinones^[103]. Further, recently developed highly sensitive LC/MS-MS procedures were used to analyze DNA from human breast tumors and their

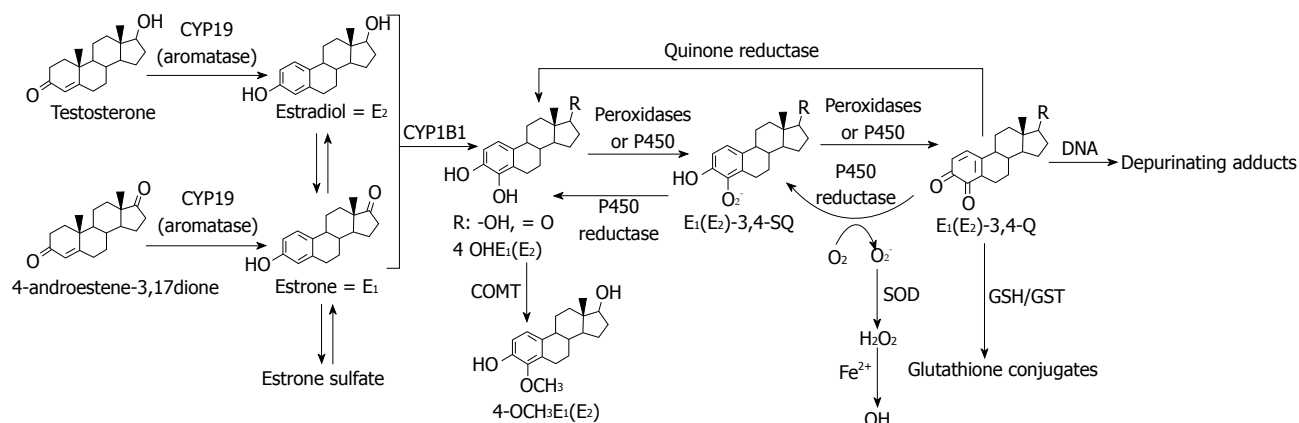


Figure 4 Estrogen oxidation to reactive metabolites that bind to DNA to generate depurinating adducts.

adjacent tissue and the DNA adducts formed from estrogen quinones were detected^[104].

All these results suggest that these mechanisms might be relevant for the carcinogenic effects of estrogens by themselves. However, there is not available in literature evidence of their formation in mammary tissue from alcohol drinking animals or humans at present.

As part of our present working hypothesis about the contribution of estrogens in the alcohol drinking effects in mammary tissue, we assumed that these estrogen quinones might be formed (Figure 4). The reasons for that hypothesis are going to be described ahead and are related to some of our results.

It is relevant to understand our “views” about the cooperative effects between alcohol metabolism to acetaldehyde, its ability to promote oxidative stress and the participation of induced levels of estrogen in carcinogenic effects in mammary tissue to describe what happens with the catechol estrogens and the estrogen quinones after their formation.

The catechol estrogens may be further metabolized by O-methylation, by reaction with glutathione, and also by glucuronidation and sulfation^[105,106].

Interestingly, catechol estrogen metabolites display a less intense ER-mediated estrogenic activity, implying that catechol estrogens are attributed to have both, direct genotoxic effects as well as ER-mediated tumor promoter actions^[107]. Further, sulfate and glucuronide conjugates seem to play a role for free estrogens and, in general, it is at present a controversy about conjugation metabolism, if they may mitigate the catechol estrogen-mediated carcinogenic properties^[107].

Notwithstanding, being the conjugated estrogens more water-soluble they seemed to be more readily excreted than the lipophilic parent estrogens^[107]. These suggest that the conjugation pathway is considered as a protection mechanism against damage caused by reactive metabolites of estrogens^[105].

An important aspect of the mechanism of generation of estrogen quinones from catechol estrogens concerns the resulting formation of ROS during the process. In effect, ROS are generated *via* the redox cycling between cat-

echol estrogens and their quinone analogs^[108-110] (Figure 4).

Catechol estrogens can be oxidized by any oxidative enzyme or metal ions such as Cu^{2+} or Fe^{3+} to give rise to semiquinones and o-quinones^[108-110]. Is our hypothesis that it might very well be that this activating pathway of the catechol estrogens was stimulated during alcohol drinking by the inductive effect of alcohol on mammary tissue XOR and lipoxygenase or even more likely, by the alcohol drinking promoted oxidative stress that was observed^[15,22,25].

The reduction of o-quinones back to semiquinones and catechols by P450 reductase provides an opportunity to generate ROS, including superoxide and hydroxyl radicals^[107].

In our experiments on the effect of alcohol drinking on rat mammary tissue we were able to detect the formation of hydroxyl radicals and lipid hydroperoxides during the metabolism of ethanol in this tissue^[16,22].

In those studies we also reported that repetitive alcohol drinking provoked the depletion of defenses against oxidative stress insult due to significantly reduced levels of glutathione, α -tocopherol and defensive enzymes such as glutathione transferase and glutathione reductase^[25]. Besides the consequences that those effects have on the oxidative stress process itself and in the promotion of the carcinogenic process^[73], this might have additional relevant consequences for the contribution of estrogens to the mammary carcinogenic process. In effect, glutathione is also able to react with the estrogen quinones and semiquinones to give glutathione conjugates in a reaction catalyzed by glutathione transferase^[107]. Further, the lowered levels of glutathione would also decrease the capacity of mammary tissue to destroy hydroperoxides since it is the necessary cofactor for glutathione peroxidase to operate even when the levels of this enzyme were not decreased^[25].

Decreased levels of two critical antioxidants like vitamin E and glutathione that we observed in mammary tissue after alcohol drinking might be not only of relevance for the promotion of oxidative stress^[25], but also to understand that their depletion might be a potential factor of the genotoxic effect of estrogens, *via* their oxidative

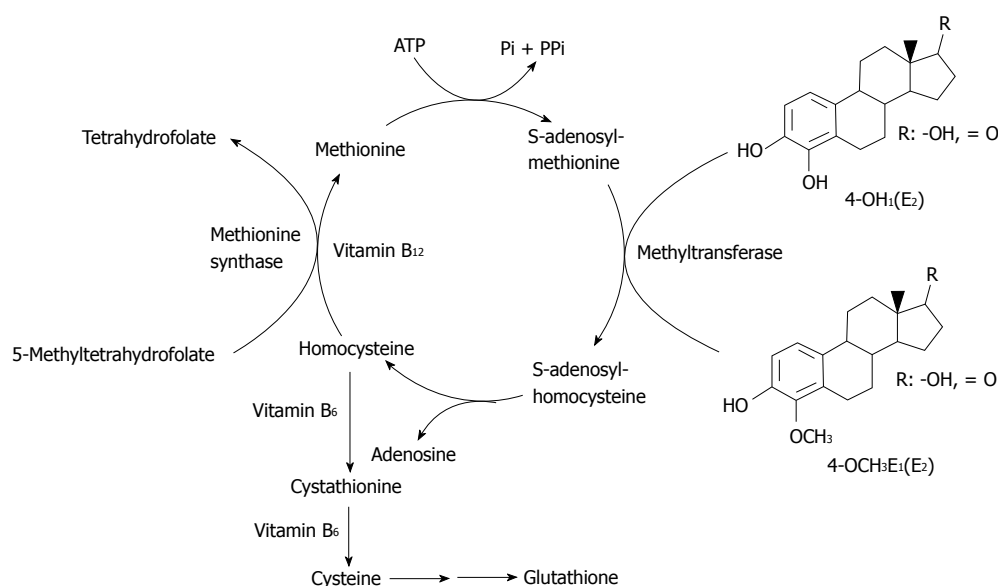


Figure 5 Pathways for catechol-estrogen methylation by carboxymethyl transferase.

metabolism that might be enhanced^[107] (Figure 4).

The increased generation of ROS produced by alcohol toxicity^[66] and the effects of estrogen^[107] might explain the decreased levels of α -tocopherol observed^[25].

In addition, the significantly decreased levels of GST observed in our experiments on the effect of alcohol drinking on mammary tissue might also be explained as attributable to the potent inhibitory effects that specific glutathione-estrogen metabolites have on the GST molecule^[111-113]; of course that remain to be proved. It might of particular relevance in light of previous observations reported by Zheng *et al.*^[114] in epidemiological studies, that alcohol consumption increased breast cancer risk in women among those who carry susceptible GST genotypes.

Not only the GSH/GST detoxicating pathway of the estrogen metabolites could be negatively modulated by alcohol drinking. Other might be the carboxymethyl transferase (COMT) pathway of catechol estrogens methylation (Figure 5). Several points of the cycle depicted in that process are already known that might be altered during alcohol drinking. For example, the S-adenosylmethionine (SAM) involved in the methyltransferase step of catechol estrogens methylation is known to be diminished because of SAM decreased hepatic biosynthesis. In alcoholic liver disease methionine adenosyltransferase capacity is affected. In fact, chronic alcoholics have hypermethioninemia and impaired methionine clearance^[115]. That leads to decreased SAM biosynthesis and in turn to impact on methylation capacity of the cell and decreased antioxidant defenses^[115].

Further, chronic exposure to ethanol has been shown not only to decrease hepatic SAM levels, but also to increase hepatic concentrations of S-adenosylhomocysteine and decrease plasma concentrations of folate in animals and humans^[115,116]. In turn, homocysteine may exert pathogenic effects largely through metabolic accumulation of SAH, which is a strong non-competitive inhibitor of

COMT-mediated methylation metabolisms of various catechol substrates, including those arising from estrogens^[117].

In addition to depleting folate concentrations, chronic ethanol exposure decreases the activity of methionine synthase, which is required to catalyze the transfer of a methyl group from folate to homocysteine to form methionine^[116,118,119].

All these facts might decrease the ability of COMT to methylate harmful catechol estrogens during chronic alcohol drinking as we describe in our working hypothesis about the concerted action of alcohol drinking, the formation of deleterious acetaldehyde, free radicals, oxidative stress and increased levels of estrogens.

WORKING HYPOTHESIS ABOUT HOW ALCOHOL DRINKING MIGHT PROMOTE CARCINOGENIC EFFECTS IN MAMMARY TISSUE

Considering the growing evidence about the relevance of acetaldehyde participation in the alcohol drinking-carcinogenic effects in target organs (for example, the aerodigestive tract) we consider that its *in situ* formation and accumulation in mammary tissue should play a role. Contribution of acetaldehyde arriving *via* blood supply should play some but a minor role.

Taking into account that oxidative stress promotion was observed and that free radical generation systems are present in mammary tissue exposed to ethanol during alcohol drinking we felt that it was relevant to consider oxidative stress as a relevant participant in the alcohol drinking promoted carcinogenic effects on mammary tissue. It is well known that it plays a role in promotion and initiation of tumors induced by other chemicals acting in other tissues as well as in mammary tissue.

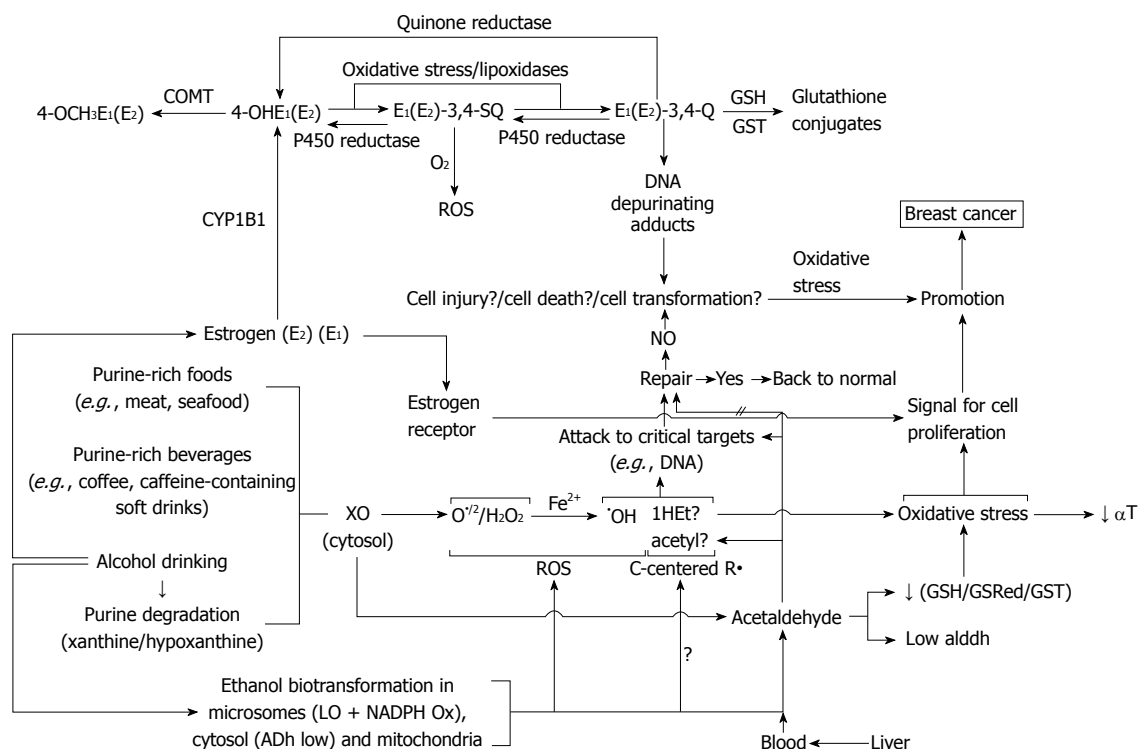


Figure 6 Working hypothesis about the mechanism of the promotion of mammary cancer by alcohol drinking.

Finally, alcohol drinking is also known to increase estrogen blood levels in animals and human beings and it is an established carcinogen for mammary tissue^[95,120]. In women, Eriksson *et al.*^[121] observed an estrogen associated acetaldehyde elevation after alcohol intake.

Alcohol is able also to provoke even on culture studies proliferative and cell transforming effects, not only *via* estrogen receptors but also as a “chemical carcinogen”, mediated by products of their oxidative metabolism^[87,122,123].

In light of the remarkable susceptibility of mammary tissue to alcohol drinking, clearly evidenced in epidemiological studies, we coined the working hypothesis that the resulting deleterious carcinogenic effects might arise from a sort of collaborative participation of these three components, making some of them more susceptible to the effect of the other. Some of those possibilities are going to be described at the time of proposing potential preventive treatments that we postulate based on existing linked literature.

Only the challenge of the hypothesis will tell whether is valid or not. Lack of hypothesis does lead to new experiments. New experiments may show the need of a new one. The present one in our hands is shown in Figure 6.

POTENTIAL PREVENTIVE TREATMENTS AGAINST ALCOHOL DRINKING DELETERIOUS EFFECTS IN MAMMARY TISSUE

The first point to mention is that being alcohol drinking one of the few causes of breast cancer promotion a

wise suggestion might be not to drink. Notwithstanding we feel that epidemiology shows that the real success of that advice may not be easily achievable. The present suggested tolerance for alcohol drinking by women is of one daily drink^[4]. The analysis of the potential preventive strategies for women deciding to moderately exceed that suggested tolerated levels will be analyzed in light of the existing knowledge and of our working hypothesis.

The preventive alternatives to be analyzed include: (1) Blockade of acetaldehyde generation and accumulation in mammary tissue; (2) Prevention of alcohol and estrogen-induced oxidative stress in mammary tissue by antioxidants; (3) Treatments increasing defenses against estrogen provoked effects; and (4) Treatments able to be preventive in different aspects of the problem.

Inhibitory effects on acetaldehyde generating enzymes present in mammary tissue

Considering that most of the acetaldehyde accumulating in mammary tissue was generated *in situ*^[15], we considered of particular interest to pay attention to inhibitors of the two pathways already evidenced by our laboratory, one present in cytosolic fraction and mediated by XOR and other, located at the microsomal fraction being linked to a lipoxygenase activity^[8,16,17,124-126].

It was of our especial interest the search for compounds present in food or drinks or available as dietary supplements, having low toxicity, potent inhibitory effects and ideally acting on both, the cytosolic plus microsomal pathways of ethanol metabolism to acetaldehyde^[127,128].

Several phytochemicals of polyphenolic nature tested appeared as potential candidates exhibiting those interest-

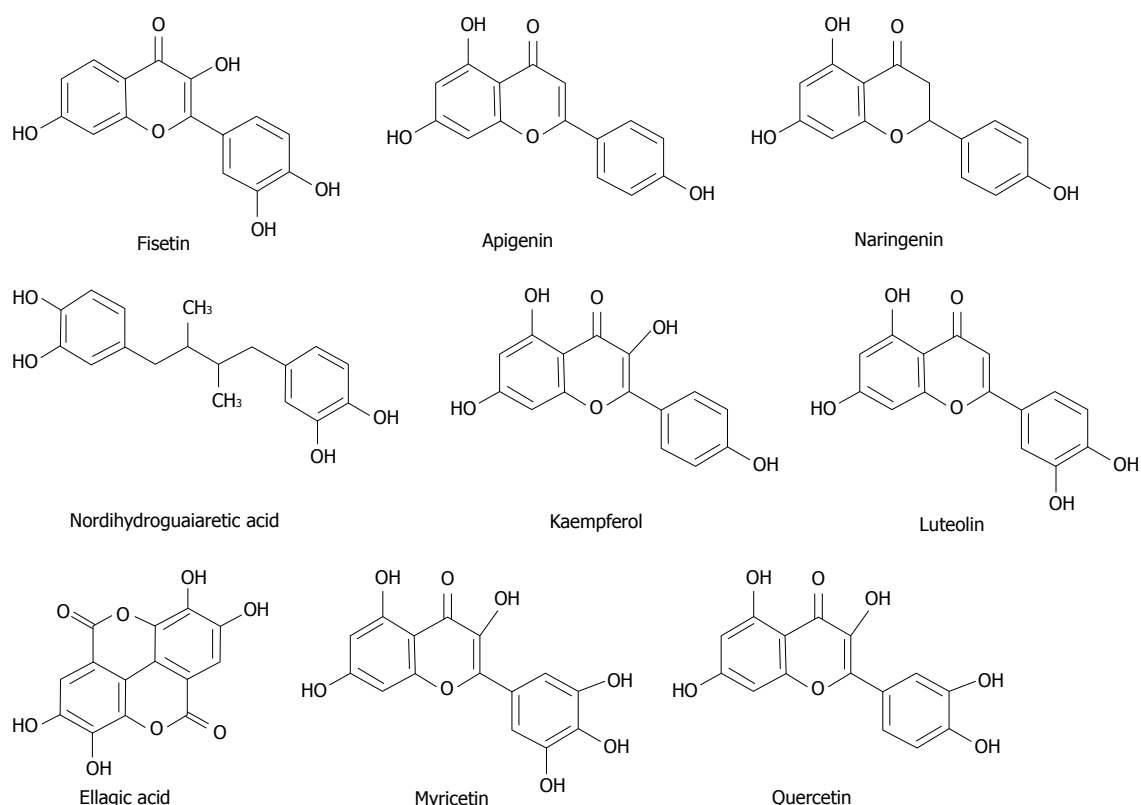


Figure 7 Structures of some of the compounds with the most powerful ability to inhibit the oxidation of ethanol to acetaldehyde in rat mammary tissue microsomal and cytosolic fractions.

ing characteristics and deserve our future attention. The most potent inhibitors among those polyphenols were flavonols quercetin, myricetin and kaempferol; also the flavones apigenin, luteolin and the polyphenols nordihydroguaiaretic acid and ellagic acid. Their structures are shown in Figure 7.

It might be important to consider at this point that several polyphenols were found to be inhibitory of carcinogenesis in laboratory investigations^[129-131].

The case of ellagic acid particularly attracted our interest because it is present in several fruit sources^[132]. This compound was able to diminish estrogen-mediated mammary tumorigenesis in ACI rats^[133]. Other authors reported anti-proliferative activities *in vitro* with several cell types^[134]. And more, it also exhibited beneficial anti-cancer effects based in a chemical study in male prostate cancer patients^[135,136].

We also found that folic acid behaved a highly potent inhibitor of acetaldehyde formation in the cytosolic fraction of mammary tissue^[124]. That suggests a preventive effect mediated not only by acetaldehyde depletion but also because it is a critical component of the overall methylation processes. Folate was found to be relevant in the prevention of breast cancer^[137].

Removal of acetaldehyde accumulated in mammary tissue

Past studies from our laboratory evidenced that acetaldehyde accumulates in mammary tissue during long periods

of time, even after single doses of ethanol given orally^[15].

Removal of acetaldehyde from mammary tissue appears far more difficult than in liver. In effect, the ALDH activity in mammary tissue subcellular fractions (cytosol, mitochondria and microsomes) was at least ten times lower than in the liver^[15]. In addition, the relevant mitochondrial ALDH activity might become irreversibly inhibited by the lipid peroxidation byproduct 4HNE, which is a potent inhibitor as well as a substrate for ALDH^[138,139]. Lipid peroxidation might occur during the oxidative stress produced by ethanol acting on mammary tissue^[22]. Further, the other potential GSH/GST system able to get rid of acetaldehyde is significantly decreased in mammary tissue after repetitive alcohol drinking^[25].

Other alternative to eliminate acetaldehyde accumulation in mammary tissue might arise from cysteine administration. Cysteine is able to ameliorate the toxicity of acetaldehyde by forming a stable adduct, the 2-methylthiazolidine-4-carboxylic acid^[140] (Figure 8). Its efficiency to lower the acetaldehyde accumulation in mammary tissue remains to be established. This alternative was suitably employed to prevent acetaldehyde accumulation in the oral cavity during alcohol drinking^[141,142].

In the case of mammary tissue, we consider N-acetylcysteine (NAC) as having more likely potential preventive action. NAC might be less effective than cysteine in extracellular environment (*e.g.*, saliva), but can be given at higher doses and during longer periods of time than cysteine because of its lower toxicity and because it is also

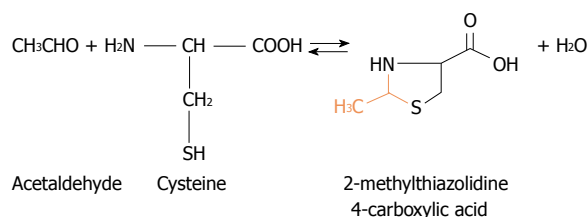


Figure 8 Reaction between acetaldehyde and cysteine to form the adduct 2-methylthiazolidine-4-carboxylic acid.

a precursor of cysteine and glutathione^[143]. In a number of studies since 1984, NAC also proved to have the potential to prevent cancer and other mutation-related diseases^[143].

Prevention of estrogen oxidation or enhancement of detoxication of estrogen reactive metabolites

The invoking hypothesis displayed in Figure 6 assumes that a significant component in the alcohol drinking promotion of mammary cancer involves de formation of catechol estrogens that might be oxidized to their quinones, which can react with DNA.

The contribution of alcohol drinking to this deleterious pathway might involve the inductive effect on CYP1B1 and provision of relevant oxidizing conditions for the generation of the estrogen reactive metabolites and the decreased detoxication of those reactive moieties.

The contribution of antioxidants if that hypothesis was likely appears attractive to prevent this factor of the alcohol-drinking *via* the oxidative estrogen metabolism component. Recent studies^[144] evidenced that the antioxidant N-acetylcysteine, a precursor of cysteine and of intracellular GSH^[145] is able to block the initial step in the genotoxicity caused by estrogen quinones.

The authors concluded that N-acetylcysteine preventive action included multiple protective mechanisms, including nucleophilicity, antioxidant activity and inhibition of DNA adduct formation^[146]. Preventive properties were also observed in human breast epithelial cells and mouse mammary epithelial cells^[147].

Further, GSH, an ubiquitous antioxidant is able to react non-enzymatically with the catechol estrogen quinones or more efficiently, with the catalytic activation of GST^[101,148].

Resveratrol, a natural antioxidant present in grapes and different plant products^[149,150] was also effective in inhibiting the formation of estrogen-DNA adducts^[146] and is also known to exert diverse anticarcinogenic effects *in vitro* and *in vivo*^[149,151].

Dihydrolipoic acid, which is formed *in vivo* when lipoic acid is administered, can also inhibit the formation of depurinating estrogen-DNA adducts^[146]. Resveratrol achieved the highest level of inhibitory effect and NAC or dihydrolipoic acid a moderate action^[146].

Interestingly, our laboratory reported that alcohol-drinking decreased levels of GSH and α -tocopherol, and GST in mammary tissue and enhanced oxidative stress in mammary tissue^[25].

These alcohol drinking-induced deleterious effects should decrease the detoxicating pathway of glutathione conjugates formation and give to the estrogen 3,4-quinones more opportunity to react with DNA to produce depurinating adducts.

Other harmful effect of alcohol drinking may also occur at the level of the COMT detoxicating pathway operating at the level of the catechol estrogens, especially the 4-hydroxylated metabolite of 17 β -estradiol. In effect, the COMT pathway to proceed requires the participation of SAM, folic acid, vitamins B6 and B12 (Figure 5).

The SAM requiring methyl transferase then methylates the 4-OH group of the catechol estrogens to give the 4-OCH₃ derivative having less toxic effects^[98,107]. In the SAM-mediated transmethylation process, S-adenosylhomocysteine (SAH) is generated as a product and is hydrolyzed afterwards by SAH hydrolase to form homocysteine and adenosine. SAH is a potent competitive inhibitor of the methylation reaction and it is important to remove adenosine and homocysteine to prevent accumulation of SAH. Re-methylation of homocysteine requires folic acid and vitamin B12. Homocysteine can also form cysteine *via* enzymatic processes requiring vitamin B6. Cysteine is, in turn, the rate determining precursor for GSH synthesis *via* a two step enzymatic process. All these processes are better reviewed by several authors^[115,116,119,152]. See Figure 5 for a summary of the SAM-mediated methylation process of catechol estrogens.

The reason for briefly introducing this matter is that this COMT detoxicating process also offers possibilities of preventive treatments.

First it is important to summarize which are the effects of alcohol drinking on the components of the above described participating molecules of the transmethylation process.

For example, it has been shown that chronic ethanol exposure decrease hepatic concentration of SAM, increasing plasma concentration of homocysteine, increase hepatic concentration of SAH and decrease plasma concentration of folate, in animal and human studies^[116].

Conversely, exogenous administration of SAM has been shown to attenuate deleterious effects of alcohol drinking in animals. SAM administration is known to restore hepatic concentrations of GSH depleted by alcohol drinking^[116].

That is, SAM treatment might also be of help to prevent alcohol drinking effects on mammary induced cancer by improving the COMT pathway of detoxication.

CONCLUSION

Our reviewed considerations about the pathogenesis of alcohol-drinking induced mammary cancer led to the working hypothesis that: acetaldehyde accumulation and its harmful effects, induced generation of ROS and oxidative deleterious effects on increased estrogen levels might cooperatively be involved in cancer promotion. That remains to be proved.

If assumptions were even partially correct, some

useful preventive alternatives might be available: inhibition of acetaldehyde forming metabolic pathways, destruction of accumulated acetaldehyde, treatments with powerful and safe antioxidants and also enhancement of estrogen quinones detoxicating pathways. Further, cocktails having mixtures of components from each type might be envisaged.

Notwithstanding, it is always important to consider that alcohol drinking induced mammary cancer is one of the very few avoidable causes of cancer and it might be possible to avoid alcohol drinking at all or more probably acceptable to drink just one drink per day (12 g of ethanol per day only).

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WJCO 5th Anniversary Special Issues (2): Breast cancer**MicroRNAs in cancer therapeutic response: Friend and foe**

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Abstract

Cancer initiation and development engage extremely complicated pathological processes which involve alterations of a large number of cell signaling cascades and functional networks in temporal and spatial orders. During last decades, microRNAs (miRNAs), a class of non-coding RNAs, have emerged as critical players in cancer pathogenesis and progression by modulating many pathological aspects related to tumor development, growth, metastasis, and drug resistance. The major function of miRNAs is to post-transcriptionally regulate gene expression depending on recognition of complementary sequence residing in target mRNAs. Commonly, a particular miRNA recognition sequence could be found in a number of genes, which allows a single miRNA to regulate multiple functionally connected genes simultaneously and/or chronologically. Furthermore, a single gene can be targeted and regulated by multiple miRNAs. However, previous studies have demonstrated that miRNA functions are highly context-dependent,

which leads to distinct pathological outcomes in different types of cancer as well as at different stages by alteration of the same miRNA. Here we summarize recent progress in studies on miRNA function in cancer initiation, metastasis and therapeutic response, focusing on breast cancer. The varying functions of miRNAs and potential application of using miRNAs as biomarkers as well as therapeutic approaches are further discussed in the context of different cancers.

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Key words: MicroRNA; Breast cancer; Therapeutic response; Biomarker

Core tip: MicroRNAs (miRNAs) have been shown to play critical roles in cancer pathogenesis and progression by modulating tumor initiation, growth, metastasis, and therapeutic resistance. In this review, we discuss the recent progress in understanding miRNA function in cancer development and therapeutic response, especially in breast cancer, as well as the potential application of using miRNA signatures as biomarkers for predicting therapeutic response and for personalizing anti-cancer regimens.

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INTRODUCTION

In the human genome, protein coding genes (around 20000) only represent approximately 1.5% of entire DNA sequences^[1]. Since little discernible function was known about the majority of non-coding sequences, they were called “junk DNA”. However, recent data provided by ENCODE project and other research progress

have revealed that a large portion of “junk DNA” may pertain to important biological functions, such as host regulatory DNA sequences, long interspersed elements, short interspersed elements and non-coding RNA genes. Non-coding RNA (ncRNA) comprises several classes of functional RNA transcripts, including ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), long non-coding RNA (lincRNA), small interfering RNA (siRNA) and microRNA (miRNA), which have been shown to exert critical roles such as regulating transcription, stability and translation of protein-coding genes^[2,3]. The most studied ncRNAs to date are miRNAs, owing to their crucial functions in control of varying biological and pathological processes, such as development, cell proliferation, differentiation, programmed cell death and stress response. Most miRNAs are highly conserved across different species, while exhibiting high specificity to tissue/cell types and developmental stages. The involvement of miRNAs in cancer initiation and progression was first reported in chronic lymphocytic leukemia^[4]. Later studies further revealed that miRNAs may serve as either tumor suppressors or oncogenes (also known as oncomiRs) in a variety of cancers, depending on which genes or pathways were regulated/dysregulated by particular miRNA in a specific cancer type^[5,6]. Meanwhile, an individual gene could be regulated by multiple miRNAs, which further underscored that the potential pathological impact of specific miRNA or miRNA cluster may vary in different types of cancer^[5,7,8]. High throughput analyses using expression microarrays or next-generation sequencing have revealed that miRNAs are dysregulated in most types of human cancer^[9,10]. Moreover, the aberrant expression signatures of miRNAs have been suggested to have diagnostic, prognostic, predictive and therapeutic values^[11-14].

As many outstanding reviews have comprehensively described the current understanding of the pathophysiological functions of miRNAs and their involvement in cancer initiation, progression and metastasis^[3,5,6,14,15], here we only summarize the recent progress in these research areas and focus on miRNA function in cancer therapeutic response, especially in breast cancer. The potential application of using miRNA signatures as predictive biomarkers for cancer therapeutic response and how these insights can be used for the development of novel anti-cancer regimens targeting miRNAs are further discussed.

MIRNA GENERATION AND FUNCTION

MiRNAs are a family of single-strand RNAs ranging from 19 to 24 nucleotides, which are predominantly transcribed from the genome as primary miRNAs by RNA polymerase II^[16,17]. The primary transcripts then undergo a multi-step process to generate precursor and mature form of miRNAs. The initial transcription of miRNA genes yields long, capped and polyadenylated primary miRNA transcripts, varying from several hundreds to several thousands of nucleotides. These primary tran-

scripts are subsequently processed by a nuclear microprocessor complex consisting of ribonuclease (RNase) III Drosha and DGCR8 (DiGeorge syndrome critical region 8, also known as Pasha), yielding precursor miRNAs (Pre-miRs)^[18]. Besides the core components of the Drosha microprocessor, several additional proteins, including the DEAD-box helicase proteins p68/DDX17, NF90 and NF45, have been shown to interact with Drosha and facilitate the processing of nearly one-third of pri-miRNAs. Alternatively, pre-miRNAs can also be generated by RNA splicing when the miRNA sequence resides in intron of a protein-coding gene and is co-transcribed with the mRNA of the host gene. Precursor miRNAs form a complex with exportin 5 (XPO5) and Ran-GTP, which is then transported into the cytoplasm. The hairpin structure of pre-miRNAs can be recognized by cytoplasmic type III RNase Dicer which further cleaves pre-miRNAs into double-stranded miRNAs. Then, the miRNA double strands are separated, and the mature miRNA can be loaded into the RNA-induced silencing complex (RISC) along with its target mRNAs, leading to posttranscriptional gene silencing^[19-21] (Figure 1).

In general, miRNAs post-transcriptionally decrease gene expression by inhibiting ribosome-dependent translation and/or destabilizing mRNAs of target genes. Typically, mature miRNAs recognize their target genes through sequence-complementarity within the 3'-untranslated region (3'-UTR) of mRNA to the miRNA seed region (nt. 2-7). However, several lines of evidence indicated that miRNAs can also bind to 5'-UTRs or open reading frames (ORFs) of a target mRNA and repress its expression^[22,23]. By comparison, ORF targeting appears less frequent and less effective than 3'-UTR targeting but still much more frequent than 5'-UTR targeting based on the computational and experimental genome-wide analyses.

The overall function of miRNAs is believed to negatively regulate gene expression. However, recent evidence suggested that certain miRNAs, such as miR-369, can enhance the translation *via* binding to the AU-rich elements of the target gene mRNA^[24]. Moreover, miR-328 was found to indirectly increase the protein output by promoting release of translation-inhibiting hnRNP E2 from the target gene mRNA, thereby de-repressing translation^[25]. In addition, miR-373 was shown to induce expression of genes with complementary promoter sequences, while miR-10a can bind to the 5'-UTR of ribosomal protein gene transcripts and augments their translation^[26]. Nevertheless, in contrast to the well-studied consensus mechanism of miRNA-dependent gene silencing, much remains to be learned about how miRNA up-regulate gene expression.

In addition to intracellular functions, abundant miRNAs are detected in the serum and other biological fluids. Circulating miRNAs are released from source cells into extracellular milieu and can be taken up by recipient cells *via* endocytosis. This process may be mediated by as yet unidentified miRNA-binding membrane receptors on

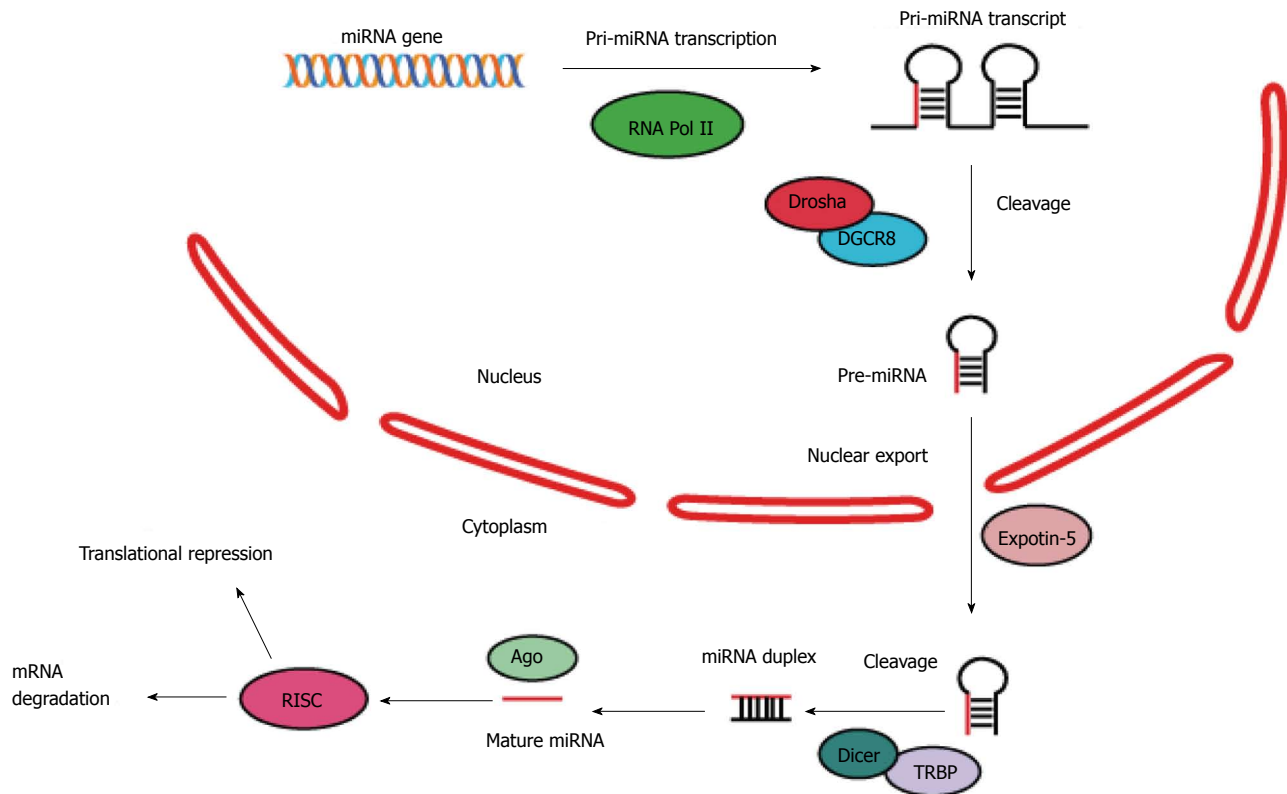


Figure 1 Biogenesis and function of microRNAs.

the recipient cells^[27]. The circulating miRNAs can act on neighboring cells as well as at remote sites in a paracrine and endocrine fashion, suggesting their roles in mediating both short- and long-range intercellular communication^[28,29]. The uptaken miRNA could regulate target gene expression *via* RISC in recipient cells, or induce pro-metastatic inflammatory response by binding to intracellular Toll-like receptors (TLRs)^[28].

MIRNAS IN CANCER DEVELOPMENT

MiRNAs have been shown to play pivotal roles in cancer initiation and progression. It was found that miRNA genes were frequently located within cancer-associated genomic regions, either amplified or deleted, and miRNAs can act either as oncogenes or tumor suppressors, depending on regulated genes and cancer types^[30]. Furthermore, a particular miRNA can exploit both tumor-suppressive and oncogenic functions depending on the cellular context of its target genes in different cancers.

The first evidence that miRNAs may be involved in human cancer pathogenesis was derived from a study aiming to identify tumor suppressors within the 13q14 region which is frequently deleted in chronic lymphocytic leukemia (CLL)^[4]. It was found that, in 69% of CLL patients analyzed, polycistronic miR-15a and miR-16-1 were deleted or downregulated by epigenetic inhibition. Since downregulation of miR-15a/16 is observed in the majority of indolent CLLs, loss of miR-15a and miR-16-1 is likely among the initiating events in the pathogenesis

of this disease^[4]. Subsequent studies revealed additional tumor suppressor miRNAs including the let-7 family and miR-34 family^[15]. These tumor suppressor miRNAs were shown to target varying protein-coding oncogenes for translation inhibition or mRNA decay^[5]. For example, miR-15a and miR-16-1 inhibit the expression of anti-apoptotic gene *BCL2* (B cell lymphoma 2) and *MCL1* (myeloid cell leukemia 1), both of which are well-established oncogenes in hematopoietic cancers.

The tumor suppressive effects of let-7 have been attributed to inhibition of a list of oncogenes, such as *KRAS*, *CCND1*, *CDK6*, *HOXA9*, *TLR4* and *MYC*^[31-33]. Let-7 was also found to negatively regulate self-renewal and tumorigenicity of breast cancer initiating cells through repressing cell proliferation and growth as well as inducing cell cycle exit and terminal differentiation. It has been proposed that cancer stem-like cells or tumor initiating cells may play important roles in breast cancer incidence. Consistently, ectopic expression of miRNAs from the let-7 family was shown to suppress mammary tumor development in mouse models^[33]. Furthermore, by comparing miRNAs in normal stem cells from the mammary gland and cancer stem-like cells from DCIS tumors, miR-140 was found to be significantly decreased in cancer stem-like cells but not in normal stem cells, indicating that miR-140 may play a critical role in preventing malignant transformation of mammary stem cells. The downregulation of miR-140 may be associated with DNA hypermethylation in breast cancer cells. Further investigation found that expression of stem cell marker

SOX9 and ALDH1, which are significantly upregulated in DCIS stem-like cells, are repressed by miR-140. Accordingly, overexpression of miR-140 decreased expression of SOX9 and ALDH1 in ER α -basal-like DCIS mammospheres, and reduced tumor growth *in vivo*^[34]. These studies indicated that tumor suppressive miRNAs may antagonize tumor formation by reducing cancer initiating cell population.

Tumor suppressive miRNAs may also work downstream of coding tumor suppressor genes, which may directly regulate miRNA expression. For instance, tumor suppressive miR-205 can be directly transactivated by the tumor suppressor p53^[35]. Moreover, a study evaluating miR-205 expression in a panel of highly aggressive triple-negative breast cancer cell lines demonstrated that miR-205 is substantially downregulated compared with normal cells. Accordingly, reconstitution of miR-205 expression strongly reduced cancer cell proliferation and tumorigenic potential in cell culture models and inhibited tumor growth in animal models. The miR-205-dependent tumor suppression is attributed, at least in part, to repressing E2F1 and LAMC1, resulting in blockade of cell cycle progression as well as reduced cell adhesion and migration^[35].

Similarly, oncogenes may also promote tumor growth by inhibiting tumor suppressor miRNAs. It was found that estrogen downregulated miR-34b in ER α -positive/p53 wild-type breast cancer cells, as well as in ovarian and endometrial cells, but not in ER α -negative or p53 mutant breast cancer cells^[36]. The negative association between ER α and miR-34b expression levels has also been validated in ER α -positive breast cancer patients. miR-34b was found to inhibit expression of cyclin D1 and Jagged-1 (JAG1) in breast cancer cells. It may also mediate tumor suppressor p53 signaling by repressing oncogenes such as cyclin-dependent kinase 4 (CDK4), MYC and MET^[37]. In addition, overexpression of miR-34b could inhibit tumor growth in an orthotopic xenograft mouse model transplanted with ER α -positive breast cancer cells, but not with ER α -negative or p53 mutant breast cancer cells^[36]. These results suggest that downregulation of miR-34b may play an important role in ER α -driven breast cancer tumorigenesis.

In contrast to the anti-tumorigenic functions described above, oncogenic miRNAs, such as miR-21, miR-17-92 cluster and miR-155, exhibit strong cancer-promoting activity in various human malignancies. As a well-characterized oncomiR, miR-21 was found to be upregulated in almost all cancer types^[38]. *In vivo* studies revealed that cancer cells showed strong addiction to miR-21, whereas inactivation of miR-21 induced complete tumor regression in a pre-B-cell lymphoma model^[39]. In breast cancer, repression of the tumor suppressors such as PTEN, PDCD4 and TPM1 by miR-21 was demonstrated to mediate cancer cell survival and proliferation. A strong correlation between the high expression level of miR-21 and advanced clinical stage, lymph node metastasis and poor prognosis has also been found in

breast cancer patients^[40-42]. It was found that the increased ratio between miR-21 and PDCD4 level in bone marrow of breast cancer patients showed a significant correlation with shortened disease-free survival (DFS) and overall survival (OS)^[43]. Meanwhile, the expression of miR-17-92 cluster, another well-studied oncomiR located within 13q22, was found to be frequently increased in a variety of cancers such as breast cancer, lung cancer, colon cancer and lymphoma, through gene amplification or transcriptional activation^[38]. Interestingly, this miRNA cluster was found to be a direct downstream target of the MYC oncogene, and the increased expression of miR-17-92 could attenuate apoptosis induced by MYC amplification, thereby promoting B cell lymphoma development in a mouse model^[44,45]. These findings suggest that up-regulating miR-17-92 may contribute significantly to the oncogenicity of MYC. In contrast, blockade of miR-17 decreased the breast cancer cell invasion/ migration *in vitro* and metastasis *in vivo*^[46]. Moreover, a series of studies have shown that miR-17-92 cluster acts as an oncogenic gene to promote breast cancer cell invasion and migration through diverse signaling cascades such as Wnt/ β -catenin pathway and miR-17-92/ZBTB4/Sp axis^[46-51]. Likewise, miR-27a was also found to play oncogenic roles through ZBTB/Sp cascade in breast cancer cells. Upregulation of miR-27a has been shown to repress ZBTB10 and Myt-1 expression in breast cancer cells, which promoted cancer cell proliferation^[52]. Inhibiting miR-27a by miR-27a antagmir or anti-cancer drugs, such as betulinic acid, resulted in increased ZBTB10 and decreased Sp family of transcription factors expression in breast cancer cells, thereby leading to consequent growth suppression^[52-54].

Recent studies also demonstrated that differentially expressed miRNAs correlated to specific pathological features in breast cancer, such as tumor grade, disease stage, proliferation index and vascular invasion^[55]. Moreover, a panel of differentially expressed miRNAs have been identified between ER⁺ and ER⁻ breast cancer patients. In this panel, miR-191 and miR-26 are the most significantly upregulated miRNAs, while miR-206 was at the opposite end of spectrum^[54]. In another study, miR-7, miR-128a, miR-210, and miR-516-3p were identified to be significantly associated with ER⁺ luminal signature and breast cancer aggressiveness^[56]. All these studies support the potential application of miRNA profiling in cancer diagnosis and subtype characterization.

MIRNAS IN CANCER METASTASIS

It has been demonstrated that miRNAs also play critical roles in tumor metastasis by regulating the migration and invasion of cancer cells^[57,58]. A pair of basal-like subtype breast cancer-specific miRNAs, miR-221 and miR-222, could promote epithelial-to-mesenchymal transition (EMT), a phenotype strongly associated with cancer invasion and metastasis, by repressing epithelial genes while enhancing mesenchymal gene expression^[59-61]. The transcription of miR-221/222 can be directly upregulated

by FOSL1 (also known as Fra-1), a RAS-activated FOS family transcription factor, and the level of miR-221/222 decreased when MEK (mitogen-activated or extracellular signal-regulated protein kinase) was inhibited, which suggest that miR-221/222 cluster is a transcription target of the oncogenic RAS-RAF-MEK signaling pathway. The miR-221/222-mediated decrease of the mesenchymal signature gene E-cadherin is dependent on repression of trichorhinophalangeal syndrome type 1 (TRPS1), which is a miR-221/222 target and a transcriptional repressor belonging to GATA family. Decrease of TRPS1 could enhance the transcription of zinc finger E-box-binding homeobox 2 (ZEB2), thereby inhibiting E-Cadherin expression while up-regulating Vimentin^[60]. Besides, miR-9 was found to directly repress E-Cadherin expression in breast cancer cells, resulting EMT transition as well as activation of β -Catenin signaling^[62]. The activated β -Catenin promotes VEGF (vascular endothelial growth factor) transcription, which in turn enhances angiogenesis during the secondary tumor formation. Therefore, these data strongly support the hypothesis that miRNAs may contribute to the aggressiveness of breast cancer by promoting EMT.

In contrast, miR-126 decreases the ability of breast cancer cells to remodel the metastatic niche by recruiting endothelial cells from the tumor microenvironment, leading to reduced metastatic colonization^[63]. Meanwhile, miR-335 was found to target the stemness transcription factor SOX4 and extracellular matrix protein tenascin C, resulting in suppression of breast cancer cell metastasis and migration. Consistently, decrease of miR-335 was found in the majority of primary tumors from breast cancer patients who eventually relapsed, which was also associated with poor DMFS^[64]. Interestingly, it was found that treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and selective AHR modulator 6-methyl-1,3,7-trichlorodibenzofuran (MCDF) could induce miR-335 expression and SOX4 downregulation in several breast cancer cell lines, which inhibited cancer cell growth and lung metastasis *in vivo*^[65,66]. In addition, it was found that significant decrease of miR-34a/c was associated with breast cancer metastasis and lymph node invasion. Overexpression of miR-34a/c leads to decreased breast cancer cell motility and invasiveness as well as reduced distal lung metastasis in animal models. Additional studies found that Fra-1 mRNA and protein levels were decreased by miR-34a/c upregulation, indicating that FOSL1 is a downstream target of miR-34a/c. Furthermore, significant decrease of miR-34a in breast cancer metastases inversely correlates with Fra-1 expression, confirming the critical role of miR-34a-Fra-1 axis in regulating metastasis^[67]. As we discussed above, FOSL1/Fra-1-dependent upregulation of miR-221/222 has been linked with aggressiveness of breast cancer^[60], and miR-221/222 may serve as indirect downstream target of miR-34a/c for its anti-metastatic activity. The similar crosstalk between miRNAs and their critical roles in cancer progression have been further supported by other studies. A recent

study showed that miR-22 may suppress expression of anti-metastatic miR-200, which increased the population of breast cancer stem-like cells and promote metastasis in mice. It was found that miR-22 directly targets the TET (Ten eleven translocation) family of methylcytosine dioxygenases, which promotes DNA demethylation of the miR-200 promoter and its transcriptional activation. The negative correlation between miR-22 and TET-miR-200 axis was also confirmed in metastatic breast cancer patients, supporting the clinical relevance of miRNA crosstalk in breast cancer progression^[68].

Some miRNAs can promote oncogenesis in one cancer type while suppressing tumor development in the other. For instance, although the miR-221 has critical roles in antagonizing breast cancer metastasis and aggressive behaviors, overexpression of miR-221 in liver cancer promoted tumor initiation by inhibiting the expression of the tumor suppressor phosphatase and tensin homolog (PTEN)^[69]. On the contrary, miR-221 acts again as a tumor suppressor in erythroblastic leukemia by reducing the expression of the KIT oncogene^[70]. The complex interactions between miRNAs and protein-coding genes in various cancers indicate that therapies targeting both miRNA and protein-coding genes may be required for effectively controlling cancer progression. However, when miRNAs are considered as emerging therapeutic targets, the selection of miRNA may be highly cancer type- and even stage-specific.

MIRNAS IN CANCER THERAPEUTIC RESISTANCE

Therapeutic resistance is the major obstacle of effective cancer treatment, and plays paramount roles in cancer relapse and cancer-related deaths. Previous studies have demonstrated that drug resistance of cancer cells can be mediated by a variety of mechanisms including the removal or detoxification of the drug, upregulation of anti-apoptotic processes, or alteration of drug transporters such that the therapeutic agent cannot gain entry into the target cells or is immediately exported. Recently, dysregulation of miRNAs was found to not only affect cellular processes involved in carcinogenesis, but also have a direct impact on the cancer therapeutic response. A number of oncogenic miRNAs (*e.g.*, miR-9, -155, -21) have been shown to induce chemoresistance *in vitro* by modulating expression of key resistance-associated genes^[5,71-73]. In breast cancer, miRNAs have been implicated to affect the response to different types of treatments, including anti-endocrine therapies, targeted therapies, chemotherapy, and radiotherapy, *via* treatment-specific or shared mechanisms.

Chemotherapy

The importance of ATP-binding cassette (ABC) transporter proteins, including multidrug resistance protein 1 (MDR1/P-glycoprotein/ABCB1), breast cancer resistance protein (BRCP/ABCG2) and multidrug

Table 1 miRNAs involved in chemotherapy response regulation in breast cancer

miRNA	Drug	Target(s)	Ref.
miR-328	Mitoxantrone	BRCP/ABCG2	[75]
miR-21	Doxorubicin	MDR1, PDCD4	[81]
miR-451	Doxorubicin	MDR1	[76]
miR-130/107	Cisplatin	RAD51D	[125]
miR-345	Cisplatin	MDR1	[77]
miR-326	VP16/Doxorubicin	MRP1	[78]
miR-128	Doxorubicin	BMI1, ABCC5	[79]
miR-34a	Docetaxel	BCL-2, CCND1	[83]
miR-125b	Doxorubicin	BAK1	[84]
	Paclitaxel	E2F3	[85]
miR-200b/c	Doxorubicin	ZEB1, PTEN	[86,87]

resistance-associated proteins 1/2 (MRP1/ABCC1, MRP2/ABCC2), in the development of chemoresistance has been well-established^[74]. Recently, miRNAs were reported as key regulators of drug transporter gene expression in cancer cells. For example, miR-328 was shown to negatively regulate BRCP by directly targeting 3'-UTR of ABCG2 and consequently influence drug disposition in breast cancer cells. miR-328-directed downregulation of ABCG2 expression significantly improved response to the chemotherapeutic agent mitoxantrone in a cell culture model^[75]. Besides, several other miRNAs have been found to target MDR1, including miR-451^[76], miR-7, miR-345^[77], and miR-326^[78], all of which increased cancer cell sensitivity to numerous chemotherapeutic agents. In addition, miR-128-dependent repression of BMI1 and ABCC5 (MRP5) has been implicated in the improved response to doxorubicin treatment, both *in vitro* and in breast cancer patients^[79]. Reduction of miR-128 leads to upregulation of Bmi-1 and ABCC5 in breast tumor initiating cells, which contributes to chemotherapeutic resistance in breast cancer. Ectopic expression of miR-128 sensitizes breast tumor initiating cells to the pro-apoptotic and DNA-damaging effects of doxorubicin, indicating the potential therapeutic value of miR-128 in reducing drug resistance. Moreover, miRNAs may indirectly repress drug resistance gene expression by repressing their upstream activators. It was reported that miR-137 was significantly decreased in cancer cells resistant to doxorubicin. Further investigation revealed that miR-137 can directly repress the expression of constitutive androstane receptor, a nuclear receptor promoting MDR1 gene transcription, thereby downregulating MDR1 expression^[80]. In contrast, miR-21 may upregulate MDR1 expression through repressing PDCD4, a miR-21 target gene, leading to reduced apoptosis and chemotherapeutic resistance^[81]. The increase of MDR1 may be associated with decreased interaction between PDCD4 and eIF4A, resulting in enhanced protein translation.

It has been recognized that one miRNA could simultaneously target multiple genes sharing the same miRNA-recognition sequence, and these genes could be involved in a specific signaling network and play additive or synergistic roles in regulating cellular processes, such as

apoptosis, proliferation or cell survival processes. For instance, chemotherapeutic drugs could upregulate miR-21 expression in breast cancer cells, which in turn inhibits chemodrug-induced apoptosis and promotes therapeutic resistance. Both PTEN and PDCD4 were identified as miR-21 target genes in this experimental setting, whose downregulation could promote cancer cell survival and proliferation^[82]. Moreover, miR-34a was found to regulate human breast cancer cell response to docetaxel through targeting BCL-2 and CCND1^[83]. Furthermore, miR-125b was found to inhibit pro-apoptotic BCL-2 antagonist killer 1 (BAK1) expression. Upregulation of miR-125b in breast cancer cells could markedly inhibit taxol-induced cytotoxicity and apoptosis, and consequently increase the resistance to taxol^[84]. In parallel, miR-125b directly targets the E2F3 gene, which regulates cell proliferation and apoptosis^[85]. Interestingly, circulating miR-125b in breast cancer patient serum may also serve as a biomarker predicting chemoresistance. Tumor samples from breast cancer patients with high levels of circulating miR-125b showed increased cancer cell proliferation and decreased apoptosis. Accordingly, ectopic miR-125b expression correlated with increased resistance to anticancer drugs, whereas suppressing miR-125b level improved breast cancer cell sensitivity to chemotherapy^[85]. Additional studies also revealed that inhibition of miR-21 and miR-200b enhanced sensitivity to doxorubicin in breast cancer cells, which may be mediated by repression of PDCD4 and PTEN, as well as upregulation of pro-apoptotic genes due to ZEB1 inhibition, respectively^[81,86,87].

All these findings indicate that miRNAs may regulate cancer cell response to chemotherapeutic drugs by targeting multiple genes, simultaneously or in parallel, which lead to complex changes in varying cellular processes, such as proliferation and apoptosis. A list of miRNAs involved in sensitivity to chemotherapy in breast cancer can be found in Table 1.

Anti-endocrine therapy

Anti-estrogen therapy is prescribed as standard treatment for ER-positive breast cancer and tamoxifen has been shown to reduce mortality in these patients by 31%^[88]. However, many patients subsequently display resistance and clinical progression, and the involved mechanisms have not been fully understood. Multiple miRNAs have been implicated in mediating anti-estrogen therapy resistance by modulating ER α expression, receptor tyrosine kinase signaling, cell survival signaling, and apoptosis^[89,90]. MiR-221/222 were shown to be key regulators of anti-endocrine resistance *in vitro*, and the resistance could be achieved by downregulation of cell-cycle inhibitor p27/Kip1 and ER α ^[91,92]. Overexpression of miR-221/222 promoted resistance to tamoxifen in MCF-7 cells. Moreover, knockdown of miR-221/222 sensitized ER⁺ breast cancer cells to tamoxifen-induced cell growth arrest and apoptosis. The protein level of p27/Kip1, which has been demonstrated as a direct target of miR-221/222, decreased along with miR-221/222 overexpression in

Table 2 miRNAs involved in antiendocrine therapy response in breast cancer

miRNA	Drug	Target(s)	Ref.
miR-221/222	Tamoxifen	P27Kip1 ER α	[91]
	Fulvestrant	TGF- β	[93]
miR-181b	Tamoxifen	TIMP3	[94]
miR-30c	Tamoxifen		[96,97]
miR-342	Tamoxifen	cyclin B1	[126]
miR-451	Tamoxifen	14-3-3 ζ	[127]
Let-7	Tamoxifen	ER- α 36	[128]
miR-128a	Letrozole	TGF α R1	[129]

MCF-7 cells. Additionally, ectopic p27/Kip1 increased cell death in the endocrine resistant cells exposed to tamoxifen. In parallel, overexpression of miR-221/222 in MCF-7 and T47D cells decreased ER α protein, but not mRNA, level, while miR-221/222 depletion partially rescued ER α downregulation in these cells^[92]. Besides, the miR-221/222-mediated resistance to fulvestrant, a drug inducing ER α degradation, was also attributed, at least in part, to the activation of β -catenin and estrogen-independent growth of breast cancer cells^[93]. In an *in vivo* study using miRNAs as therapeutic targets, it was found that when anti-miR222 and -181b were directly delivered into mammary tumor xenografts, the growth of tamoxifen-resistant xenografts was suppressed^[94]. The authors subsequently found that miR-221, -222, and -181b all target TIMP3 directly, and knockdown of TIMP3 in MCF7 cells enabling tumors to grow in the presence of tamoxifen. A significant association was found between the expression level of miR-221 and hormone receptor (HR) status in breast cancer patients. High plasma miR-221 level was associated with HR-negative genotype as well as poorer overall response rate to neoadjuvant chemotherapy and shorter overall survival. Additional studies also found that plasma miR-221 may have predictive value in assessing chemoresistance in patients with breast cancer who received neoadjuvant chemotherapy^[95].

In contrast, miR-30c was shown to be associated with a better response to endocrine therapy in advanced ER+ breast cancer. Higher expression level of miR-30c was found in patients with longer progression-free survival (PFS) after tamoxifen treatment, which may be attributed to inhibited HER and RAC1 signaling pathways^[96]. It was also found that miR-301 was upregulated in mammary tumor *vs* normal tissue. Moreover, miR-301 expression increased again in patients that relapsed following tamoxifen treatment, but not in those who remained relapse-free^[97]. Consistently, downregulation of miR-301 in tamoxifen-resistant cells restored the sensitivity to the drug. A summary of miRNAs involved in sensitivity to antiendocrine therapy in breast cancer can be seen in Table 2.

Targeted therapy

Targeted therapy has brought great success in treating

selected subtypes of breast cancer, but the subsequent drug resistance is not rare. Compared with chemotherapy and endocrine therapy, much less was known about the mechanisms involved in resistance to targeted therapies. It has been shown that hyperactivation of the PI3K-Akt pathway played a critical role in HER2⁺ breast cancer resistance to trastuzumab (a therapeutic antibody targeting HER2/ERBB2)^[98], and miR-21-mediated PTEN repression may contribute to the increased Akt activation in trastuzumab-resistant breast cancer cells^[99]. Resistant cells can be re-sensitized to the trastuzumab treatment by blocking miR-21 with antisense oligonucleotides, which leads to proliferation inhibition and G₁-S cell cycle arrest. Consistent resensitizing effect of miR-21 inhibitor can be also observed in breast cancer xenograft models, while miR-21 mimics promoted resistance to trastuzumab. Importantly, upregulation of miR-21 was found in tumor biopsies obtained from patients receiving trastuzumab treatment, which associated with poor therapeutic response, indicating a potential role of miR-21 level in predicting trastuzumab response. On the contrary, restoration of miR-205 expression, which is downregulated in breast tumors, was found to sensitize breast cancer cells to gefitinib and lapatinib, two tyrosine kinase inhibitors targeting downstream signaling of HER2^[100]. The authors further identified HER3 as a direct target of miR-205 in breast cancer cells. HER3 is a critical partner of HER2 to activate downstream tumorigenic signaling pathways, and HER3 upregulation has been implicated as a potential mechanism in breast cancer resistance to HER2-targeting therapies^[101-103]. Therefore, reintroduction of miR-205 likely could improve the response to HER2-targeted therapy by silencing HER3^[100]. Moreover, plasma miR-210 has been found to correlate with sensitivity to trastuzumab, tumor presence, and lymph node metastases in breast cancer patients. High baseline circulating miR-210 level in patients before receiving neoadjuvant chemotherapy combined with trastuzumab significantly correlated with pathologic complete response, suggesting that miR-210 may serve as a predictive marker for better response to regimens combining trastuzumab^[104].

PARP1 [poly (ADP-ribose) polymerase] inhibitors hold high promise as a novel targeted therapy for treating cancers harboring BRCA1/2 mutation, such as breast and ovarian cancers^[105]. Recent studies have demonstrated that inhibiting PARP1 results in synthetic lethality in cancers with defect in DNA repair due to BRCA1/2 mutation, which highly overlap with basal-like triple negative breast cancer (TNBC)^[106]. Interestingly, miR-182 has been shown to sensitize breast cancer cells to both radiotherapy and to PARP1 inhibitors by repressing BRCA1 expression and inhibiting DNA repair^[107]. Antagonizing miR-182 in breast cancer cells increases BRCA1 protein level, leading to reduction of IR-induced cell death and resistance to PARP1 inhibitors. These results suggested that inhibiting miR-182 may further enhance the therapeutic efficiency of PARP1 inhibitors in combination with genotoxic therapies.

Radiotherapy

Besides targeted therapy and chemotherapy, miRNAs may also modulate cancer cell response to radiotherapy. For example, inhibition of miR-155 was shown to sensitize breast cancer cells to ionizing radiation^[108]. Likewise, anti-miR-21 treatment decreased breast cancer cell survival after irradiation, which was associated with failed G₂/M checkpoint upon DNA damage^[109]. Cell cycle arrest at the G₂/M checkpoint is essential for DNA repair machinery after DNA damage, whose failure likely will increase apoptosis upon genotoxic stress. It was found that miR-21 expression was transiently increased after irradiation in T47D cells and MDA-MB-361 cells, which was required for proper cell cycle arrest in these cells after irradiation. These observations indicated that miR-21 may contribute to radiation resistance in breast cancer cells through enhancing cell cycle checkpoint and subsequent DNA repair^[109]. Similarly, increased miR-34a has been linked to poor response to radiotherapy in breast cancer cells^[110]. It appeared that miR-34 promotes apoptosis while antagonizes non-apoptotic cell death in nematodes upon radiation, which was also confirmed in human breast cancer cell lines. Moreover, it has been shown that miR-34 is a target gene of p53 and can be upregulated in response to radiation^[111]. Thus, inhibiting miR-34 with antagonist may be of potential therapeutic utility for sensitizing p53-mutant breast cancer to radiotherapy.

MIRNA AS CANCER THERAPEUTICS

miRNA therapeutics can be devised to downregulate or block the function of pathogenic miRNAs as well as to upregulate the expression of disease-defensive miRNAs. For instance, miravirsin is a locked nucleic acid-modified DNA phosphorothioate antisense oligonucleotide that forms a highly stable heteroduplex with mature miR-122, resulting in its inactivation. It is one of the earliest miRNA-targeting therapeutics approved for clinical trials and has recently shown positive results in treating hepatitis C infection in a phase II trial^[112]. In chronic HCV infection patients, miravirsin treatment reduced HCV RNA level in blood samples in a dose-dependent manner without obvious viral resistance.

Since the pathogenesis of cancer involves a number of gene mutations, amplifications and deletions, usually an effective cancer therapeutic regimen needs to target multiple genes involved in same and/or parallel functional networks as well as several signaling cascades. A significant advantage of using miRNAs as therapeutic targets lies in that one miRNA generally has multiple coding genes or non-coding RNAs as targets, which may be involved in a single pathway or in parallel pathways regulating cancer progression^[6]. Compared with the strategy using siRNAs, which are usually aiming to repress one specific target gene, miRNA therapeutics appear to be superior to a mixture of siRNAs. For example, several genes associated with the epidermal growth factor recep-

tor (EGFR) signaling pathway, such as p38 mitogen-activated protein kinase (p38), signal transducer and activator of transcription 3 (STAT3) and AKT2, are miR-124 targets^[113]. As aberrant activation of EGFR signaling has been found in a number of human malignancies, such as lung and breast cancers^[114], it is plausible that enhancing miR-124 expression may concurrently repress the expression of p38, STAT3 and AKT2, thereby effectively inactivating the EGFR signaling pathway.

Antagomirs are synthetic RNAs with a 2' -O-methyl linkage and phosphorothioate modification and conjugated to cholesterol. They can be used as miRNA antagonizers by complementarily binding to the targeted miRNA, thereby deblocking other endogenous miRNA target gene expression. It was found that miR-10b antagomirs remarkably reduced the lung metastasis formation in a breast cancer xenograft model. Further investigation demonstrated that silencing of miR-10b with antagomirs significantly decreased miR-10b levels and enhanced expression of Hoxd10, a miR-10b target gene playing critical roles in breast cancer metastasis^[115]. Another type of miRNA inhibitors, miRNA sponges, are RNA traps that are constructed with tandem binding sites complementary to the seed sequence of the miRNA of interest. It was shown that a single sponge inhibitor can block an entire miRNA family sharing the identical seed sequence. In a study using miRNA sponges targeting miR-9, it was found that sponge-dependent miR-9 inhibition dramatically reduced mouse breast cancer cell lung metastasis, potentially by alleviating miR-9-modulated E-Cadherin downregulation and inhibiting EMT^[62].

Although the preclinical studies implied high promise of using miRNA therapeutics in cancer treatment, there are also significant challenges remaining to be overcome before practical clinical applications of miRNA-targeted therapy^[6]. First of all, miRNA activity highly depends on the cellular context and cancer types. Different target genes may be repressed by the same miRNA in different cell types and consequently opposite biological effects may occur within the same organism. Consequently, targeting a specific miRNA may be beneficial in one cell type but harmful in another. Thus, selective delivery of miRNA therapeutics to target tissues is a major obstacle for effective miRNA-based therapy and minimal side effects. A specific and efficient delivery system that targets only cancer cells has yet to be developed. Moreover, double-stranded RNAs (≥ 21 base pairs) can elicit a sequence-independent interferon response^[116]. Since macrophages and monocytes were found to remove complexed RNAs from extracellular spaces, systemically delivered miRNAs or RNA-based miRNA inhibitors might trigger and be eliminated by the host immune response^[117]. Nevertheless, the recent advance of using antibody-conjugated nanoparticles to encapsulate miRNA agents may represent a promising future of cancer cell-selective delivery approach with minimal adverse immune response in patients^[118].

Beyond being directly delivered as pharmacologi-

cal intervention, miRNAs may also serve as mediators for carrying out tumoricidal effect of other anti-cancer agents. For instance, Enoxacin, an antibacterial compound, was found to inhibit cancer growth through upregulating tumor suppressive miRNA production^[119]. Similarly, curcumin analogue CDF was shown to inhibit pancreatic tumor growth *in vitro* and in xenograft models, which involved CDF-induced EZH2 downregulation and consequent activation of tumor suppressor miRNAs, such as let-7, miR-26a, and miR-101^[120]. It is worth noting that induction of miRNAs may lead to complicated pathological responses in cancer cells, which can be exemplified by the observation that retinoid (ATRA)-induced miR-21 upregulation in MCF-7 cells reduced cell motility but promoted cell proliferation^[121]. Meanwhile, general inhibition of miRNA biogenesis and function by small molecules was shown to reverse tumorigenesis^[122]. Therefore, the pharmacological effects of conventional drug-induced miRNAs in cancer treatment are highly drug- and cancer type-specific.

As combination therapies have been proved to be more effective than single agents in various cancer regimens, it is not surprising that combinations of varying miRNA agents as well as along with conventional cytotoxic drugs or molecularly targeted agents have been studied in treating cancers. It was shown that overexpression of miR-30c, which is a favorable prognostic marker in breast cancer, sensitized TNBC cells to doxorubicin treatment in an animal model. The chemo-sensitizing effect of miR-30c was attributed to the downregulation of miR-30c target gene, twinfilin 1, which promotes EMT and induces drug resistance by upregulating IL11^[123]. Similarly, adenoviral delivery of miR-145 along with 5-fluorouracil achieved greater tumor reduction than using 5-fluorouracil alone in breast cancer models^[124]. These preclinical studies suggest that selective miRNA therapeutics may be applicable as sensitizing agents in clinic when used in combination with conventional therapies.

CONCLUSION

Dysregulated miRNAs in breast cancer play critical roles in the cancer initiation and progression. However, the signatures of miRNA alteration may also help to stratify patients into different risk groups as well as to predict therapeutic response. Accumulating evidence support the notion that miRNAs may serve as both therapeutic targets and tools in anticancer therapy. We envision that novel miRNA therapeutic approaches will be developed and approved for clinical application in the near future, which are combined with chemotherapy, anti-endocrine therapy or radiotherapy, based on small RNAs regulated pathway. To this end, better understanding of the regulatory mechanisms involved in miRNA biogenesis and function is pivotal for developing successful application of miRNA therapeutics. Although there are still big challenges in developing miRNA-based therapy, such as the effective delivery with high selectivity and safety evaluation,

miRNAs have emerged with great potential as diagnostic/prognostic biomarkers as well as promising therapeutic targets in cancer treatment.

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Update on prevention and screening of cervical cancer

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Abstract

Cervical cancer is the third most common cause of cancer in women in the world. During the past few decades tremendous strides have been made toward decreasing the incidence and mortality of cervical cancer with the implementation of various prevention and screening strategies. The causative agent linked to cervical cancer development and its precursors is the human papillomavirus (HPV). Prevention and screening measures for cervical cancer are paramount because the ability to identify and treat the illness at its premature stage often disrupts the process of neoplasia. Cervical carcinogenesis can be the result of infections from multiple high-risk HPV types that act synergistically. This imposes a level of complexity to identifying and vaccinating against the actual causative agent. Additionally, most HPV infections spontaneously clear. Therefore, screening strategies should optimally weigh the benefits and risks of screening to avoid the discovery and needless treatment of transient HPV infections.

This article provides an update of the preventative and screening methods for cervical cancer, mainly HPV vaccination, screening with Pap smear cytology, and HPV testing. It also provides a discussion of the newest United States 2012 guidelines for cervical cancer screening, which changed the age to begin and end screening and lengthened the screening intervals.

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Key words: Cervical cancer; Cancer screening; Pap smear; Human papillomavirus; Papillomavirus vaccines

Core tip: Screening is the best method to prevent cervical cancer. Screening strategies should weigh the benefits and risks of screening to avoid discovery and needless treatment of transient human papillomavirus (HPV) infections. Current United States guidelines recommend Pap smear screening with conventional or liquid-based method no frequent than every 3 years, or every 5 years in women greater than age of 30 if done in conjunction with HPV testing. Screening is not recommend in females younger than 21 years, regardless of age at initiation of sex. In this population, options for prevention include HPV vaccination and decreasing other risk factors associated with HPV infection.

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INTRODUCTION

The World Health Organization estimates that yearly, about 530000 women worldwide are identified with cervical cancer and 275000 women die from the disease^[1]. Cervical cancer is heralded as being the third most common cause of cancer among women in the world and

the second most common form of cancer in women in the developing world^[2]. Cervical cancer is responsible for the largest cause of mortality in women due to cancer in most developing countries.

There has been a large decline in the incidence and death rate of cervical cancer in industrialized countries observed during the past few decades. This unfortunately, has not been mirrored by a similar decline in developing nations. An example of this is illustrated by the 70% decrease in mortality caused by cervical cancer in the United States from 1955 to 1992. Each year this initial decline in death caused by cervical cancer has been sustained at a rate of a 3% decrease in the incidence of cervical cancer^[2]. Similarly, in the United Kingdom there has been a 70% decline in the mortality caused by cervical cancer recorded in 2008 than was reported 30 years prior^[2]. In industrialized nations the age-adjusted incidence of cervical cancer is 10 out of 100000 per year; however in developing nations the incidence of the disease can be as high as 40 out of 100000. By 2030, it is expected that cervical cancer will be responsible for the death of 474000 women annually with over 95% of these deaths anticipated to occur in low- and middle-income countries (LMICs)^[3].

HPV infection and cervical cancer

Infection with HPV is the main causative agent in cervical cancer. The latest estimation of the number of genotypes of HPV was 200 with 18 genotypes that are directly related to cervical cancer^[4,5]. The fifteen HPV types that have a strong oncogenic potential include HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82. These high-risk HPV types account for 95% of all cervical cancer.

It has been found that greater than one HPV type can exist in pre-invasive and invasive cervical cancer^[6]. This imposes a level of complexity in identifying which one is the actual causative agent, with various genotypes depending on geographical regions. While high-risk HPV 16 and 18 are accountable for around 90% of all cervical cancer^[7], there is greater than average presence of subtypes 31 and 45 detected in the developing world^[8]. There is also a prominent presence of HPV 58 associated with pre-invasive lesions in women in various countries, including Thailand, Uganda, Zambia and Cameroon^[9-12].

The most carcinogenic HPV genotype is HPV 16, which mostly causes squamous cell carcinoma. HPV 18 mostly causes adenocarcinoma, a cancer that is less frequently found but more aggressive, resulting from the endocervical glandular^[13]. However, cervical carcinogenesis may arise from infections with many high-risk types that act synergistically^[14]. The Bethesda classification encompasses the biological behavior of cervical squamous intraepithelial lesions (SILs)^[8]. The classification system partitions abnormal squamous epithelial cells into four categories: (1) atypical squamous cells of undetermined significance (ASCUS); (2) low grade squamous intraepithelial lesions (LSILs), including light dysplasia/cervical intraepithelial neoplasia (CIN) 1 in addition to HPV associated cell changes; (3) high-grade squamous intraepithelial le-

sions (HSIL), encompassing moderate dysplasia/CIN 2, severe dysplasia, and carcinoma *in situ*/CIN 3; and (4) squamous cell carcinoma^[8]. Almost 90% of infections with HPV clear on its own within 1-2 years^[15]. High-grade cervical intraepithelial lesions that are classified as CIN 2 have a 40% chance of regression. High-grade cervical intraepithelial lesions that do not regress are categorized as CIN 3. These lesions have a 30% probability of progression to invasive cervical cancer^[16]. HPV 16 is the most persistent infection and the type that is most likely to progress to CIN 3, carcinoma *in situ*, and invasive cervical cancer. HPV negative cervical cancer is extremely rare, but it has been found. This form of cervical cancer is believed to be due to an artifact caused by limitations in the current detection methods or perhaps due to the loss of HPV DNA during the progression to cancer.

Risk factors for cervical cancer

Sexually transmitted infection with HPV is the strongest risk factor for development of cervical cancer. There are multiple risk factors that have been connected with the acquisition of HPV infection and cervical cancer (Table 1). HPV acquisition is most dependent on genital contact. This prominent risk increases with higher number of sexual partners of a woman or her partner^[17-19]. Other sexual and reproductive risk factors associated with HPV infection and cervical cancer include: initiation of sexual activity at an early age (≤ 18 years), earlier age at first full-term pregnancy (< 18 years), high parity (4 or greater vaginal deliveries), use of combined hormonal oral contraceptives for longer than 5 years, and a history of other sexually transmitted infections [e.g., chlamydia, human immunodeficiency virus (HIV), herpes simplex 2]^[18,19]. The use of tobacco, both current and past, increases the risk of squamous cell cervical carcinoma, and the risk rises with quantity of cigarettes smoked per day and number of years smoked^[19]. Infection with HIV is strongly associated with incidence and persistence of HPV infection, and advancement to invasive cervical cancer from squamous intraepithelial lesions^[19]. In fact, cervical cancer is one of the acquired immunodeficiency syndrome (AIDS)-defining illness, *i.e.*, a person with HIV who develops cervical cancer is considered to have AIDS. The acquisition of HPV is most dependent on contact with the genital skin and condom use is associated with reduced cervical cancer risk^[19]. However, condom use is only 70% effective in averting the transmission of HPV since there is remaining contact with genital skin that is not covered by the surface of the condom^[17]. In summary, counseling for tobacco cessation, delaying initiation of sexual intercourse, using condoms, and decreasing number of sexual partners may prevent HPV infection and help to reduce the risk of cervical cancer.

CERVICAL CANCER PREVENTION WITH HPV VACCINATION

Another potential way to prevent cervical cancer is the

Table 1 Cervical cancer risk factors

Cervical cancer risk factors ^[17-19]
Genital Infection with high risk human papillomavirus
HIV infection
Smoking
Younger age at first sexual intercourse
Greater number of sexual partners
Oral contraceptives use greater than 5 yr
Having 4 or greater full-term pregnancies
History of sexual transmitted diseases

HIV: Human immunodeficiency virus.

use of HPV vaccination to prevent high risk HPV infection and subsequent cervical carcinogenesis. The Food and Drug Administration (FDA) approved in 2006, Gardasil, a recombinant quadrivalent HPV vaccine. This vaccine has the capability of preventing infection with HPV 16 and 18 in addition to HPV 6 and 11, and it is targeted for use in females 9-26 years of age^[20]. It has been marketed as having the ability to prevent genital warts as well as cervical cancer when given in three vaccinations, at months 0, 1 to 2, and 6^[21]. Gardasil also has the capability to convey protection against vulvar, vaginal cancer and intraepithelial neoplasia, and recently, for the deterrence of genital warts in males age 9-26 years^[6]. Short to medium clinical studies show the capability of Gardasil to protective against HPV-16 and 18 infections and its associated precancerous lesions for up to 5 years post vaccination^[6,8,13].

In 2008, a second vaccine, Cervarix, the HPV bivalent vaccine targeting HPV 16 and 18 was approved^[22]. Cervarix is indicated for use in females aged 10 to 25 years when given in three vaccinations at months 0, 1 to 2, and 6^[23]. Cervarix is effective against anogenital warts caused by HPV, precancerous lesions, and cervical cancer^[6]. Short to medium clinical studies show Cervarix conveys protection against HPV-16/18 and its associated precancerous lesions for 6.4 years post vaccination^[2,10,15,20].

The two HPV vaccines, Gardasil and Cervarix, are currently approved in over 100 countries. In their individual trials, the efficacy of Cervarix in protecting against cervical cytologic abnormalities in HPV-naïve women is slightly higher than Gardasil^[19]. Cervarix also seems to have higher cross-protection against other nonvaccine HPV types, as evidenced by its higher reduction in excisional treatments for CIN 2/3 disease compared to Gardasil, and its efficacy in decreasing incidence of genital warts caused by HPV 6, 11, and 74^[19]. However, clinically significant differences in efficacy of Gardasil *vs* Cervarix is difficult to discern and will not be apparent for many years. Researchers believe that the differences will be revealed with longer-term evaluations of women that were vaccinated in countries with population-based registries that can track HPV associated cervical lesions^[8].

There are a cluster of symptoms that have been reported most frequently in correlation to administration

of the HPV vaccines including pain where injected (78%), ecchymosis (17%), fainting (15%), and swelling (14%). These side effects have been reported most commonly in younger than older girls^[24].

Routine HPV vaccination of girls is recommended by the Centers for Disease Control and Prevention's Advisory Committee on Immunization Practices (ACIP) at 11 to 12 years of age with catch-up vaccinations at 13 to 26 years of age^[25] (Table 2). However, the American Cancer Society has not found enough research evidence to recommend for or against routine vaccination of females age 19 to 26 years^[24]. Young women are the targeted group because immunological response is greatest in girls aged 10-15 years, and the vaccine has greatest efficacy in girls who haven't initiated sex^[26]. Estimations have been made that only 7% of students in United States high schools report having started sexual intercourse prior to 13 years of age^[26]. In the developing world there is a much variation in the prevalence of virginity and the age which women marry. Therefore, international vaccination programs may have to change according to their country's conditions and traditions^[27].

Barriers to implementation of HPV vaccine

The acquisition of immunity of the entire population or herd immunity has been met by a great deal of challenges. Advocates for the vaccine estimate that approximately 70%-80% of girls that are pre-pubertal are required to be vaccinated to obtain herd immunity. This level of immunity will be hard to reach in light of the fact that many conservatives in the US have described the drug as "the promiscuity vaccine" and have imposed their fears that inoculating preteen girls will disrupt their message of abstinence from pre-marital sexual intercourse *via* what they have called the "disinhibition effects"^[28]. All of this political rhetoric has resulted in a shift in public opinion of the vaccine and resulted in a decline in the percentages of parents that are in favor of the vaccine. Interestingly, the intention to vaccinate with HPV is greatest when the vaccine is depicted that it is free or cheaply available and that it prevents cancer, rather than preventing an infection that is sexually transmitted^[25]. Studies show that there are still realist barriers in place as it pertains to the cost of the vaccine as well as the stigma that is attached to it^[27].

Advocates for the HPV vaccination also believe that herd immunity will only authentically be obtained when there is the existence of a gender-inclusive vaccination policy^[28]. There is a belief that men play a pivotal role as carriers of HPV. However, there has been a limited amount of clinical trials that have been carried out on boys as it pertains to HPV vaccinations. This fact is even reflected in the lack of attention given to administering HPV vaccines to boys and men in United States newspapers^[29]. Positive strides have been made with regards to boys and men immunization when the ACIP approved the non-routine vaccination of Gardasil in boys age nine to 18 years for the purpose of preventing genital warts^[29]. While it is known that males represent a reservoir for fe-

Table 2 Recommendations for human papillomavirus vaccination by the Advisory Committee on Immunization Practices

Population	Recommendation for HPV vaccination
Females 11-12 yr of age	Routine vaccination with 3 doses at 0, 1-2, and 6 mo of either HPV2 or HPV4. Can be initiated as early as age 9 and be given up to age 26
Females 13-26 yr of age	Catch up immunization with 3 doses at 0, 1-2, and 6 mo of either HPV2 or HPV4
Males age 11-12 yr	Routine vaccination with HPV4 with 3 doses at 0, 1-2, and 6 mo. Can be initiated as young as age 9 and be given up to age 26
Female or males with inadequate dose of HPV vaccine	Minimum time between 1 st and 2 nd vaccine doses is 1 mo. Minimum time between the 2 nd and 3 rd vaccine doses is 3 mo. Insufficient receipt of HPV vaccine due to shorter than the recommended dosing interval should be re-administered
Females or males with interrupted vaccine schedule	HPV vaccination does not need to be restarted. The 2 nd dose should be administered as quick as possible if delayed after the 1 st dose. The 2 nd and 3 rd dose should be separated by 3 mo. If just the 3 rd dose is late, it should be given as soon as possible

HPV: Human papillomavirus vaccine; HPV2: Bivalent human papillomavirus vaccine (Cervarix); HPV4: Quadrivalent human papillomavirus vaccine (Gardasil).

male HPV infections, HPV vaccination in boys is controversial because there is no proof that it is cost-effective^[29].

While the controversy over the cost-effectiveness of the vaccine in males as well as the debate surrounding the use of the vaccine in young girls continue, some question the true effectiveness of the HPV vaccine. The Females United to Unilaterally Reduce Endo/Ectocervical Disease (FUTURE) trials that validated the effectiveness of the vaccine were only conducted over a three-year timeline^[21]. However, the average time from carcinogenic HPV infection to invasive cervical cancer, if it happens, is at least 25-30 years^[22,30]. Furthermore, it takes approximately five to seven years from acquisition of HPV infection and the first incidence of a pre-invasive cervical lesion^[22]. As a result of this reasoning, some argue that to declare that the vaccine averted the occurrence of cervical lesions after only a few years of follow-up has the potential to be misleading.

Another factor that concerns the international community is the presence of serotypes that are not targeted by the two HPV vaccines^[31,32]. For example, the quadrivalent vaccine prevents infection from HPV 16, 18, 6 and 11, and the bivalent vaccine targets HPV 16 and 18, however, there are other genotypes of HPV that are prevalent in other geographical regions. Consequently, a daunting question is imposed on the effectiveness of the current vaccines in these other regions.

CERVICAL CANCER SCREENING

The ultimate objective of cervical cancer screening is to find high-grade cancer precursor lesions and early asymptomatic invasive cervical cancer, while avoiding the discovery and needless treatment of fleeting HPV infection and its resultant benign lesions. Since the majority of HPV infections and many CIN 1 and CIN 2 cases are transient, there is a large margin for harm that is associated with discovering these fleeting lesions, including mental stress, physical discomfort incurred from extra diagnostic and treatment measures (*e.g.*, vaginal pain, bleeding, infection), and a higher risk of maternity complications such as preterm delivery after treatment^[33,34].

The systemic screening with the Papanicolaou cy-

tological test (Pap smear) to find pre-invasive cervical lesions and early stage cancer has drastically reduced the incidence and death from cervical cancer in the United States and other industrialized nations^[34]. However, cervical cancer still produces much morbidity and mortality in certain sub-populations. In the United States, approximately one-half of cervical cancer is diagnosed in women who were never screened. Groups of the population that participate least frequently in Pap smear include: women who are less educated, older, uninsured, or homeless; migrant workers who face language barriers; and lesbians^[24]. The segment of the United States population at highest risk for cervical cancer is Hispanic and African American women. Fortunately, these populations have benefited from community-based awareness raising programs, which have successfully resulted in a decline in their prevalence of cervical cancer^[35]. It is then practical to reason that programs similar to the ones implemented on Hispanic and African-American women should be applied to the various groups of the population where the women are at greater risk to having cervical cancer due to their lack of compliance with Pap smear screening.

Cervical cytology tests

There are two forms of Pap smears, conventional and liquid-based cytology. In the conventional method cells are obtained from the neck of the cervix and then the cells are spread on a glass slide. In the liquid-based cytology method, the cells are obtained from the neck of the cervix, but instead of being spread on a glass slide, they are placed in a small glass vial that contains preserving fluid. There has been much debate with regards to which form is superior. Current evidence indicates that no clinically important differences in sensitivity or specificity exists when comparing liquid-based and conventional cytology^[36]. The United States Preventive Services Task Force (USPSTF) considers both of these methods to be of substantial net benefit when they are administered in the appropriate age groups at the recommended interval^[37].

HPV testing

Although the Pap test has proven to be a greatly effec-

tive tool for screening in countries that have the capacity to implement it to the majority of its population, one problem with the test is its high rate of false positive cytology^[38]. The higher understanding of the correlation between HPV and cervical cancer led to the development of molecular tests for HPV with greater sensitivity (approximately 90 percent)^[39]. However, it has slightly reduced specificity for CIN2 and CIN3 when compared with cytology. The currently available DNA test detects only the high-risk HPV types, and has greater reproducibility than cytology. The HPV test is a solution hybridization that has the capacity to amplify the DNA signal in the assays of the 13 HPV high-risk types^[14]. The HPV test should be performed only in women age 30 years or more because women less than 30 years have a high prevalence of transient infection and a low prevalence of underlying high-grade lesions^[37]. Therefore, HPV DNA testing in women under the age of 30 can lead to unneeded evaluation and overtreatment^[37].

At the present time HPV DNA testing has the highest sensitivity, which can additionally be used with Pap smears (co-testing) for optimizing diagnosis of high-grade cervical intraepithelial neoplasia^[39]. In women with mild or borderline abnormal Pap results, a Pap-plus-HPV test may be better, since a negative HPV DNA test has the potential to assure women that their Pap smear result is probably untrue; whereas treatment for a positive HPV DNA test may begin quicker in these women due to the high sensitivity of this test^[40].

Visual inspection with acetic acid

Low-and-middle-income countries (LMICs) are faced with a lack of critical resources for health in general and often an even larger deficit for preventative health initiatives for women. To combat this, LMICs pursue screening options that work within the various societal confounds faced by women in their countries. The majority of these LMICs do not have the current capacity to sustain cytology-based cervical cancer prevention programs^[41]. In these societies, the Pap test is hindered by numerous operational factors that inhibit quality, including the follow-up challenges of multiple visits for screening and later post-diagnosis therapy, inefficient recall and referral systems, inadequate resources for screening and treatment, and competing priorities in the healthcare systems^[41]. A viable alternative to the Pap test has been developed due its low cost and ability to “see-and-treat” in one visit. This screening method, known as visual inspection with acetic acid (VIA), partnered with cryotherapy-based treatment of VIA-positive lesions is a testing method that has been readily mastered by non-physician providers and has been extensively studied as a viable alternative to the Pap smear^[41,42]. A method of screening that is gaining increasing popularity in LMICs is the combination of VIA-based “see-and-treat” platforms with HPV DNA testing, given that they have the benefit of same-visit benefit of triage by VIA-based screening^[43-45]. This opportunity is made possible with the

ongoing development of low-cost, rapid molecular-assay technologies for HPV that may function optimally in the field^[46,47].

CURRENT GUIDELINES FOR CERVICAL CANCER SCREENING

In the United States, there has recently been a shift in the way that screening for cervical cancer is being conducted with recognition that yearly screening was unnecessary and caused higher rate of harms. This is due to greater understanding of the pathological development of cervical cancer and the discovery of the HPV DNA test and HPV vaccines that have occurred in the last decade. Consequently, screening guidelines have evolved rapidly, and many of the organizations that develop screening guidelines now agree on the screening recommendations^[27,48,49]. The American Cancer Society (ACS), American Society for Colposcopy and Cervical Pathology (ASCCP), and the American Society of Clinical Pathology (ASCP) all tasked expert panels within the past five years to review the available evidence on cervical cancer screening and jointly produce a new cervical cancer screening guideline. At the same time, the United States Preventive Services Task Force (USPSTF) developed an updated systematic review of cervical cancer screening. On March 14, 2012, The ACS/ASCCP/ASCP group^[33] and the USPSTF^[36] released their updated guidelines. The American Congress of Obstetrics and Gynecologist (ACOG) issued their updated guidelines for cervical cancer screening shortly thereafter in November 2012^[50]. The consensus of recommendations made by these organizations, as it pertains to cervical cancer screening, are for the general population only. The guidelines are not for women that are at a high risk, as they may need more frequent screenings, including women with a history of cervical cancer, who are immunocompromised, or were exposed in utero to diethylstilbestrol^[50].

Table 3 presents the current guidelines for specific age groups. These differ from previous recommendations most notably in when to begin screening and the screening intervals. Women before the age of 21 years should not have Pap smears, irrespective of the age when they initiated sexual activity^[37,48,49]. The previous guidelines by the ACS in 2002 and 2003 stated that Pap smears should start 3 years following the initiation of sexual intercourse^[37,51]. There has been a call for lengthening the screening intervals in two of the age classifications. The updated ACS/ASCCP/ASCP and ACOG guidelines^[51,52] have increased the time between Pap smears to 3 years in females between ages 21 to 29. Their previous guidelines recommended screening be done every 2 years. The reason behind this change in the guidelines is because 2-3 year screening of women before age 30 carry similar predicted lifetime risk of cervical cancer mortality (0.05 per 1000 women); however screening women every 2 years increases the risk of colposcopies by 40% compared with screening every 3 years^[49]. Hence, 3 year screening in

Table 3 Comparison of cervical cancer screening guidelines

Population	Current Guidelines ACS/ACOG/USPSTF 2012	Prior ACS guideline 2002/2003	Prior ACOG guideline 2009	Prior USPSTF guideline 2003
Females younger than 21 yr of age	Begin screening at age 21	Begin 3 yr following the onset of vaginal intercourse, but no later than 21 yr	Begin 3 yr following the onset of vaginal intercourse, but no later than 21 yr	Begin within 3 yr of onset of sexual activity or age 21, whichever is earliest
Females age 21–29 yr	Conventional Pap or liquid based cytology alone every 3 yr	Conventional Pap: Annually; every 2–3 yr for females ≥ 30 with 3 negative cytology tests Liquid-based cytology: Every 2 yr; every 2–3 yr for females ≥ 30 yr with 3 negative cytology tests If HPV testing used: Every 3 yr if HPV negative and cytology negative	Cytology every 2 yr	Conventional Pap: At least every 3 yr Liquid-based cytology: Insufficient evidence If HPV testing used: Insufficient evidence
Females age 30–65 yr	HPV and Pap smear co-testing every 5 yr or Pap smear alone every 3 yr. Do not use HPV testing alone.		HPV and cytology co-testing every 3 yr	
Women older than 65	Stop screening if adequate prior negative screening result and women not at high risk	Stop screening in Women ≥ 70 yr with 3 or more recent, consecutive negative tests and no abnormal tests in previous 10 yr	Stop between 65 and 70 yr of age after > 3 consecutive negative cytology tests over the past 10 yr	No screening if adequate prior negative screening result and women not at high risk
Women after hysterectomy	No screening if removal of cervix and no prior high grade pre-cancer or cervical cancer	Discontinue if hysterectomy for benign reasons and no previous high-grade CIN	Stop screening	Discontinue if hysterectomy done for benign reasons
Women who were immunized with HPV	Same as non-immunized women	No vaccines recommended for use at this time period	Same as non-immunized women	No vaccines recommended for use at this time period

ACS: American Cancer Society; ACOG: American Congress of Obstetricians and Gynecologists; USPSTF: United States Preventive Services Task Force; HPV: Human papillomavirus; CIN: cervical intraepithelial neoplasia.

women younger than age 30 years has the optimal benefit to risk ratio. In women 30 to 65 years of age, screening can be done every 5 years if the woman's result on co-testing with Pap smear and HPV testing are negative, since co-testing increases the sensitivity of screening, and co-testing every 5 years results in fewer colposcopies and comparable cancer risk than Pap smear screening every 3 years^[52,53]. Cytology testing only at 3-year intervals is also satisfactory in this patient population. The new guidelines also recommend a decrease in the age that screening is stopped, from 70 to 65 years^[49,50,54]. The reason for this is that studies show in women age 65 or older, new high-risk HPV infection is associated with a extremely low absolute risk of HPV persistence and progression to CIN3^[55,56].

There are some special circumstances that require specific recommendations in the screening guidelines. The new guidelines maintain previous recommendations to not screen women that have received hysterectomies with excision of the cervix for a benign cause and who do not have prior history of cervical cytology higher than CIN2^[37,48,49]. This recommendation has been made in part based on evidence produced by a large study of 5330 screening Pap smears in women with previous hysterectomy where there was just one person found with dysplasia and none with cervical cancer^[56]. Another unique circumstance that has arisen since 2006 was the advent

of the HPV vaccine. Current guidelines recommend the same screening strategy in individuals that have received the vaccine as in individuals that have not had the vaccine because it will be another decade or more before modeling studies predicting the effectiveness of the vaccine will be available^[57]. The guidelines also address the situation when women have a negative Pap smear but a positive HPV test. The ACS/ASCCP/ASCP and ACOG recommend genotyping of HPV 16/18 and if positive, immediate colposcopy^[49]. However, evidence for HPV 16/18 genotyping is sparse; therefore, an acceptable alternative option is to perform the combined HPV and cytology testing again within 12 mo^[49,58]. These recommendations are based on results found in large cohort studies showing that the risk of CIN 3 approximates 10% over 1 to 4 years when a woman's test is evident for HPV 16, and over 2 to 5 years if the woman's test shows HPV 18^[59,60].

DISCUSSION AND FUTURE PERSPECTIVE ON CERVICAL CANCER PREVENTION AND SCREENING

With the advent of the HPV vaccine and the limitless screening possibilities that have been afforded by the growing understanding of HPV and the role that it plays in the evolution of cervical cancer, there is a real possibil-

ity that cervical cancer can be eliminated in the future. However, for that vision to become a reality there are numerous complexities that have to be resolved with regards to prevention as well as to screening for cervical cancer. The innovative strides that have been made at the present time must be met by global efforts that are tailored to various societal confines.

In the United States there has been a push by health care providers for immunization with HPV vaccine routinely in young women. This effort has not only been met by opposition created from those challenging the morality and questioning the effectiveness of the vaccine; it has also been met by exclusion of male counterparts in the dissemination of this vaccine, as well as the ever present lack of access of certain populations to adequate health care. Individuals that are at higher risk of acquiring cervical cancer are those that demonstrate less knowledge of HPV and the HPV vaccine. Therefore, educational outreach and program funding is needed that are targeted at reaching the subgroups of the population with low health care literacy and who are at risk of succumbing to the morbidity and mortality of this preventable cancer.

The call to local and governmental officials to enhance the educational outreach and program funding as a means to decrease the incidence of morbidity and deaths due to cervical cancer is also at the frontline of the dialogue in LMICs. Immunization of women with HPV vaccine to potentially prevent cervical cancer in these regions may take a back seat to other health care issues in light of the cost and the unique blend of genotypes that are present based on the geographical region. Fortunately, officials in these regions are becoming more knowledgeable of the advantages of implementing innovative cervical cancer see-and-treat programs. There is a continued need for industrialized nations to lend aid to these countries. This aid should not only be sent in the form of the monetary contributions that have been made by vaccine manufactures; they should continue sending aid via providing the service of individuals that can train their non-physician workforce who do a great deal to treat the masses of women in their countries.

In both LMICs and developed countries, the advent of HPV DNA testing has had a tremendous impact on the way that screening for cervical cancer is conducted. Affordable versions of this test are being developed, non-physician providers can perform it independently, and the results can be obtained the same day. More research needs to be done to see if testing with this technology should be conducted as the primary testing method, especially in hard to reach populations, since compared to cytology, it offers extended safety after a negative result. Some experts argue that because testing for HPV has greater sensitivity than Pap smear, while Pap smear screening has greater specificity, HPV testing should be performed initially and then obtain Pap smear screening for patients testing positive for HPV. The potential advantage to this was seen in a Canadian trial that found that HPV testing followed by Pap smear caused lower re-

ferrals for colposcopy than did either alone (1.1% *vs* 2.9% with only Pap smear or 6.1% with just HPV testing)^[59].

The greatest effect on mortality rates from cervical cancer is on women that are unscreened or under screened. There is a huge need to continue with the innovative strides that have been made to overcome the health care barriers crippling this population. If this population is able to benefit from low-cost screening and vaccinations subsidized by the government and continued efforts that are being made possible by the growing dialogue surrounding cervical cancer, it is possible that women in future generations will no longer succumb to cancer of the cervix.

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WJCO 5th Anniversary Special Issues (3): Cervical cancer**Role of IL-10 and TGF- β 1 in local immunosuppression in HPV-associated cervical neoplasia**

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and transforming growth factor (TGF)- β 1 to produce a local immunosuppressive environment, which, along with altered tumor surface antigens, forms an immunosuppressive network that inhibits the antitumor immune response. In this review we analyzed the available data on several deregulated cellular immune functions in patients with NIC I, NIC II and NIC III and cervical cancer. The effects of immunosuppressive cytokines on innate immune response, T-cell activation and cellular factors that promote tumor cell proliferation in cervical cancer patients are summarized. We discuss the functional consequences of HPV E2, E6, and E7 protein interactions with IL-10 and TGF- β 1 promoters in the induction of these cytokines and postulate its effect on the cellular immune response in squamous intraepithelial cervical lesions and cervical cancer patients. This review provides a comprehensive picture of the immunological functions of IL-10 and TGF- β 1 in response to HPV in humans.

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Key words: Cervical cancer; Immunosuppression; Interleukin-10; Transforming growth factor- β 1; Human Papillomavirus

Abstract

Cervical cancer is a worldwide disease that constitutes a significant public health problem, especially in developing countries, not only due to its high incidence but also because the most affected population comprises women who belong to marginalized socio-economic classes. Clinical and molecular research has identified immunological impairment in squamous intraepithelial cervical lesions and cervical cancer patients. Human Papillomavirus (HPV) has several mechanisms for avoiding the immune system: it down-regulates the expression of interferon and upregulates interleukin (IL)-10

Core tip: Human Papillomavirus (HPV) persistence is a key event for cervical cancer development. HPV proteins E2, E6 and E7 induce the transcription of immunosuppressive cytokines as a means of evading HPV the host immune system. We postulate that interleukin-10 and transforming growth factor- β 1 induce HPV immune system evasion through an immunosuppressive state in the local microenvironment of the cervix in HPV-infected women. These findings allow us to gain insight in our understanding of how HPV persist in the cervix and favor cervical lesions and cancer development, with potentially strong impact on vaccine development and the

design of new targeted immunotherapies for women with cervical neoplasia.

Torres-Poveda K, Bahena-Román M, Madrid-González C, Burguete-García AI, Bermúdez-Morales VH, Peralta-Zaragoza O, Madrid-Marina V. Role of IL-10 and TGF- β 1 in local immunosuppression in HPV-associated cervical neoplasia. *World J Clin Oncol* 2014; 5(4): 753-763 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i4/753.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i4.753>

INTRODUCTION

Cervical cancer is the second most common cancer in women worldwide^[1]. Cervical cancer and its precursor, squamous intraepithelial lesion (SIL), arise as a result of an uncontrolled and persistent infection with a high-risk human Papillomavirus (HPV)^[2]. Based on molecular epidemiological studies that provide risk estimates of specific HPV types in cases and controls, as well as the evidence of the oncogenic potential of the different HPV types, 13 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, and 58, 68, 82) have been identified, with three considered probable (26, 53, 66). Ten types were classified as low-risk (6, 11, 40, 42, 43, 44, 54, 61, 72, 81CP6 108) and three of indeterminate risk (34, 57, and 83)^[3]. The most prevalent high-risk viral types in the general population are 16 and 18. These two types are responsible for approximately 70% of all cervical cancer cases^[4].

Most of the HPV infections are of a transient and intermittent nature, especially among women under 30 years of age^[5]. Around 70% of infections disappear within approximately one year. After two years 90% of the infections will have disappeared. Only 10% of the women studied remained infected and developed cervical epithelium alterations and eventually cervical cancer^[5,6]. The majority of women clear HPV infections spontaneously through the antiviral host immune response. The mechanistic explanation for HPV clearance is by specific immunological reactions, which require competent humoral and cell-mediated immune mediators^[7,8]. Among the factors influencing viral persistence are host factors (genetic or acquired, such as age, immunosuppression, oral contraception, smoking) and viral factors (genotype, variants, viral load and viral integration)^[6,9].

In this review we discuss the mechanisms that allow HPV and human cervical cancer cells to evade immune surveillance through soluble immunosuppressive factors produced in the tumor microenvironment, such as interleukin (IL)-10 and transforming growth factor (TGF)- β 1, and we dissect the molecular events underlying tumor immune escape.

NATURAL HISTORY OF CERVICAL CANCER

The natural history of cervical cancer has been well

established by a large number of prospective cohort studies. Figure 1 summarizes the molecular events and changes at tissue level related to HPV infection and cancer establishment.

The development of a precancerous lesion and cancer involves several events. Exposure to high-risk HPV causes an initial infection of squamous epithelium in the transformation zone. This is followed by persistent infection, viral genome integration into the host cell genome, genomic alterations, immortalization and transformation of epithelial cells (Figure 1)^[10].

As the understanding of the natural history of the disease has improved, the classification of these lesions has received different names [PAP I to V; moderate dysplasia, carcinoma *in situ* and severe, cervical intraepithelial neoplasia (CIN) I, II, III; low squamous intraepithelial lesions (LSIL) that include CIN I or mild dysplasia, condyloma and koilocytosis and high squamous intraepithelial lesions (HSIL) that include CIN II and CIN III or moderate or severe dysplasia and carcinoma *in situ*], as shown in Figure 1. The development of cervical cancer is preceded by a series of cellular abnormalities characterized by cytological and histological maturation variations and irregularities in nuclear cytoplasm^[5]. LSIL have a diploid DNA content or polyploid. This is correlated with their tendency to revert. In contrast, HSIL type CIN III is often aneuploid, have a greater degree of cellular atypia and are more likely to persist and progress^[6].

The immune system plays a key role during HPV-carcinogenesis since the majority of high-risk HPV infections (90%), as well as most of low-grade lesions (75%), regress. Upon infection, on average 2-3 years are necessary to develop CIN 1/2 and/or high-grade intraepithelial lesion (CIN3) and nearly one third of untreated HSIL may progress to cancer in about ten years^[5,6].

HPV enters the mitotically active basal cells through micro abrasions in the epithelium of the cervix. After infection, early HPV genes E1, E2, E4, E5, E6 and E7 are expressed in infected cells and the viral genome is maintained episomally. The virus can lie latent until the negative regulation of viral transcription in these cells by cellular factors is released. The viral genome is replicated with greater intensity and late genes L1 and L2 are expressed in the upper layers of the epithelium. Capsid ruptures allow the release of the viral genome, the formation of new virions and the start of a new infection^[11,12].

The long latency period between initial infections and the emergence of cancer suggests that HPV can evade recognition by the immune system. Indeed, the infection cycle of HPV is characterized by the absence of viraemia, very low expression levels of viral protein, no inflammation and no danger signal to alert the immune system^[13-15]. Host immune responses to HPV are generally low-level because the virus, being confined to basal epithelial cells, is shielded from the circulating immune cells during initial stages of infection. In this location there is only a limited expression of viral proteins. Other factors contributing to the low level of host immunity are that HPV infection

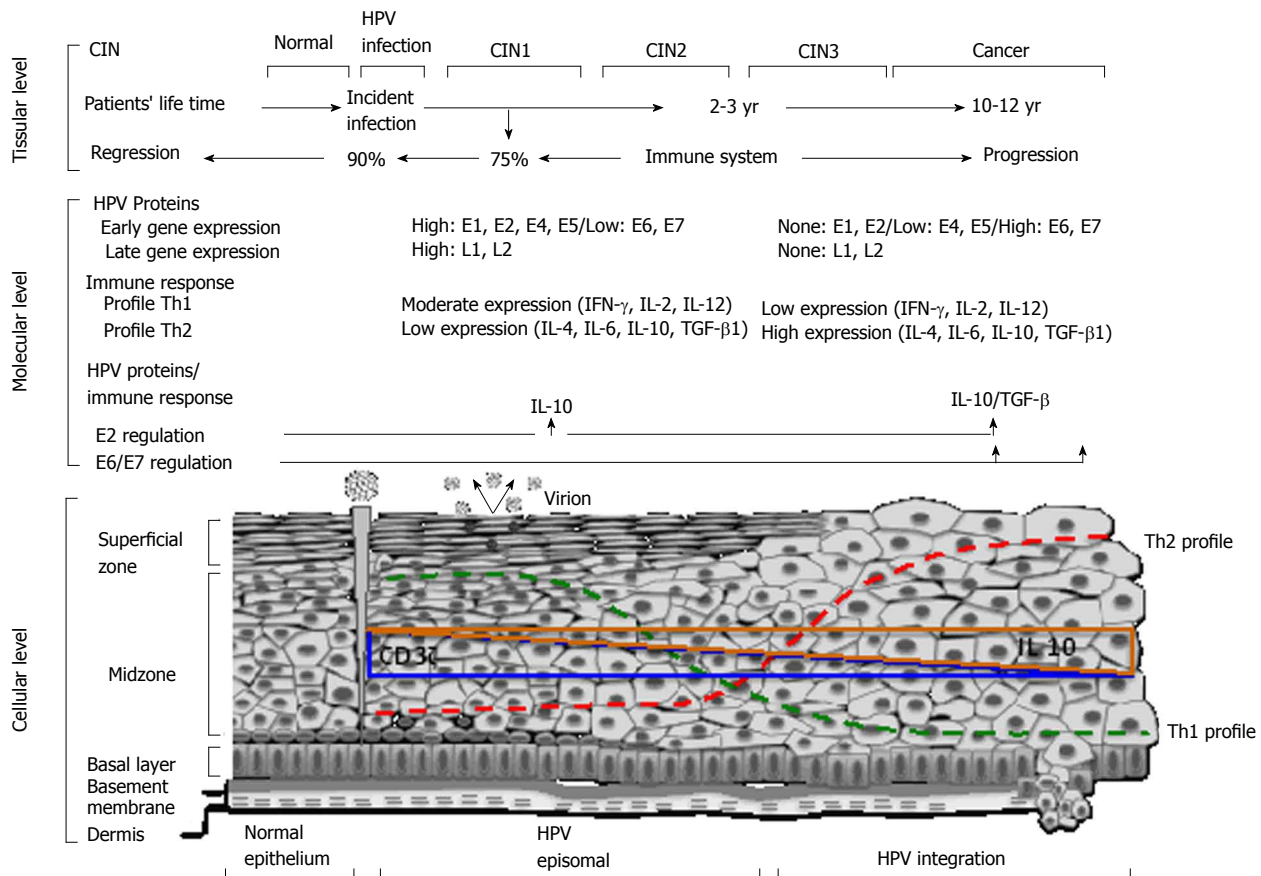


Figure 1 Systematic representation for cervical human Papillomavirus-carcinogenesis: Role of immune response. The main event is the human Papillomavirus (HPV) infection of epithelial cells in the basal membrane. HPV can turn into episomal bodies, which will be in charge of, on one part, producing infective virions and, on the other, integrating into the genome of epithelial cells. Upon infection, an average of 2-3 yr are necessary to develop of CIN 1/2 and/or high-grade (CIN3), often characterized by the integration of the viral genome, one key event for disease progression as this frequently triggers the deregulation of the E6 and E7 oncogenes, major chromosomal alterations and cellular immortalization^[10]. The immune system plays a key role during HPV-carcinogenesis since the majority of high-risk HPV infections (90%), as well as most of low-grade lesions (75%) regress^[5,6]. Due to this, and the long periods of time between viral infection and the progression to invasive disease, the fail of immune response is necessary to cancer development. Since the beginning of HPV infection, there are augment in the immunosuppressive cytokine IL-10 at the cervical level, and this increased according with the grade of lesion, being the highest concentration at cancer stage^[25]. A shift to Th2-type cytokines in the course of development of cervical cancer is reflected with an increased serum concentration of Th2-type cytokines^[22,23]. Inversely, the presence of CD3zeta chain in the tumor infiltrating T-lymphocytes, decreases as the grade of lesion progress, this is due to a reduction of Th1-type cytokines at the late stages of the disease^[24]. These are key elements for the impairment of immune responses that allows HPV-persistence, viral integration into the genome epithelial cells, and cellular transformation and immortalization.

is non-lytic; that a functionally active immune response is generated only at later stages of HPV infection; and that only in suprabasal keratinocytes has the HPV DNA been sufficiently amplified to be detected by the host immune-surveillance cells^[16].

Although most HPV infections are transient and sub-clinical, progression is strongly associated with HPV persistence. This process often leads to disruption of viral E1/E2 regions and integration in the genome of the host cell. E2 rupture releases E6/E7 viral promoters and increases expression of these oncogenes, as show in Figure 1^[10-12,17]. Infection with one high-risk HPV acts as a trigger for the cascade of events in which the mechanisms of repair or correction of cell replication, mediated by p53 and pRb, are altered. Thus, the cell cycle is controlled by the virus, which triggers cellular changes that culminate in the transformation and immortalization of epithelial cells, in consequence establishing conditions for the onset

of cancer^[18-20].

During cancer progression, the pattern of changes in expression of viral oncoproteins changes; in CIN I, the order of events is similar to that observed in productive lesions. In CIN II and III, however, the event occurrence is delayed and virions production is restricted to smaller areas near the epithelial surface. Integration of HPV sequences in the genome of the host cell may accompany these changes and may lead to a further deregulation of the expression of E7 (and the loss of replication proteins E1 and E2). In cervical cancer, the production stages of the viral cycle are not permanent and the viral episomes are lost^[11,12].

A predominance of the Th2 cytokine profile, in association with a diminished Th1 profile, has been demonstrated in patients with cervical cancer^[21]. A shift to Th2-type cytokines in the course of development of cervical cancer is reflected by an increased serum concentration

of Th2-type cytokines^[22,23]. Inversely, the presence of CD3zeta chains in the tumor-infiltrating T-lymphocytes decreases as the grade of lesion progresses. This is due to a reduction of Th1-type cytokines in the late stages of the disease^[24]. This shift from Th1 to Th2 might be responsible for facilitating tumor progression by subverting various cellular immune surveillance mechanisms.

In addition, an impaired cellular immune response induced by immune suppressor cytokines, such as interleukin (IL)-10 and transforming growth factor beta (TGF- β 1), has been involved in high risk HPV persistence and cervical cancer development^[21]. Since the beginning of HPV infection there are increases in the immunosuppressive cytokine IL-10 at the cervical level. The increase is proportional to the grade of lesion, with the highest concentration occurring at the cancer stage^[25]. These are key elements for the impairment of immune responses that enable HPV-persistence, viral integration into the genome epithelial cells, and cellular transformation and immortalization.

IMMUNE EVASION IN HPV INFECTION, SIL AND CERVICAL CANCER

Innate immune response, which involves macrophages, natural killer (NK) cells, and natural killer T cells, plays a critical role as the first line of defense against HPV infection^[26]. However, effective evasion of innate immune recognition seems to be the hallmark of HPV infections^[27]. Several alterations of natural immunity have been documented in HPV infection. The interferon (IFN) response, a key antiviral defense mechanism^[28], is actively suppressed by the E6 and E7 proteins of high-risk HPVs, which inhibit the interferon receptor signaling pathways and prevent activation of the interferon response genes^[29,31]. Macrophages are activated by binding to viral components, such as single-stranded DNA, and by cytokines, which can kill HPV-infected cells via tumor necrosis factor α (TNF- α) secretion. HPV16 E6 and E7 proteins inhibit the translocation of macrophages to the site of HPV infection^[32].

Likewise, NK cells are a subset of lymphocytes that kill virally infected cells or tumor cells lacking the surface expression of major histocompatibility complex (MHC) class-I molecules. Low NK cell receptor expression and reduced cytotoxic activity of NK cells were recently observed in cervical cancer and precursor lesions. A previous study suggests that NKp30, NKp46 and NKG2D down-regulation represents an evasion mechanism associated with low NK cell activity, HPV-16 infection and cervical cancer progression^[26,33]. Recent studies have demonstrated that CD1d on the surfaces of cells infected with HPV6 or 16 is down-regulated by E5 protein, and that this may be a mechanism for immune evasion^[34]. Additionally, augmented serum level of major histocompatibility complex class I-related chain A (MICA) protein and reduced NKG2D expression on NK and T cells in patients with cervical cancer and precursor lesions have

been reported^[35,36].

Similarly, Toll-like receptors (TLRs) play a key role in the innate immune system against HPV infection. The activation of the TLR9 pathway by CpG motives is impaired severely in human keratinocytes expressing HPV16 E6 and E7 oncoproteins. This event is due to the ability of the viral oncoproteins to down-regulate TLR9 mRNA. This phenomenon has been observed in HPV16-positive cancer-derived cell lines and in primary cervical cancers. TLR9 promoter down-regulation is less significant for high-risk HPV 18 compared with HPV 16 and is completely absent in cells expressing E6 and E7 from the low-risk HPV 6. Thus, the efficiency of HPV16 in persisting appears to correlate with its ability to down-regulate the transcription of TLR9^[37].

In high-risk HPV-infected women there is a decrease in the levels of Langerhans DCs in the human female genital tract (reduction of E-cadherin by E6)^[38]. CD4 regulatory T cells (Treg) subsets act as major mediators of peripheral immune tolerance by regulating Th1 and Th2 immune responses^[39,40]. Treg cells contribute to the induction of peripheral tolerance via expression of inhibitory cell-surface molecules (CD4⁺/CD25⁺ T cells) or production of IL-10 and TGF- β 1. IL-10 produced by Treg cells can impair TAA cross-presentation by DCs, thus potentially preventing T cells from mounting an effective immune response against malignant cells. IL-10 hinders the antigen-presenting properties of DCs by reducing their expression of *human leukocyte antigen* (HLA) class II molecules, inter-cellular adhesion molecules (*e.g.*, ICAM-1), co-stimulatory molecules (*i.e.*, B7-1/CD80 and B7-2/CD86), and Th1 cytokines (*e.g.*, IL-12), which correlates with the ability of IL-10 to impair primary alloantigen-specific T cell responses^[41]. IL-10 inhibits the host inflammatory response and favors tumor development. Thus, the predominant secretion of Th2 cytokines in innate immunity attenuates the antitumoral response^[42].

In addition, HPV effectively evades the innate immune response to delay the activation of adaptive immunity in patients with SIL^[43,45]. HPV16 E5-mediated immune evasion also involves suppressing the expression of MHC class I and Ag processing via the TAP pathway, reflecting the lack of Ag presentation to cytotoxic T lymphocytes (CTLs)^[13,46]. Immune function alterations that have been described in HPV infection further include impairment of CD4⁺ T-cell-mediated immunity and cytokine dysregulation in the blood of women with precancerous lesions or cervical cancer^[22,44]. At the systemic level, there is a report that shows a dysregulated CD28 and CTLA-4 expression in peripheral blood T cells of cervical cancer patients, which may lead to impaired function of these lymphocytes and a systemic immunosuppression related to disease progression^[47]. A lack of dendritic cells^[48,49] and a decrease in helper T cell type 1 immune infiltrates have been described in the presence of HPV-associated lesions^[50,51]. Therefore, all these findings provide solid evidence that altered immune responses are a crucial step involved in the carcinogenic events medi-

ated by HPV.

IL-10 and TGF- β 1 cytokine expression in SIL and cervical cancer: Evidence for the generation of local immunosuppression

It is well known that antiviral and anti-tumor immunity in cervical cancer is activated by Th1 cytokines and inhibited by Th2 cytokines. The transformation zone of the cervix, the region most sensitive to SIL and cancer development, is associated with above-average levels of type II cytokines (IL-4/IL-6) or immunosuppressive cytokines (IL-10 and TGF- β 1) produced by various types of cells, including macrophages, dendritic cells and keratinocytes^[52]. Since both cytokines have the ability to interfere with the efficient induction of a type I response by antigen-presenting cells (APC), these cytokines may contribute to the predisposition of this region to cervical carcinogenesis^[52].

We hypothesize that the changes in the immune response observed during different stages of the HPV infection can support the idea that development of immunosuppressive environment in cervix correlates with the progression of lesions into more aggressive neoplasia. IL-10 (UniprotKB/Swiss-Prot: P22301) is a Th2 anti-inflammatory cytokine that participates in the regulation of the immune response at several levels and can have pleiotropic effects on cell mediators of adaptive and innate immunity^[53]. Human IL-10 is produced by CD4⁺ T cells, activated CD8⁺ T cells, Epstein-Barr virus-transformed lymphoblastoid cell lines, fibroblasts and monocytes. It has potent inhibitory effects on T cell proliferation and inflammation. IL-10 also depresses the production of a number of Th1 cytokines normally synthesized by activated macrophages and mononuclear cells^[53]. More recent studies have clarified that IL-10's immunosuppressive effect on T cells is mainly indirect and is mediated by two other immune cell types: DCs and Treg cells^[54]. Additional evidence supports the hypothesis that DCs are the major target of IL-10's immunosuppressive effect. It has been shown that IL-10 reduces the expression of antigen-presenting and costimulatory molecules and interferes with the maturation of monocytes to DCs^[55]. These properties support the role of IL-10 as a strong immunosuppressive cytokine and a potent negative regulator of immunoproliferative and inflammatory responses.

A previous study reported a high tendency of IL-10 expression associated with the frequency of HPV types and the severity of the disease^[25]. Additionally, Arany *et al.*^[56] described a mechanism by which IL-10 might enhance persistence and progression of HPV-related lesions under certain conditions (*e.g.*, dysplastic progression, HIV infection) when the cytokine expression in the cervical microenvironment changes. It has been shown that peripheral blood mononuclear cells (PBMC) from patients with both SIL and cervical cancer produce decreased amounts of IL-2 and IFN- γ and higher levels of IL-4 and IL-10 following mitogenic stimulation, compared

with the control group^[57]. High levels of IL-10 have been detected in serum and at the cervical level of patients at early stages of the disease^[58-60]. The systemic IL-10 mRNA expression level and the IL-10 protein level in serum are significantly higher in SIL compared to women without lesions. The IL-10 expression level is determined by whether a person is a carrier of the allele A of the SNP at -592 nt of the human IL-10 gene^[23]. Therefore, the presence of IL-10 appears to be an important factor in the progressive development of the impaired immune response against cervical lesions.

Most cervical tumors expressed IL-4 and IL-10 mRNA and, most importantly, all of them expressed TGF- β 1 and IFN- γ mRNA^[21,22,61]. IL-10 has been identified by immuno-histochemical analysis in tumor cells and koilocytic cells, but not in tumor-infiltrating lymphocytes, suggesting that the IL-10-producing cells are those transformed by HPV. Similarly, a correlation between immunostaining for IL-10 protein and the level of IL-10 mRNA expression has been reported and supernatant from HPV-transformed cell lines has been found to contain IL-10 and TGF- β 1. These findings show a predominant expression of immunosuppressive cytokines, which help down-regulate tumor-specific immune responses in the tumor microenvironment^[21,22,61].

A recent study examined T cell functions of PBMC and tumor infiltrating T lymphocytes (TIL) from women with SIL or cervical cancer, including proliferation, cytokine mRNA expression (IL-2, IFN- γ , IL-4, IL-10, TGF- β 1), and CD3 ζ expression^[24]. The distribution of T cells in the epithelium and stroma of biopsies from women with SIL and from women with microinvasive carcinoma of the cervix was determined by immuno-histochemical analysis. There were more TIL in the stroma than in epithelium in advanced stages of the disease where CD8⁺ T cells prevailed. Consistent with other reports^[62], it was found that CD8⁺ T cells predominate but lack activity compared with CD4⁺ T cells in women with cervical cancer^[24]. To better understand T cell behavior during the course of cervical cancer disease, the proliferation of PBMC and TIL from patients with SIL or cervical cancer with phytohemagglutinin (PHA) or immobilized anti-CD3 was examined and compared to those of healthy donors. For PHA, PBMC from cervical cancer patients proliferated less than those from SIL patients and healthy women, although the differences between SIL patients and healthy women were not significantly different^[24]. Moreover, a significant difference was found in anti-CD3-stimulated PBMC between cervical cancer patients and healthy donors. When the proliferation of PBMC *vs* TIL was compared in women with cervical cancer, only PBMC proliferation was statistically higher than that of PHA-stimulated TIL. Thus, women with cervical cancer have a deficient PBMC proliferative response, with no response observed for TIL^[24].

Additionally, it has been suggested that reduced T cell function may be associated with alterations in CD3 ζ protein expression in cervical cancer patients^[63]. A previ-

ous study demonstrated that *in vivo* suppression of CD3 ζ chains in patients with CIN can occur as the result of a circulating factor^[64]. This circulating factor is composed of IL-10 and TGF- β 1, which reduce CD3 ζ expression *in vitro*, as show in Figure 1^[24]. We therefore propose that IL-10 and TGF- β 1 play an important role in generating an immunosuppressive state in the tumor microenvironment which allows the tumor to evade the host cellular immune responses in cervical cancer^[24]. A significant correlation between low T cell proliferation and decreased CD3 ζ mRNA expression by anti-CD3 stimulated T cells has been reported^[24]. Thus, decreased T cell function appears to correlate with cervical cancer progression, which corresponds to a decreased T cell proliferation in cervical cancer patients^[24,62,64]. Similarly, a significant positive association between CD3 ζ /IL-2 and CD3 ζ /IFN- γ expression has been found, indicating that an optimal expression of CD3 ζ is associated with the expression of IL-2 and IFN- γ ^[24,64].

A separate role in immune response escape may be played by TGF- β 1 (UniprotKB/Swiss-Prot: P36897), a multifunctional cytokine that prevents cellular immune responses by inhibiting T cell proliferation and differentiation into cytotoxic T cells and helper T lymphocytes. It achieves this by inhibiting stimulatory functions induced by APC. An additional T cell subset, known as Th3, secretes TGF- β 1, which can suppress cytotoxic T cell function. Animal and human models suggest that TGF- β 1 can promote tumor progression by facilitating extracellular matrix invasion and angiogenesis and by inhibiting immune surveillance^[65]. Direct inhibitions of the proliferation and effector functions of CTLs are the immunosuppressive functions of TGF- β 1. TGF- β 1 may polarize APC activity to favor a Th2 type immune response. In animal models, neutralization of TGF- β 1 with monoclonal antibodies or antisense oligonucleotides results in tumor regression or decreased invasiveness^[66,67].

After HPV infection of basal epithelia cervical cells, E6 and E7 oncoproteins are expressed (Figure 1), and induce cytokine expression of TGF- β 1 throughout the Sp1 transcription factor. The E6-Sp1 and E7-Sp1 complex formation can migrate into the nucleus and induce the TGF- β 1 gene expression^[68]. TGF- β 1 down-regulates IL-2 receptor signaling in T cells and IL-12 expression by APC and induces the expression of IL-10 by macrophages, contributing to immunosuppression during cervical carcinogenesis^[68]. Reports have shown increased TGF- β 1 expression by cervical cancer, consistent with previous reports of TGF- β 1 expression in 94.1% of the stroma surrounding invasive cancer^[69]. TGF- β 1 gene expression is normally constitutive; however, during tumor progression there is an upregulation of TGF- β 1 gene expression, which induces favorable conditions for tumor development^[70].

Finally, the presence of IL-10 and TGF- β 1 in supernatants from human cervical carcinoma cell lines HeLa and SiHa after 24 h of incubation without FBS has been reported. HPV-positive cervical cell lines produce higher

quantities of both cytokines than the HPV-negative C-33A cell line^[24]. It was found that the inhibitory effect of rhTGF- β 1 is totally counteracted with neutralizing anti-rhTGF- β 1 whereas the inhibitory effect of rhIL10 is blocked with neutralizing α -rhIL-10. Thus, both cytokines independently produce a profound inhibitory effect on T cell proliferation^[24]. Furthermore, loss of the inhibitory effect of TGF- β 1 plays a role in the transformation of normal cervical epithelial cells to dysplastic and malignant cells. Following development of malignancy the TGF- β 1 acts to facilitate aggressiveness and tumor progression^[71].

Expression of IL-10 and TGF- β 1 may be induced by HPV

We hypothesize that the expression of some cytokines, such as IL-10 and TGF- β 1, may be induced by HPV, and that IL-10 and TGF- β 1 cytokines may be produced by the transformed cell as a mechanism to escape the immune response. Several human heterologous promoters are regulated by HPV proteins. Particularly, HPV-16 E5, E6, and E7 oncoproteins, *trans*-activate a large variety of viral and cellular gene promoters^[72]. On the other hand, the presence of HPV E2 regulatory recognition site was reported in the position from -2203 nt to -2191 nt in the IL-10 gene regulatory region^[73]. HPV E2 protein is a sequence-specific DNA-binding protein that recognizes the specific sequence ACCN₆GGT present in the viral long control region (LCR) of all HPV genomes^[73].

To understand the potential trans-activation ability of HPV E2 protein on the human IL-10 gene expression in cervical cancer, the effects of HPV E2 protein on the promoter activity of human IL-10 gene were evaluated in cervical tumor cell lines, using the luciferase gene reporter assay^[74]. The human IL-10 gene regulatory region was obtained by PCR and several construct plasmids that contain different fragments of the IL-10 promoter region were generated in order to transfect HPV-negative C-33A cells. C-33A cells were transfected with the constructs containing the IL-10 regulatory region alone and co-transfected with pCMV16E2 expression plasmid, which expresses HPV E2 protein. For comparison, a plasmid containing the IL-10 complete promoter (pGIL10VB1 plasmid from -2534 nt to +132 nt) and the HPV E2 recognition site was used. The reporter IL-10 gene activity was detected as relative promoter activity, in cells that were transfected with IL-10 complete promoter. This promoter activity increased 6-fold when the cells were co-transfected with pCMV16E2. Similar promoter activity to that of the IL-10 complete promoter was found when the cells were transfected with plasmid that did not contain the HPV E2 recognition site. Thus, we concluded that HPV E2 is able to transactivate human IL-10 gene expression throughout the HPV E2 recognition site into the IL-10 promoter^[74].

A synthetic DNA probe containing the HPV E2 recognition sequences present in the IL-10 promoter was designed, and the ability of HPV E2 protein to interact with the E2-binding site was determined using an EMSA assay, to corroborate the effects of the HPV E2 protein

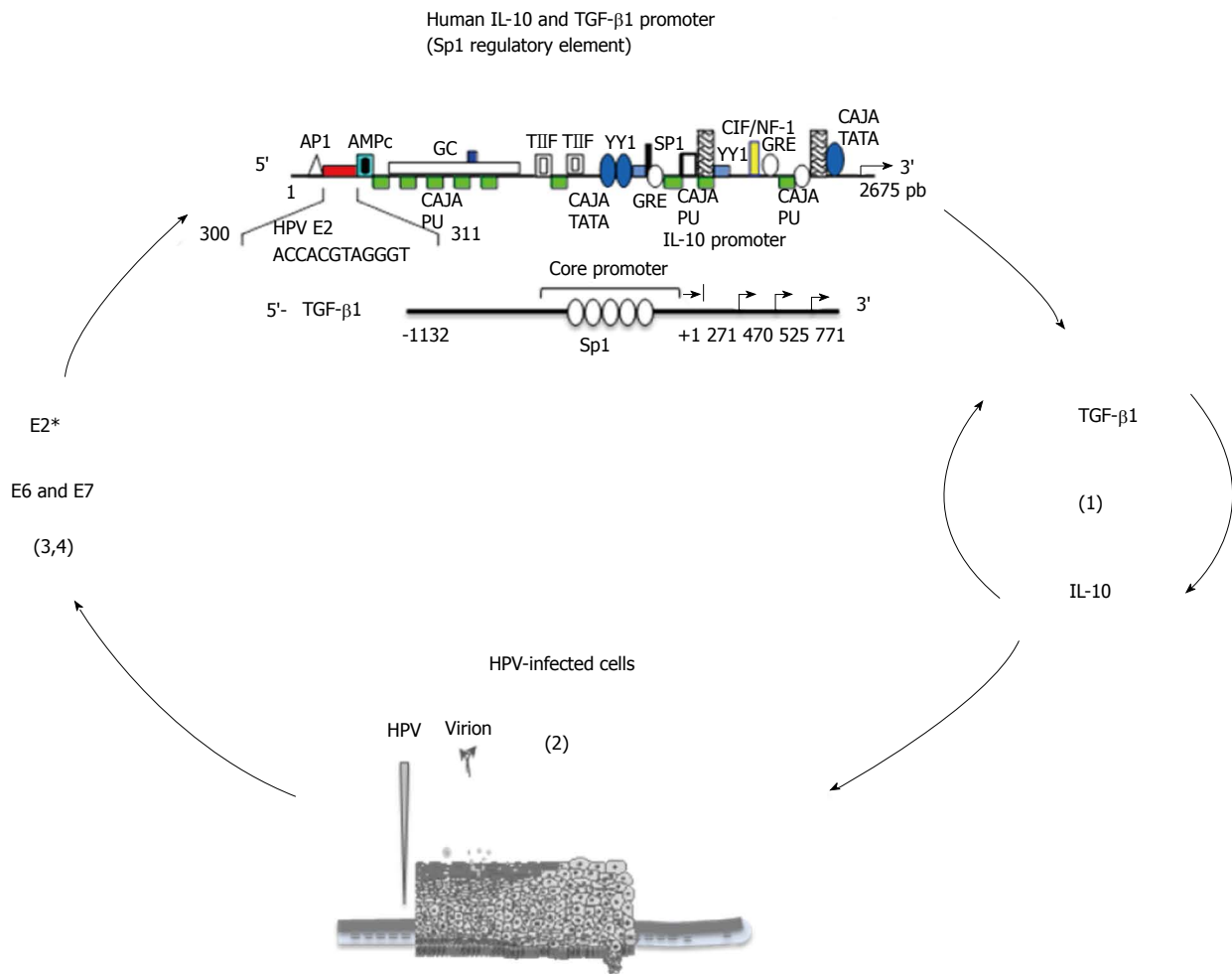


Figure 2 Vicious cycle between Interleukin-10, transforming growth factor- β 1, and human papillomavirus. (1) IL-10 enhances TGF- β 1 expression and vice-versa^[76]; (2) IL-10 induces transcription of HPV-16 E6 and E7^[56]; (3) HPV E6 and E7 proteins induce activation of TGF- β 1 promoter through Sp1 recognition sequence^[21]; (4) Human gene IL-10 regulatory regions possess Sp1 regulatory elements, which suggests that HPV E6 and E7 proteins may bind to IL-10 regulatory region to activate IL-10 gene transcription, just as HPV E2* protein does^[74], and may induce IL-10 expression in HPV-transformed cervical cells. HPV can enhance the expression of cytokines TGF- β 1 or IL-10, which inhibit T cell functions and augment viral proteins, contributing to the persistence of HPV infection and progression of HPV-related cervix lesions to cervical cancer. The production and activity of these two cytokines, IL-10 and TGF- β 1, are interrelated and likely involve a positive feedback loop in which IL-10 enhances the expression of TGF- β 1 and vice-versa^[76]. In fact, IL-10 not only enhances the production of TGF- β 1 but also controls the ability of target cells to respond to TGF- β 1. This involves the IL-10-mediated restoration of the expression of TGF- β 1 receptor 2 in recently activated T cells, which usually down-regulate this receptor and become desensitized to the inhibitory effects of TGF- β 1. Conversely, TGF- β 1 can promote the production of IL-10^[76]. IL-10: Interleukin-10; TGF- β 1: Transforming growth factor- β 1; HPV: Human papillomavirus.

on the transactivation mechanism of the IL-10 promoter and the importance of physical interaction with the HPV E2 recognition site. HPV E2 protein is able to bind to the IL-10 gene promoter, particularly with in the HPV E2 recognition site. The evaluation of IL-10 gene expression in HPV E2 transfected cells, measuring IL-10 mRNA by semiquantitative RT-PCR analysis, demonstrated that HPV E2 is able to induce IL-10 gene expression. This evidence contributes to the knowledge of the IL-10 gene expression molecular mechanism induced by HPV E2 protein, and is crucial to understanding the role of IL-10 in the process of HPV cervical carcinogenesis^[74].

These results lead us to propose a novel molecular pathway through which HPV E2 protein may stimulate IL-10 gene expression. This finding suggests a potential mechanism through which HPV proteins regulate IL-10

gene expression during cervical cancer development, which may represent an immune response regulation strategy mediated by HPV proteins^[74]. Additionally, it has been demonstrated that IL-10 induces transcription of the E7 early promoter, through the segment of the upstream regulatory region^[56]. Also, cervical keratinocytes express IL-10 receptor mRNA, which may explain IL-10's autocrine activity on tumour cells and HPV transcription in a dose-dependent manner. IL-10 expression by cervical cells after HPV infection is associated with enhanced viral persistence and progression of HPV- lesions to cancer^[75].

To gain insight into the potential TGF- β 1 gene regulation mechanism produced by the high-risk HPV E6 and E7 oncoproteins, the TGF- β 1 promoter activity induced by these oncoproteins was analyzed. When the TGF- β 1

core promoter in HPV E6- and E7-expressed tumor cells was evaluated, an increase in the reporter gene expression was found, compared with E6 and E7 non-expressed tumor cells. The TGF- β 1 promoter *trans*-activation was slightly elevated in E7 compared to E6 expression cells. These findings support the notion that the TGF- β 1 promoter activation by HPV-16 E6 and E7 could be physiologically relevant, particularly in cell transformation and immortalization^[68].

A cluster of five DNA binding motifs 100% homologous to Sp1 recognition sites was found in the TGF- β 1 core promoter sequence (-650 nt to +36 nt). DNase Footprinting assays *in vitro* were used to examine the profile of DNA-protein interaction at the TGF- β 1 core promoter to identify the target sequences responsible for TGF- β 1 promoter *trans*-activation by HPV-16 E6 and E7 oncoproteins. A differential protection pattern in the TGF- β 1 core promoter was found which suggests that HPV-16 E6 and E7 oncoproteins may induce varied interactions in tumor cells and may favor the recruitment of co-activators and/or transcription factors. This finding indicates that HPV-16 E6 and E7 oncoproteins expression may induce the formation of novel molecular transcription complexes throughout Sp1 recognition sites in tumor cells during cervical cancer development^[68].

In addition, the *trans*-activation caused by E6 and E7 can be abolished by mutation in the Sp1e (-108 to -102) recognition site. The data shows that HPV-16 E6 and E7 oncoproteins bind first to the Sp1 transcription factor and then the Sp1-E6 or Sp1-E7 complex binds to the TGF- β 1 regulatory element site (GGGGCGG). They do not bind directly to DNA^[68]. The physical interactions and functional cooperation between HPV E6 and E7 oncoproteins and cellular regulatory elements at the TGF- β 1 promoter explain the contribution of HPV-16 to TGF- β 1 gene expression in cervical cancer^[68]. Similarly, human gene IL-10 regulatory region possesses this Sp1 regulatory element (GGGGCGG). This suggests that HPV E6 and E7 proteins may bind to the IL-10 regulatory region to activate IL-10 gene transcription, just as the HPV E2 protein does, which may induce IL-10 expression in cervical HPV-transformed cells, as shown Figure 2.

CONCLUSION

The presence of IL-10 and TGF- β 1 in high-risk HPV cervical infections and patients with SIL may constitute an early event that promotes a microenvironment in the lesion with negative effects on the cellular immune response. Such a microenvironment could favor virus persistence and progression to cervical cancer. These cytokines have been detected in serum and cervical tissues from patients with high-risk HPV infection, with low grade SIL, high grade SIL, and cervical cancer. Their levels increase in correlation with the severity of the lesions^[23]. Results discussed in this review suggest that cervical cancer is characterized by local immunosuppression

dependent on Th2/Th3 cytokines. This data is in agreement with findings in cervical biopsies in which there is a pattern of Th2/Th3 cytokine expression present in cervical cancer but absent in normal cervix, suggesting that HPV infection induces the transcription of immunosuppressive cytokines as a means of evading the host immune system^[21,22,61].

IL-10 is a potent immunosuppressive cytokine that induces and is induced by TGF- β 1 expression. It also induces the HPV-16 E6 and E7 proteins, which induce TGF- β 1 transcription activation and IL-10 gene expression to create a vicious cycle. IL-10 and TGF- β 1 also down regulate CD3 ζ expression, which plays a crucial role in T-cell activation. Finally, IL-10 and TGF- β 1 induce the recruitment of Treg cells, which produce a profound peripheral tolerance. In summary, we postulate that IL-10 and TGF- β 1 induce immune system evasion through an immunosuppressive state in the environment of the cervix in women infected with HPV. This information is highly relevant in the areas of HPV vaccine generation and the design of new targeted immunotherapies for women with LGSIL, HGSIL, and cervical cancer^[77].

The future of research on the immune system in the context of HPV-associated cervical cancer has more questions than answers. Since it has been demonstrated that T-lymphocytes from patients with cervical lesions and cervical cancer are partially activated and had reduced expression of several signal transduction molecules which are involved in the complete activation of T-lymphocyte, it is of great interest to investigate whether the expression of these molecules can be reversed by type Th1 cytokines and whether they can turn these HPV-specific T-lymphocytes fully functional.

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WJCO 5th Anniversary Special Issues (3): Cervical cancer

Brachytherapy in cancer cervix: Time to move ahead from point A?

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Abstract

Brachytherapy forms an integral part of the radiation therapy in cancer cervix. The dose prescription for intracavitary brachytherapy (ICBT) in cancer cervix is based on Tod and Meredith's point A and has been in practice since 1938. This was proposed at a time when accessibility to imaging technology and dose computation facilities was limited. The concept has been in practice worldwide for more than half a century and has been the fulcrum of all ICBT treatments, strategies and outcome measures. The method is simple and can be adapted by all centres practicing ICBT in cancer cervix. However, with the widespread availability of imaging techniques, clinical use of different dose-rates, availability of a host of applicators fabricated with image compatible materials, radiobiological implications of dose equivalence and its impact on tumour and organs at risk; more and more weight is being laid down on individualised image based brachytherapy. Thus, computed tomography, magnetic-resonance imaging and even positron emission computerized tomography

along with brachytherapy treatment planning system are being increasingly adopted with promising outcomes. The present article reviews the evolution of dose prescription concepts in ICBT in cancer cervix and brings forward the need for image based brachytherapy to evaluate clinical outcomes. As is evident, a gradual transition from "point" based brachytherapy to "profile" based image guided brachytherapy is gaining widespread acceptance for dose prescription, reporting and outcome evaluation in the clinical practice of ICBT in cancer cervix.

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Key words: Cancer cervix; Intracavitary brachytherapy; Point A; Image guided brachytherapy; Computed tomography-guided brachytherapy; Magnetic resonance imaging-guided brachytherapy; Ultrasound guided brachytherapy

Core tip: Traditionally, intracavitary brachytherapy in cancer cervix is based on dose prescription at point A. However, with the availability of computed tomography-magnetic resonance imaging compatible applicators, various imaging techniques, treatment planning systems for dose computations and evaluation, there is a gradual shift towards image based brachytherapy. The article reviews the evolution of dose prescription concepts from "point" to "image" based brachytherapy in the current clinical practice of intracavitary brachytherapy of cancer cervix. This could enable prescribing doses to conform the target and avoid normal structures based on individualized applicator geometry, tumour architecture and anatomy of the organs at risk, thereby improving the therapeutic outcome.

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INTRODUCTION

Radical treatment of cancer cervix with radiotherapy is incomplete without the use of brachytherapy. The present standard of care for treatment of locally advanced cancer cervix is concurrent chemoradiation followed by brachytherapy. The probability of local control and development of normal tissue toxicity is related to the dose of radiation delivered. Since brachytherapy delivers a significant proportion of the dose, it is imperative to properly estimate the tumour extension and place the applicators optimally to decrease normal tissue toxicity and improve local controls.

The practice of external beam radiotherapy (EBRT) has seen radical changes with the advances in the field of imaging, radiation treatment planning and treatment delivery. Standard fields bounded by just “four lines” have given way to treatment portals encompassing the tumour in all its “four dimensions”. Thus individualized target delineation, dose computation and dose delivery dose has become the routine for any EBRT treatments.

However, practice of brachytherapy has not changed much and continues to be based on systems that were developed in early 20th century. Brachytherapy practices in cancer cervix had perhaps lesser scope for development, given the relatively fixed configuration of the applicators, poor visualisation of the standard applicators on computed tomography (CT) scans and the limited scope of dose optimisation with a single uterine tandem. With the advent of CT/magnetic resonance imaging (MRI) compatible applicators, availability of three-dimension (3D) cross sectional imaging and brachytherapy treatment planning software, it is time to explore the arena of cervical brachytherapy beyond the “point” based dose prescriptions.

This article summarises the evolution of brachytherapy in cancer cervix, focusing specifically on the intracavitary brachytherapy (ICBT), an integral component of radiotherapeutic management in cancer cervix. It analyses the problems inherent with the traditional point A based prescriptions and evaluates the present day scenario to explore the future approaches and possibilities that could provide a definitive dose prescription and reporting parameters on an individualized basis.

POINT A: THE TRADITIONAL BASIS OF INTRACAVITARY BRACHYTHERAPY

Use of radium sources in ICBT of cancer cervix started in 1903 and dose prescription at that time was largely empirical or subjective, on account of lack of data regarding biological effects of radiation on tumour and the surrounding normal tissues. To surmount this problem, dosimetric systems were formulated that provided guidelines regarding loading, arrangement and duration

of treatment with a set of specific radioisotopes in a designated manner to deliver the desired dose. The three dosimetric systems in use were-Paris system, Stockholm system and the Manchester system. With ICBT prescriptions being specified in terms of mg-hours, only the amount of radium required and the time duration were specified; doses to the normal tissues were neither calculated nor quantified.

To overcome these issues, Tod *et al*^[1,2] formulated the Manchester system in 1938 and they subsequently modified it in 1953. Within this system, they attempted to define the treatment in terms of dose to a point, which they believed to be representative of the target itself and was reproducible from patient to patient. To better define the actual dose that was delivered with specific “mg-hr systems”, Tod and Meredith calculated the dose (in Röntgen) at multiple points in the pelvis. They showed that the initial lesion of radiation necrosis was due to the high dose effects in the area at the medial edge of the broad ligament, within the pyramidal shaped area (paracervical triangle), where the uterine vessels cross the ureter. Keeping this triangle in mind as the dose limiting region, the authors defined point A, lying within the paracervical triangle that was 2 cm lateral to the center of the uterine canal and 2 cm superior to the mucosa of the lateral fornix, in the plane of the uterus. Additionally, applicator design, loading and arrangements were specified so as to deliver the same dose-rate at point A, regardless of the combination of applicators used for treatment.

The strong point of Manchester system point A prescription was the constancy of dose rate at the point A, irrespective of the combination of tandems and ovoids used. Point A prescription was easily taken up into clinical practice on account of its simplicity and was most suitable at that time with limited imaging modalities available (*i.e.*, orthogonal radiographs). As the originally defined point A could not be visualised on a radiograph, it was modified and redefined in relation to the applicator itself that could be visualized on radiographs. As per the new definition, point A was defined as a point 2 cm above the external os of uterus and 2 cm lateral to the uterine tandem in the plane of the uterus^[2].

HOW WELL DOES BRACHYTHERAPY POINT A REPRESENT THE ANATOMICAL POINT A?

The point A, defined in the paracervical triangle was based on the assumption that the region represented the tolerance limits due to crossing of the uterine artery and ureter. Wang *et al*^[3] correlated the anatomical point A and the brachytherapy point A in 11 patients undergoing ICBT. The anatomical point As-both right and left, were marked with radio-opaque clips and their positions compared with the brachytherapy point A. During the 64 brachytherapy applications, it was observed that the mean distances between the brachytherapy point A and anatomical point A were 5.2 cm (SD: ± 1.0) on right and 5.4

cm (SD: ± 1.1) on left. Furthermore, the dose received to the anatomical point A on right and left were 35.2% and 30% of the doses prescribed to the right and left brachytherapy point as respectively. This, questions the basic assumption of point A (the so called crossing point of the uterine artery and ureter) as being the dose limiting point. Lewis *et al*^[4] too, have demonstrated that the location of point A was far from the ureter in 93% of their observations and at a distance of 0.8 cm or more.

INTERNATIONAL COMMISSION ON RADIATION UNITS AND MEASUREMENTS REPORT 38 RECOMMENDATIONS AND ITS IMPLICATIONS

To provide an uniformity in the reporting of ICBT of cancer cervix practiced at different centres, International Commission on Radiation Units and Measurements (ICRU) proposed reporting guidelines in 1985 in its ICRU Report 38^[5]. As per the report, the type of source, the applicator, total reference air kerma (TRAK), dimensions of the reference volume (60 Gy or other dose equivalent volume), description of the dose distribution, dose rate and/or treatment time, absorbed dose at reference points and regional structures and organs at risk (OAR) were to be reported when using the 2D method of treatment planning.

Pötter *et al*^[6] reported their outcomes in 189 patients of cancer cervix stages I a to IVb treated between 1993-1997 in Vienna using the ICRU Report 38. They used a combination of a box technique for EBRT and a high-dose-rate ICBT using ring-tandem applicator. Small tumours were treated with 50 Gy of EBRT (25 Gy in brachytherapy reference volume) and 5-6 fractions of 7 Gy at point A (isoeffective at 76-86 Gy at point A) while the larger tumours received 3-4 fractions of 7 Gy after 50 Gy EBRT with open fields, (isoeffective to 82-92 Gy at point A). The 60 Gy ICRU volumes for the irradiation of small tumors ranged from 240 to 407 cm³ (mean: 337 cm³) and for larger tumors from 452 to 785 cm³ (mean: 607 cm³). At a mean follow-up of 34 mo, depending on the disease stage, their actuarial pelvic control and disease-specific survival rates varied from 52.7% to 100% and 52.1% to 100% respectively. The actuarial late grades 3 and 4 complication rate (LENT/SOMA) was 2.9% for the bladder, 4.0% for the bowel, 6.1% for the rectum and 30.6% for the vagina (shortening and obliteration). The authors felt that, in future the outcomes could be further improved using image based ICBT for a highly individualized treatment planning based on the topography of the actual tumour and OARs.

DOSE PRESCRIPTIONS IN HDR ERA USING POINT A AND ICRU REPORT 38

Over the years, the practice of ICBT has changed from

low-dose-rate (LDR) to high-dose-rate (HDR), taking into considerations several logistics and technical advantages of HDR over LDR. The use of HDR brachytherapy has increased substantially in the last decade all over the world. As per the recent Quality Research in Radiation Oncology (formerly Patterns of Care) survey (2007-2009), 62% facilities in United States were using HDR as compared to 13% in the 1996-1999 survey^[7]. During a recent global survey, HDR was found to be practiced by 85% of the respondents^[8].

Three randomized controlled trials have proved HDR brachytherapy to be comparable to LDR brachytherapy in terms of loco-regional control and complication rates^[9-11]. A meta-analysis showed that there were no significant difference in terms of outcomes between LDR and HDR^[12].

However, depending on the institutional protocols HDR ICBT requires multiple applications. This could lead to a variation in the applicator geometry and its spatial position in relation to the pelvic organs, pelvic bony anatomy and the organs at risk^[13-17]. These have been reported in terms of changes in the uterine axis, uterine length, slippage of tandem, colpostat separation and vaginal packing, resulting in fluctuations in spatial location of the applicator in craniocaudal axis, lateral and antero-posterior rotation as well as variation in coronal, transverse and saggital planes (Figure 1)^[18]. This has been attributed to mainly patient movement, vaginal packings and tumour regression during the interval between multiple fractions of HDR ICBT.

The variation in the applicator position in successive brachytherapy sessions, results in varying location of point A, as it is primarily defined in relation to the applicator itself^[14,19,20]. Multiple HDR applications, thus result in different set of point As for both right and left sides, each set corresponding to each application. Thus, the resultant of multiple ICBT in HDR brachytherapy could lead to multiple point As on right and left side, each corresponding to a particular application. This eventually results in loss of the geometrical definition of a point (a dimensionless entity), as multiple points would result in a volume encompassed by these multiple points on both sides of the intrauterine tandem (Figure 2)^[21]. Multiple point As, thus tend to change into "volume A" for multiple HDR applications in a single patient.

As a consequence to the above, in a given patient, multiple HDR ICBT could result in multiple ICRU volumes, with different volumes of common intersections depending on the variability of the applicator positions during these multiple ICBT applications (Figure 3)^[21]. All these could result in variation in the doses to various ICRU Report 38 reporting parameters-OARs, ICRU volumes, total reference air kerma in the same patient during the course of multiple HDR ICBT^[14,18,19].

Continuing to report dose to point A or ICRU Report 38 parameters, is therefore fraught with uncertainty. Apart from the ease of defining these points based on orthogonal radiographs, it is quite imperative that the extent of tumour coverage within the prescribed dose would still

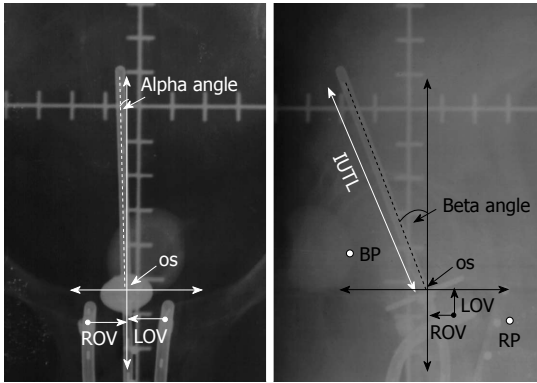


Figure 1 Schematic representation of antero-posterior and lateral radiographs with the applicator for estimation of various applicator components. IUTL: Intrauterine length; VDL: Vertical displacement; ADL: Antero-posterior displacement; ROV: Right ovoid to os; LOV: Left ovoid to os; BP: ICRU bladder point; RP: ICRU rectal point; ICRU: International Commission on Radiation Units and Measurements. Reproduced with permission^[18].

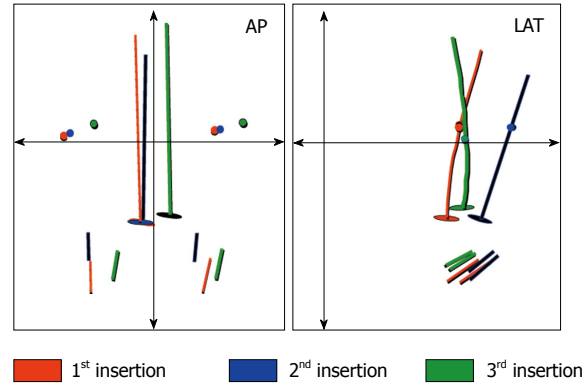


Figure 2 Fusion of standard Fletcher-Suit application positions of three insertions in a given patient with respect to the bony pelvis depicted in antero-posterior and lateral projections. Reproduced with permission^[21]. LAT: Lateral; AP: Antero-posterior.

most workers, although point A still continued to be the most common reporting parameter.

A NEED TOWARDS IMAGE GUIDED BRACHYTHERAPY: CT BASED INTRACAVITARY BRACHYTHERAPY

The limitations of orthogonal radiographs and dose prescriptions based on point A has mandated exploration of brachytherapy based on the actual position of the tumour and OARs in relation to the applicator. In the 90's, changes in brachytherapy practice started owing to the availability of CT scans and applicators that were CT/MRI compatible^[24]. Major changes in the design of the applicators were incorporated in keeping with the requirements of the tumour volume to be treated, *viz* a combined intracavitary/interstitial approach using Vienna ring applicator^[25] or Utrecht ovoid applicator^[26]. Shin *et al*^[27] performed CT based intracavitary brachytherapy and compared them with conventional point A based treatment plans. For CT based plans, dose was prescribed to the outermost point that covered all CTVs. In 30 treatment plans with HDR ICBT, the mean target volume coverage index, conformal index, significantly improved with CT based treatment plans. However, the mean values of bladder and rectal point doses and volume fractions receiving 50%, 80% and 100% of the reference dose did not differ between the plans based on CT or point A.

In another study, reported by Datta *et al*^[28], patients underwent an ICRT application and thereafter underwent a CECT scan with the CT compatible applicator *in situ*. On the scans, the target was delineated by including the entire cervix mass along with any parametrial or intrauterine or vaginal extension. The plans were generated with doses prescribed at point A and the tumour coverage within the prescribed isodose was evaluated on the axial CT images. It was observed that in FIGO stages II and III, when prescribing doses at point A, the mean percent-

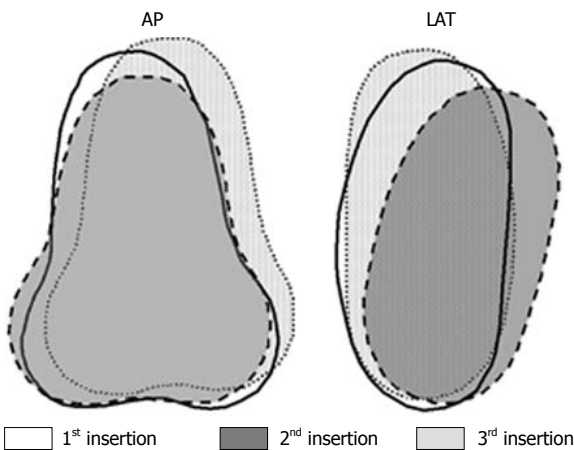


Figure 3 Fusion of three 6 Gy International Commission on Radiation Units and Measurements 38 dose distribution in a given patient with respect to the cervical os depicted in antero-posterior and lateral projections. Reproduced with permission^[21].

remain ambiguous. Moreover, the outcomes in cancer cervix have not been found to correlate with the point A doses. Katz *et al*^[22], evaluated the outcomes for tumour control and bladder and rectal morbidity in 808 applications in 396 patients with respect to the dose at point A and also the ICRU Report 38 rectal and bladder points. They reported a lack of correlation between the reference doses and outcomes^[22]. Thus, point A as a panacea of reporting or dose prescription for ICBT is questionable.

Although ICRU Report 38 recommended reporting of the 60 Gy reference isodose dimensions and other parameters including the ICRU reference volumes and doses to OARs, a survey by Pötter *et al*^[23] showed that these guidelines are usually not followed, nor are they reported by most centres in clinical practice or in the literature related to HDR ICBT. The variations in the ICRU Report 38 reference volumes with multiple HDR applications could have further added complexity to the reporting parameters and therefore might not have been favoured by

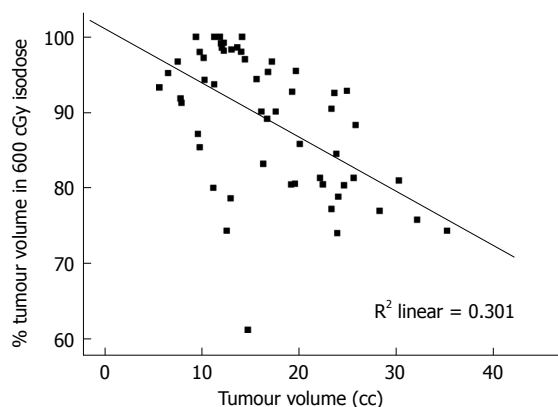


Figure 4 Scatter-plot for tumour volume vs percentage of tumour enclosed within the 6 Gy isodose lines. Reproduced with permission^[28].

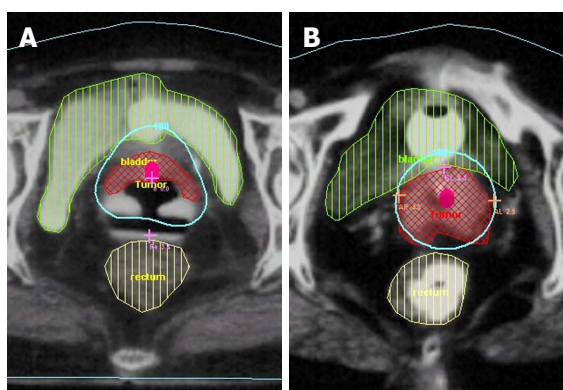


Figure 5 Tumour well covered within 6 Gy isodose volume (A) and tumour lying outside the 6 Gy isodose volume (B). Reproduced with permission^[28].

age tumour volume encompassed within the prescribed isodose (of 600 cGy) ranged from 60.8% to 100% with a mean \pm SD) of 88.8% (\pm 9.2). The extent of target coverage was inversely correlated with the target volume delineated at the time of brachytherapy (Figures 4 and 5)^[28]. Apart from this, the true maximal doses to bladder and rectum were underestimated when compared to the ICRU Report 38 reference points for these OARs and represented the 90th and 95th percentile of the maximum doses to these organs respectively.

Impact of use of 3D image based (mainly CT based) brachytherapy on outcomes was reported by Charra-Brunaud *et al.*^[29]. For the radical radiotherapy treatment arm, the local relapse free survival at 24 mo was 73.9% and 78.5% while the grade 3-4 toxicity rate was 22.7% and 2.6% in 2D *vs* 3D plans, respectively. Clearly improved outcomes both in terms of local control rates and toxicity rates have been demonstrated with use of image based brachytherapy planning.

Soft tissue imaging is best done with MRI. However, CT scans are widely available in most of the radiation oncology departments and could be used more frequently for logistic reasons. It has been shown that although CT scans are adequate for contouring the OARs, the CT based contours could significantly overestimate the tumour width^[30]. The results of the CT contouring could

be improved by contrast enhanced images for both bladder and rectum. Intravenous contrast could enhance the central areas of the cervix more than the peripheral areas and may also help to identify the uterine artery, thereby assisting the delineation of the upper border of cervix. The guidelines using CT scans for ICBT have been detailed using a comparative study with MRI^[30].

CT GUIDED INTERSTITIAL BRACHYTHERAPY

Apart from intracavitary brachytherapy, availability of CT compatible interstitial needles, permits CT guided interstitial brachytherapy. This approach could facilitate a better assessment of the target volumes and its delineation and dose adaptation leading to improved outcomes. Wang *et al.*^[31] reported the use of CT guided HDR interstitial implantation in 20 patients and had achieved a median 90% dose of 45 Gy for high risk clinical target volume by brachytherapy alone. Together with external radiotherapy, the median 90% dose reached to 94 Gy. At a median follow-up of 15 mo, only 2 patients experienced local failure. In a similar report, by Lee *et al.*^[32], 68 patients with both primary and recurrent disease were treated with CT guided interstitial brachytherapy. A median cumulative equivalent dose of 78.4 Gy was delivered by interstitial brachytherapy. At a median follow-up of 17 mo, the actuarial local control reported was 86% with grade 3 late toxicities in nine patients.

MRI BASED IMAGE GUIDED BRACHYTHERAPY AND GUIDELINES

MRI with its superior soft tissue contrast and visualization is able to detect subtle abnormalities that may not be appreciated on CT. It has been found to accurately estimate tumour size to within \pm 5 mm and correctly identify the parametrial invasion in 88% of cases^[33]. In contrast to CT which is needed for EBRT treatment planning as it requires tissue electron density data for computation, brachytherapy calculations rely on the inverse square law. Thus, MRI based treatment planning represents the current state of the art in ICBT of cancer cervix.

The Groupe Européen de Curiethérapie-European Society for Therapeutic Radiology and Oncology (GEC-ESTRO) published their detailed guidelines in 2005 and 2006 on the 3D image-based treatment planning in ICBT for cancer cervix^[34,35]. Recently, the American Brachytherapy Society (ABS) has also framed their recommendations and adopted the GEC-ESTRO guidelines for contouring, image-based treatment planning, and dose reporting^[24,36]. The guidelines provides clear recommendations for tumour delineation-gross target volume (GTV), high risk (HR) clinical target volume (CTV), intermediate risk (IR) CTV and OARs (rectum, bladder, sigmoid colon, and any adjacent bowel loops). The GTV, HR-CTV and IR-CTV represent a declining tumour cell density and thus are expected to have different radiotherapy dose

requirements. As per the recommendations, parameters for reporting are based on 3D image based dosimetric evaluation of ICBT and these include the reference volume, TRAK, prescribed dose, point A dose, D90 CTV (dose in 90% of CTV), D100 CTV (minimum target dose), D100 GTV (minimum dose in GTV), V100 CTV (CTV volume receiving > 100% of the prescribed dose) and dose volume parameters for OAR's. The recently published ABS guidelines provides dose limits for target and OARs for ICBT based on both radiographs and image based brachytherapy. The guidelines also suggests doses for template based interstitial HDR brachytherapy following 45-50.4 Gy of EBRT^[24].

The transition from 2D to 3D CT to 3D MRI based brachytherapy has been rapid. Radiation oncologists need to get accustomed to correctly interpreting the MRI data before delineation of the GTV and the high risk CTV. There is a learning curve involved and interobserver variations with MRI have been found to be lesser as compared to CT scans^[37,38]. GEC-ESTRO recommends to start with the standard method of prescription and then adjust the loading pattern and dwell times for optimisation. So, the starting point could either be point A prescription or the 60 Gy reference volume. The ABS recommends cautious use of optimisation based exclusively on dose volume histogram parameters as changes in spatial dose distribution may be significant and if not carefully analysed there may be unfavourable results^[24,36]. Using the response adapted CTV defined at the time of brachytherapy, there is an option of using dose escalation and delivering much higher dose than is feasible with EBRT^[39].

Apart from usual T2 images used for MRI based ICBT, diffusion weighted imaging (DWI) and the derived apparent diffusion coefficient (ADC) values, could also add additional biological information on tumour cell density. Haack *et al*^[40] have demonstrated significant differences in ADC values for the three GEC-ESTRO target. The mean ADC values were lowest for the IR-CTV, followed by HR-CTV and highest for GTV of the GEC-ESTRO target volumes.

The GEC-ESTRO working group recently issued guidelines for the MRI for 3D image guided cervical cancer brachytherapy^[41]. They recommended pelvic MRI scanning prior to radiotherapy and at the time of ICBT with one MR image. Multiplanar (transversal, sagittal, coronal and oblique image orientation) T2-weighted images with pelvic surface coils have been considered as the golden standard for delineating the topography of the tumour and the critical organs, while the use of complementary MRI sequences (*e.g.*, contrast-enhanced T1-weighted or 3D isotropic MRI sequences) was considered as optional.

Beriwal *et al*^[42] investigated the dosimetric consequences of brachytherapy planning using individualized MRI/CT based 3D-treatment plans for each ICBT application *vs* plans based on a single scan for all fractions. They observed that a single-plan procedure achieved

acceptable dosimetry in most patients but individualized planning at each application improved dosimetry as it took into account the variation in the applicator geometry and position of critical organs during each HDR ICBT.

Nesvacil *et al*^[43] evaluated the feasibility of adaptive 3D image based ICBT using a combination of MRI for the first BT application and planning the subsequent fractions on CT. They reported that such an approach is feasible, especially in small tumours for HR CTV coverage and OARs. However, for larger tumours, MRI based ICBT was preferred for all BT applications. This could be required in departments with limited access to MRI.

Tanderup *et al*^[44] in 72 consecutive patients compared the point doses to 3D dose volume parameters for tumour and OARs. They reported that the HR CTV90 was highly variable in standard plans with point A dose prescriptions. Although for small tumours (< 31cc), HR-CTV were well covered by standard plans in 94% patients, the OAR constraints exceeded in 72% of the cases. This was improved by MRI based optimization. On the contrary, optimization resulted in full coverage of the HR-CTV90 in 72% of the patients as compared to 25% with standard plans. The authors, concluded that point A was a poor surrogate of HR-CTV doses and MRI based image guided adaptive brachytherapy could improve target coverage and OAR doses.

OUTCOMES WITH MRI BASED IMAGE GUIDED ADAPTIVE BRACHYTHERAPY

The practice of image guided adaptive brachytherapy (IGABT) is gradually gaining momentum and the number of centres opting for IGABT is increasing. In United Kingdom, 71% of the centres in 2011 had embarked on IGABT compared to just 26% in 2008^[45]. In Canada, although point A is still the most common dose prescription point, but 73% of the centres have expressed their desire to change to 3D IGABT^[46]. Most of these centres are either using 3D imaging and planning or are in transition towards 3D IGABT.

The data on outcomes with MR based treatment planning is emerging gradually^[47-49]. Lindegaard *et al*^[48] have demonstrated that a point A based non-optimised plan will result in discrepancy to the target doses ranging from 50% to 150%. The ICRU Report 38 bladder reference point underestimated the 2 cm³ bladder dose by 75%-300%, while it overestimated the rectal dose in 75% patients. 3D MRI based planning, improved the optimization to the various dose-volume parameters^[48]. Pötter *et al*^[50] have reported excellent local control rates of 95%-100% at 3 years in limited/favourable tumours (stage I B1/ II B proximal, less than 4-5 cm) and 85%-90% in larger tumours (stage II B-IV) with acceptable treatment related morbidity (< 5%). Pelvic recurrences in this series had decreased by 65%-70%, as compared to historical series. They attributed this to the practice of MRI guided dose volume adaptation that enabled dose escalation in larger tumours (prescribed D90 > 85 Gy) often with in-

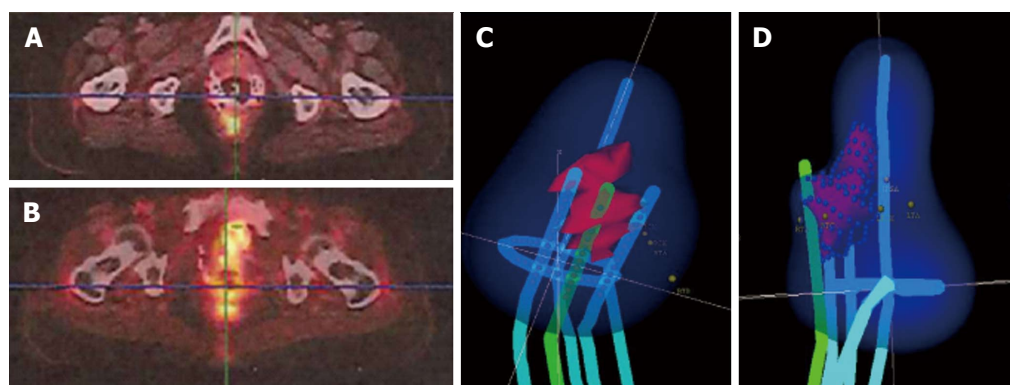


Figure 6 Positron emission tomography-computed tomography explore for brachytherapy planning in cervical cancers. A and B: Positron emission tomography-computed tomography with the Vienna applicator; C and D: Depiction of the 6 Gy isodose volume. The tumour in relation to the applicator is visualized within the 6 Gy volume.

terstitial needles as used in Vienna applicators.

In the Nordic study, Lindegaard *et al.*^[51] reported comparative results of outcome of cohort of patients treated at 2 different time periods with orthogonal radiograph based point A dose prescription (99 patients in NOCECA study, between 1994-2000) and those with MRI based IGABT (140 consecutive patients, 2005-2011). With IGABT, the actuarial local control reported was 91% at 3 years. MRI based IGABT significantly improved both overall and cancer specific survivals by 16% ($P < 0.005$) and 19% ($P < 0.001$) at three years, respectively. The improved survival was also seen in stage II B to IV stages ($P = 0.01$). Additionally, significant reduction in moderate gastrointestinal and vaginal morbidity was reported with MRI based plans ($P < 0.001$). There were also few patients who reported severe morbidity and grade 3 urological and gastrointestinal morbidities were reduced by more than 50% with MRI based IGABT over point A dose prescription brachytherapy ($P = 0.02$). This study further highlights the improved outcomes both in terms of local control, survival and reduced toxicities with MRI based IGABT in comparison to point A based conventional brachytherapy.

Studies from Vienna explored feasibility of dose escalation with CT based *vs* MRI based treatment planning^[52]. They found that compared to conventional radiograph based treatment planning, it was possible to escalate the dose to 95% of the target volume by a mean factor of 1.2 (range 1-1.7). The doses to the OARs, rectum and bladder could be maintained within the prescribed tolerance limits of 71% of the prescribed dose. Further, in a subgroup of 10 patients, MRI based ICBT permitted a dose escalation to 138% compared to 124% by CT based planning.

Using 3D IGABT with MRI, Tharavichitkul *et al.*^[53] reported their intermediate term results in 47 patients of locally advanced cancer cervix. Patients received a combination of 45-46 Gy EBRT followed by 6.5-7 Gy of 4 fractions of HDR IGABT. At 26 mo, the local control, disease free survival and overall survival were reported to be 97.9%, 85.1% and 95.6% respectively. The grade 3-4 bladder and rectum morbidity was 2.1% for each of these

OARs.

Results of a retrospective analysis of 46 patients using MR based 3D IGABT along with chemoradiation was reported from University Medical Centre, Utrecht, The Netherlands^[54]. At a median follow up of 41 mo, 3 year local control, progression free survival and overall survival was 93%, 71% and 65% respectively. Late grade 3-4 gastrointestinal and vaginal toxicities were observed in 4 patients (9.5%).

A multi-centric collaborative study, (intErnational study on MRI guided BRACHytherapy in locally advanced Cervical cancer) was launched in 2008 to validate these results prospectively^[55]. The study attempts to use MRI based IGABT in locally advanced cervical cancers in a multi-centric setting to establish benchmark for local control, overall survival, morbidity and quality of life. It also would correlate local control with GEC-ESTRO dose volume parameters for GTV, HR CTV, and IR CTV as well as late morbidity and dose volume parameters for OARs. With an expected sample size of more than 600 patients and a proposed long term follow up of 3-6 years, the outcomes from this study are keenly awaited to reconfirm the utility and efficacy of MRI based IGABT.

POSITRON EMISSION TOMOGRAPHY-CT BASED IMAGE GUIDED ADAPTIVE BRACHYTHERAPY

Positron emission tomography-CT (PET-CT) has been used increasingly for initial tumour evaluation and also during follow up in cancer cervix. PET-CT has also been explored for brachytherapy planning in cervical cancers (Figure 6). PET-CT is superior to other modalities for ruling out any positive regional lymph node or distant metastasis. The additional information could also be used to assess the target volumes for image based brachytherapy^[56,57]. Olsen *et al.*^[58] recently reported on a comparative evaluation of PET-CT and MRI. They compared the ADC maps on DWI MRI to evaluate the concordance of two functional imaging techniques and observed a good correlation of functional imaging between FDG-PET

and DWI for cervical cancer. Tumor subvolumes with increased metabolic activity on FDG-PET also were found to have greater cell density by DWI. With the availability of PET-MRI in clinics, it could be expected that in near future the IGABT could be based not just on anatomic imaging as evident on MRI or CT, but on an anato-metabolic-imaging using PET- CT/MRI.

FUTURE CONSIDERATIONS AND PRACTICAL ISSUES FOR CLINICAL ADAPTION OF IGABT

Even though GEC-ESTRO recommends 3D MRI image-based treatment planning, point A continues to be used as a starting point of optimisation and dose to point A is still reported as a bridge between the 2D and 3D treatment planning. The superiority of MRI based treatment planning has been documented in several publications. Without contesting the superiority of MRI based planning at each brachytherapy application, there is much trepidation in accepting it in routine clinical practice worldwide, especially in resource constraint settings on account of the additional cost, extra manpower and infrastructural requirement. Recently several publications have explored alternative techniques to acquire 3D image data without escalating the cost of the treatment^[59-62].

Trans-rectal ultrasound and MRI have been demonstrated by Schmid *et al*^[59] as having high correlation in accurately measuring the target width and thickness at the time of brachytherapy, when the target itself was defined as the complete macroscopic tumour mass and the remaining cervix. Trans-abdominal ultrasound too has been used to delineate the uterus, cervix and the central disease and has been demonstrated to have a fairly strong correlation with MRI^[60].

In absence of MRI, CT scans alone when used for brachytherapy planning can ensure OAR doses to be kept within acceptable limits. However, CT based target volumes have been overestimated as compared to MRI volumes^[30,63]. In limited resource setting, MRI based pre-planning at the first brachytherapy application and consecutive CT/MRI data fusion has been demonstrated to be safe and feasible with acceptable inaccuracy of soft tissue registration by Dolezel *et al*^[64].

Incorporation of successive clinical examination to CT based delineation has been explored by Hegazy *et al*^[65]. The study concluded that target delineation accuracy can systematically improve through incorporation of additional information from comprehensive 3D documentation of repetitive gynaecological examinations and can improve accuracy of dose optimization in settings with limited imaging facilities. With availability of CT images alone, a minimum two-third uterine height may be a good surrogate for height of HR CTV.

Considering the practical logistic issues, that could arise during practice of IGABT, one may have to judiciously select the patient population who may benefit

with MRI based IGABT vs CT based technique. For early and favourable disease cases, excellent local control rates have been reported with CT based planning^[45]. For these cases, dose escalation may not be required and may not need MRI based IGABT. For locally advanced diseases, the standard point A based prescriptions can result in under dosage or geographic misses as has been documented on CT images^[28].

The best way forward will be to perform a 3D cross sectional imaging prior to brachytherapy to correctly estimate the residual disease and thereafter proceed either with CT based or preferably MRI based planning^[34,35,63,64]. An alternate in form of an ultrasound based planning as evident from some of the recent studies could also be explored^[61,62,66]. It would still need some more time till we can integrate PET-CT/MRI into IGABT in cervical cancers.

CONCLUSION

Continuing to use point A dose prescriptions in ICBT at a time when the practice of EBRT has moved away from point prescriptions towards biological target based planning, amounts to an inequality in the fundamental approaches to planning for EBRT and ICBT. This is neither acceptable nor desirable. Point A prescription has for long served as the workhorse of intracavitary brachytherapy. Now, it may be time to honour it with a well-deserved place in the archives of ICBT of cancer cervix and move ahead and adapt image based intracavitary brachytherapy for an individualized and evidence based adaptive brachytherapy.

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WJCO 5th Anniversary Special Issues (3): Cervical cancer**Cervical cancer: Can it be prevented?**

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Abstract

Cervical cancer prevention requires a multipronged approach involving primary, secondary and tertiary prevention. The key element under primary prevention is human papilloma virus (HPV) vaccination. So far, only prophylactic HPV vaccines which prevent HPV infection by one or more subtypes are commercially available. Therapeutic HPV vaccines which aid in clearing established infection are still under trial. Secondary prevention entails early detection of precancerous lesions and its success is determined by the population coverage and the efficacy of the screening technique. A number of techniques are in use, including cytology, visual inspection (using the naked eye, magnivisualizer, acetic acid and Lugol's iodine), HPV testing and a combination of these methods. Updated screening guidelines have been advocated by the American Cancer Society in light of the role of HPV on cervical carcinogenesis. Recent research has also focussed on novel biomarkers that can predict progression to cancer in screen positive women and help to differentiate those who need treatment from those who can be left for follow-up. Last but not the least, effective treatment of precancerous lesions can help to reduce the incidence of invasive cervical cancer and this constitutes tertiary prevention.

A combination of these approaches can help to prevent the burden of cervical cancer and its antecedent morbidity and mortality, but all of these are not feasible in all settings due to resource and allocation constraints. Thus, all countries, especially low and middle income ones, have to determine their own cocktail of approaches that work before we can say with certainty that yes, cervical cancer can be prevented.

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Key words: Cervical cancer; Prevention; Screening; Human papilloma virus; Pap smear

Core tip: While cervical cancer is not new, approaches to prevent the burden of this deadly disease are constantly being re-invented, be it human papilloma virus (HPV) testing or screening strategies. Novel biomarkers than can predict which HPV positive lesions will progress into cancer are the need of the hour. Along with early diagnosis of pre-invasive lesions, the other preventive aspect includes prophylactic vaccines which have flooded the scene, but their true impact remains to be gauged as the precancerous phase of cervical cancer is longer than the vaccine has been around. Only time can answer the question: can we truly prevent cervical cancer?

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INTRODUCTION

Cervical cancer is the most common cause of deaths due to any cancer in developing countries and the number of these is ten times that in developed countries. It is the third most common cancer in women after breast and

Table 1 Risk factors for cervical cancer

Causal agent	Relative risk
Low socio-economic class	1.5
Low educational level	2-3
Early age at first coitus	2-4
Multiple sexual partners	2-5
Early age at first pregnancy	2-4
Multiparity	2-4
Long term use of oral contraceptives	1.5-2
History of sexually transmitted infections	4-10
History of genital warts	18
Cigarette smoking	2-4
Diet low in folates, carotene and vitamin C	2-3
Lack of routine cytological screening or prior abnormal smears	2-6
HIV	2.5
Immunosuppression	5.7

HIV: Human immunodeficiency virus.

colorectal cancer, with more than 530000 cases in 2008, 85% of these in developing countries. The mortality: incidence ratio is 52% and there were 275000 deaths in 2008, 88% of them in developing countries^[1,2].

The case for prevention of cervical cancer is thus a strong one that would serve to prevent mortality in many women and morbidity in many others. We know that cervical cancer is preventable, but the bigger question is, can it be prevented?

Prevention of any condition is described in terms of primary, secondary and tertiary prevention. While primary prevention deals with modification of risk factors to prevent disease occurrence, secondary prevention essentially signifies early diagnosis and treatment, while tertiary prevention seeks to limit the disability caused by the condition^[3]. Cervical cancer is particularly amenable to prevention as it has a long pre-clinical phase and the natural history of cervical carcinogenesis is well researched. In addition, treatment of pre-invasive lesions has been shown to reduce the incidence of invasive cervical cancer.

PRIMARY PREVENTION

In terms of cervical cancer, primary prevention involves education about safe sexual practices and *human papilloma virus* (HPV) vaccination^[4]. This is because HPV is known to be a necessary cause of cervical cancer and it is the persistence of this infection over decades which can lead to precancerous changes in the cervix and eventually cancer. The first vaccine licensed was Gardasil (Merck, Pennsylvania) in 2006 which protects against HPV 6, 11, 16 and 18, while Cervarix (Glaxo Smith Klein, Belgium) which was licensed in 2009 protects against HPV 16 and 18. Both of these are prophylactic vaccines which have now been introduced in over 80 countries. Short term data indicates that they are safe, immunogenic and efficacious in preventing HPV infection and hence precancerous lesions by the respective HPV types. There is

also some evidence of cross-protection against closely related HPV types. They are less effective in established infection. The best evidence of HPV vaccination success comes from Australia, where introduction of nationwide HPV vaccination resulted in a decrease in the incidence of high grade cervical abnormalities within 3 years after vaccination^[5]. Recent data indicates that two doses of vaccine may be as protective as three doses^[6] and this has implications for use where the utilization is limited by costs.

There is another kind of vaccine, the therapeutic vaccines which help in the clearance of HPV infections by generating T-cell mediated immunity against the HPV E6 and E7 antigens. These have been shown to be efficacious under trial conditions^[7].

However, although HPV infection is the most common, it is not the only causal agent of cervical cancer. As understood by the model for cervical carcinogenesis, there are various other behavioral and demographic risk factors that increase the relative risk of developing cervical cancer. These are given in Table 1.

Others factors like older age, racial factors and genetic predisposition are non-modifiable risk factors^[8]. There is some data to indicate that consumption of high amounts of whole fruits and vegetables, fish and nuts which provide a rich source of antioxidants like vitamin C, E, carotene, lutein and lycopene, and vitamin A, calcium and poly-unsaturated fatty acids can significantly reduce the risk of CIN^[9]. The mechanism of action is purported to be by enhancing HPV clearance while still in the transient phase, inhibiting the expression of E6 and E7, preventing DNA damage and reducing immunosuppression^[9].

SECONDARY PREVENTION

Secondary prevention involves screening asymptomatic patients or carrying out definitive tests in symptomatic or screen positive patients to pick up precancerous lesions before they turn into cancer. A number of methods are available for cervical cancer screening. Observational studies have shown that introduction of any regular cervical cancer screening program results in a fall in the incidence of invasive cervical cancer and cancer deaths. The Nordic countries are a prime example where introduction of organized cervical screening reduced the incidence of cancer deaths between 10%-80%.

Various modalities employed for screening preinvasive disease are: (1) cervical cytology - both conventional and liquid-based; (2) direct visual inspection (DVI); (3) visual inspection using 3%-5% acetic acid (VIA); (4) visual inspection using 3%-5% acetic acid and magnification (VIAM); (5) visual inspection using Lugol's iodine (VILI); (6) HPV DNA testing; (7) speculscopy; and (8) polar probes. Other modalities like colposcopy, cervicography and microcolpohysteroscopy can be used for further evaluation of abnormal results.

Cervical cytology is the globally preferred screening method and has been shown to reduce the incidence

Table 2 American College of Obstetricians and Gynecologists guidelines for cervical cancer screening

Commence	Frequency of smears (Pap or LBC) and HPV testing	Discontinue	HPV DNA
21 yr	1 3 yearly smears for < 30 yr 2 3 yearly smears or 5 yearly co-testing for > 30 yr (if 2 previous smears normal) 3 3 yearly smears or 5 yearly co-testing for those previously treated for CIN2/3 or cancer (up to 20 yr)	1 > 65 yr 2 After hysterectomy for benign disease with no history of CIN	For women > 30 yr, two options to manage Positive test: Repeat co-testing at 12 mo; Test for HPV 16/18 and colposcopy if positive

HPV vaccination does not change these guidelines. HPV: Human papilloma virus; LBC: Liquid based cytology.

of invasive cervical cancer by up to 80%^[10], while other methods of screening have generally been used in project settings. However, the fall in incidence with Pap smear based screening is directly linked to the frequency of screening and the proportion of population covered by screening. The conventional Papanicolaou test is done to examine exfoliated cells from the ectocervix and the endocervix using a wooden Ayre spatula and cytobrush. The smear is prepared and fixed with 95% ethylene glycol. Slides are stained using the Papanicolaou method and graded according to the revised Bethesda system. Although cytology has a high specificity of 95%-99%, pooled data has shown the sensitivity of a single Pap smear to be as low as 51%^[11]. For liquid based cytology (LBC), the method involves using a cytobrush which is rotated by 360 degrees five times around the cervix and the exfoliated cells are stirred in a proprietary solution. This reduces specimen inadequacy (which can cause false negative smear results) by 80% but adds to the cost. In addition, a single specimen may be used for HPV, chlamydia and gonorrhea testing. Screening guidelines have been advocated by various societies.

The latest screening guidelines^[12] by the American College of Obstetricians and Gynecologists (ACOG) published in 2012 are given in Table 2.

Where resources are limited, the World Health Organization recommends that the highest priority group for screening is those aged 35 or over and that screening every 5 years for a total of 3 tests in a lifetime will achieve a major impact. In low resource settings, screening using HPV as a primary test followed by further triaging for treatment using cytology or VIA of HPV positive women can cut costs while maximising benefits of screening.

Direct (unaided) visual inspection (DVI) is advocated in low resource settings where no other method is available as the incidence of CIN in a clinically unhealthy cervix may be as high as 9% compared to 0.9% in a healthy cervix^[13]. DVI can detect cancer early and improve survival rates and thus should be done in all patients, even if pregnant.

Aided visual inspection methods which include VIA, VIAM and VILI are simple, low tech approaches that are minimally reliant on infrastructure, assuming that basic facilities for performing a speculum examination are available. Non-physicians can perform the procedure if they receive adequate and ongoing training. Furthermore, results of the procedure are available immediately, making

it possible, in principle, to provide treatment during the same visit (screen-and-treat or single-visit approach). VIA involves swabbing the cervix with 5% acetic acid and inspecting the cervix in good light after 1 min to look for acetowhite lesions. For VIAM, the cervix is inspected as before but by using a self-illuminated hand held device. The disadvantages are low specificity compared to cytology, potential for over-diagnosis and over-treatment, observer dependency and usefulness in detecting ectocervical disease only. Our own institutional data (including over 1200 patients) found the sensitivity and specificity of VIA to be between 91%-96% and 31%-82% respectively^[13,14]. The large variation in specificity indicates that several variables affect the test characteristics of VIA, including light source, observer training, criteria for test positivity and the presence of co-existing infection, inflammation and metaplasia. VIAM offers the advantage of 4 × magnification using a hand held battery powered device and improved the specificity over VIA from our data^[14]. It can supplement VIA in doubtful cases where colposcopy is not available as a secondary triage. In low resource settings, primary screening by VIA/VIAM is more cost effective compared to universal Pap smear and can be used to take a guided biopsy and endocervical curettage.

HPV DNA testing

Two types of tests for HPV DNA are currently in use; one is a nucleic acid hybridization assay with signal amplification for the qualitative detection of high risk HPV types in cervical specimens (Digene Hybrid Capture 2 High Risk HPV DNA TestTM; Cervista HPV HR TestTM); the other is a polymerase chain reaction based assay (HPV DNA Nested Polymerase Chain Reaction Detection KitTM). Detection of high risk HPV DNA increases the sensitivity of detection for both squamous and glandular abnormalities; however, it does not have the analytical specificity that can help to decide which lesions need treatment and those that will regress on follow-up.

A new test for HPV E6/E7 mRNA (PreTect HPV-ProoferTM assay and APTIMATM assay) is under research and is based on the fact that mRNA levels are directly correlated to the severity of the lesion and can predict progression to cancer with higher specificity than HPV DNA testing alone. This can be used to stratify high risk HPV positive women that need treatment. The reported sensitivity and specificity range from 0.41-0.86

and 0.63-0.97 respectively for PreTect based on pooled data^[15]. FDA approval is awaited.

In both triage (investigation of minor abnormalities detected by cytology) and screening studies (when both cytology and HPV testing are jointly performed) the cross-sectional sensitivity of HPV test is high and so is the negative predictive value ($> 97\%$). The combination of the high sensitivity of HPV DNA testing and the high specificity of cytology can increase the screening interval for testing in women negative by both methods. Such a combined test was approved by the FDA in 2003 for primary screening of low risk women aged ≥ 30 years^[16]. In low and middle income countries, integrating the highly accurate HPV testing with the triaging capacity of VIA in “screen and treat” protocols can offer the dual benefits of maximizing detection using HPV and then using VIA to triage them for treatment^[17]. Despite the high cost of HPV testing compared to VIA alone, this can turn out to be cost effective in the longer run due to the costs saved on diagnosis and treatment of cancer. The advantages of HPV DNA testing are the objectivity of the test, possibility of complete automation, built in quality control, opportunities for self sampling and high sensitivity. Its disadvantages are cost, dependence on a single manufacturer (so far only HC2 is FDA approved and validated), requirement of a molecular diagnostic lab, low specificity in younger women and populations with significant HIV seropositivity, and follow up visits for test results and treatment.

Speculoscopy refers to direct observation of the cervix under 4-6 \times magnification using a blue-white chemiluminescent light source to enhance visualization of abnormal tissue after acetic acid application. This is a variant of the VIA designed to increase its specificity but it is marginally more expensive.

Polar probe is a pen-sized device (which is moved across the cervix) of electro-optical systems to identify cancer or pre-cancerous cells in cervical tissue by measuring the response of cervical tissue to light together with tissue capacitance of epithelial, basal and stromal layers. In a multicentric study, it was found to be as sensitive as a top quality Pap smear. It also has a high accuracy with instant report which prevents loss to follow up. Due to the objective, self-checking digital system, there is no subjective error of interpretation or the need for trained personnel to read the smear.

Colposcopy provides a magnified (up to 40 \times) stereoscopic view of the cervix and vagina and is a sensitive method for diagnosing CIN and invasive cancers. It helps in localizing abnormal areas from where a biopsy can be taken and accurate grading and conservative management of CIN is possible. It can supplement cytology and also triage cases that are a doubtful positive on VIA/VIAM. Its disadvantages are bulky and costly equipment and the need for experienced personnel.

Cervicography is a technique that attempts to reproduce colposcopy photographically. A photograph of the cervix (cervigram slide) is taken with a specially designed

camera (cerviscope) after the application of acetic acid and sent to an expert for interpretation. Cervicography has a better sensitivity than cytology (89% *vs* 52%), with similar specificity (94% *vs* 92%)^[18]. Cervicography is a highly sensitive tool for evaluating the ectocervical transformation zone but is unable to evaluate the endocervical canal. The expense of the instrument and the costs of photographs make it unlikely to be used for population screening, although it can be used in combination with a Pap smear to facilitate the selection of therapy for patients with an abnormal Pap test.

Microcolpohysteroscopy permits a naked eye view of the endocervix to evaluate the cervical canal *in situ*, thus obviating the need for a cone biopsy. It focuses on cells that have not been desquamated within their topographic and architectural context. A magnification of 20 \times gives visualization comparable to colposcopy, while 150 \times gives visualization comparable to cytology.

Recent research has centered on identifying the host genes up regulated in association with HPV infection, determining their suitability as “surrogate markers” for HPV infection, and using them to identify HPV-associated epithelial lesions in tissue or cytological specimens^[19]. These can help to increase diagnostic accuracy of cervical tissue specimens and provide information on risk of progression. These are given in Table 3.

Other newer technologies like optical imaging, spectroscopy and high-resolution imaging methods provide *in vivo* diagnosis with high sensitivity and specificity and are anticipated to improve conventional cervical cancer screening. They are based on the concept of morphological and biochemical alteration in the properties of cervical tissue in response to malignant transformation. In addition, contrast agents that target against specific neoplastic biomarkers can enhance the effectiveness of this new technology^[20].

The cycle of testing (using a sensitive test at regular intervals), diagnosis (using a highly specific test), treatment (with effective methods and by trained staff) and follow-up (as a part of an organized program with high population coverage) should be completed to ensure the success of screening.

TERTIARY PREVENTION

Tertiary prevention seeks to limit disability and promote rehabilitation. As cervical cancer has a long history in the form of precancerous lesions, diagnosis in the early phase and proper management (by cryotherapy or large loop excision of the transformation zone (LLETZ)) will prevent the progression to invasive cancer. Both can be done in the outpatient setting. While cryotherapy is useful for lesions involving maximum 1-2 contiguous quadrants of the cervix and no endocervical involvement, LLETZ can treat the entire transformation zone as well as a lesion extending not more than 1 cm into the endocervical canal. It also has the advantage of removing the specimen for histological analysis^[21]. Complication rate is less

Table 3 Biomarkers in cervical dysplasia

Biomarker	Significance
L1 capsid protein	Represents approximately 90% of the total protein on the virus surface and is generally detectable during the reproductive phase of HPV infection. The L1 protein is abundant in productive infections (CIN 1), found only in rare cases of CIN2/3, and not produced in carcinomas
p16INK4a (CINtec™)	Surrogate marker of HPV E7-mediated pRb catabolism, providing evidence of transformation of the cervical mucosa. On immunohistochemistry, diffuse staining for p16INK4a is present in almost all cases of CIN2, CIN3, squamous cell carcinoma and endocervical glandular neoplasia; however, it is rarely detected in benign squamous mucosa or CIN 1 lesions caused by low risk HPV types
Ki-67	Proliferation marker confined to the parabasal cell layer of normal stratified squamous mucosa but shows expression in the stratified squamous epithelium in CIN lesions in correlation with the extent of disordered maturation, but cannot discriminate HPV-mediated dysplasia from proliferating cells in benign reactive processes
DNA Aneuploidy	HPV infection leads to DNA hypermethylation, disruption of the normal cell cycle, and chromosomal aberrations, all of which may lead to changes in DNA content. Aneuploidy increases progressively from CIN1 to CIN3
MCMs (ProExC test™)	MCMs are required for the origination of DNA replication and are overexpressed in cervical high-grade dysplasia and carcinoma, but can also be seen in some benign cycling squamous and glandular cells
FISH technology	One of the most consistent chromosomal abnormalities in cervical carcinoma is gain of chromosome arm 3q (in about 70%), which can be detected by FISH. TERC gene in this region is amplified in progression to CIN3

FISH: Fluorescent *in situ* hybridization; MCAs: Minichromosome maintenance proteins; TERC: Telomerase RNA component.

Table 4 Management of preinvasive cancer (American Society for Colposcopy and Cervical Pathology 2012 guidelines)

Lesion on biopsy	Other features	Management
CIN 1	Preceding cytology of ASC-US, ASC-H, LSIL	Follow up with cytology (6, 12 mo) and HPV testing (12 mo)
CIN 1	Preceding cytology of HSIL, AGC-NOS	Either of these: Diagnostic excisional procedure or review of findings or observation with HPV and cytology (12 and 24 mo) (only if colposcopy satisfactory and ECC negative)
CIN 1	Adolescent (< 20 yr)	Follow up with cytology (12 mo)
CIN 1	21-24 yr	Follow up with cytology and colposcopy (6 monthly, up to 2 yr)
CIN 2/3	Satisfactory colposcopy	Either excision or ablation of transformation zone
CIN 2/3	Unsatisfactory colposcopy or recurrence or endocervical disease	Diagnostic excisional procedure
CIN 2/3	Adolescent (< 20 yr) and young women (21-24 yr)	Observation with cytology and colposcopy (only if colposcopy satisfactory) or treatment using excision or ablation of transformation zone
Adenocarcinoma <i>in situ</i>	Specimen from diagnostic excisional procedure	Hysterectomy preferred (rarely conservative management if margins negative and future fertility desired)

HPV: Human papilloma virus. CIN: Cervical intraepithelial neoplasia, ASC-US: Atypical squamous cells - undetermined significance; ASC-H: Atypical squamous cells - cannot exclude; LSIL: Low-grade squamous intraepithelial lesion; HSIL: High-grade squamous intraepithelial lesion; ECC: Endocervical curettage.

with cryotherapy and includes watery discharge for 3-4 wk, cervical stenosis (< 1%) and vaginal bleeding (very rare). It has no adverse effects on fertility and pregnancy and can be carried out by the average gynecologist. LLETZ on the other hand, requires more technical skill, a ready supply of electricity and is associated with severe perioperative bleeding (< 2%), cramping abdominal pain and effects on future fertility (infertility, preterm labor, cervical stenosis and dystocia).

The American Society of Colposcopy and Cervical Pathology has issued guidance for management of preinvasive cervical lesions diagnosed on biopsy^[22]. These are given in Table 4. Similar guidelines are also in place for a diagnosis based on Pap smear.

Pregnancy constitutes a special situation where the only indication for treatment is suspected invasive cancer. Both CIN 1 and CIN 2/3 require follow up during pregnancy (no more frequently than 12 wk) as the risk of progression to invasive cervical cancer is minimal and the rate of spontaneous regression postpartum is relatively high. Re-evalua-

tion is recommended no sooner than 6 wk postpartum.

Thus, prevention of cervical cancer involves a multi-pronged approach of education, creating awareness, advocacy, public-private partnerships for HPV vaccination, screening and early treatment of precancerous lesions before they develop into cancer. The extent of focus on each of these measures may vary between communities and countries based on the availability of resources and healthcare commitments. A holistic approach to prevention involving locally effective measures and treatment protocols and evaluating their adherence and success over time can help to tailor programs and policies to maximize the benefits for cervical cancer prevention.

Cervical cancer is preventable. Cervical cancer can be prevented. The extent to which we achieve this goal depends on us.

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WJCO 5th Anniversary Special Issues (4): Head and neck cancer**Clinical and scientific impact of human papillomavirus on head and neck cancer**

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Abstract

Head and neck cancer (HNC) arises from the skull base to the clavicles and is the fifth most common cancer in the world by incidence. Historically, in the developed world HNC was associated with tobacco use and alcohol consumption, and the combination of the two produced a synergistic increase in risk. However, beginning in 1983, investigators have found a significant and growing proportion of HNC patients with human papillomavirus-positive (HPV) tumors who neither drank nor used tobacco. Since that time, there has been increased interest in the molecular biology of HPV-positive HNC. Multiple studies now show that HPV has shifted the epidemiological landscape and prognosis of head and neck squamous cell carcinoma (HNSCC). These studies provide strong evidence for improved survival outcomes in patients with HPV-positive HNSCC compared to those with HPV-negative HNSCC. In many reports, HPV status is the strongest predictor of locoregional control, disease specific survival and overall survival. In response to these findings, there has been significant interest in the best management of HPV-positive disease. Discussions within major cooperative groups consider new trials designed to maintain the current strong survival outcomes while reducing the long-term treatment-re-

lated toxicities. This review will highlight the epidemiological, clinical and molecular discoveries surrounding HPV-related HNSCC over the recent decades and we conclude by suggesting how these findings may guide future treatment approaches.

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Key words: Human papilloma virus; Head and neck cancer; Squamous cell carcinoma; Chemotherapy; Radiation; Molecular biology

Core tip: Head and neck squamous cell carcinoma (HNSCC) is the fifth most common cancer, and historically, in the developed world, was associated with tobacco and alcohol. However, beginning in 1983, investigators have found a growing proportion of HNSCC patients with human papillomavirus-positive (HPV) tumors who neither drank nor used tobacco. HPV has shifted the epidemiology and prognosis of HNSCC and HPV status is the strongest positive prognostic marker in patients with oropharyngeal SCC. This review will highlight the epidemiological, clinical and molecular discoveries surrounding HPV-related HNSCC over the recent decades and how these findings will guide future treatment approaches.

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INTRODUCTION

Head and neck cancer (HNC) describes a broad range of tumors that arise from the skull base to the clavicles. Worldwide, the incidence of HNC exceeds half a million

annually, making it the fifth most common cancer in the world^[1]. In the year 2013, it is estimated that there will be 53640 new cases of HNC and 11520 deaths attributed to this disease in the United States alone^[2]. Anatomic sites of the head and neck include: the sinuses, orbits, nasopharynx, oropharynx, oral cavity, hypopharynx, and larynx. The primary risk factors for HNC include tobacco, alcohol, human papillomavirus (HPV) infection (for oropharyngeal cancer) and Epstein-Barr virus (EBV) infection (for nasopharyngeal carcinoma). The relative prevalence of these risk factors contributes to the various distributions of disease in different areas of the world.

Over the past 20 years there have been marked improvements in the diagnosis and treatment of HNC. Three-dimensional imaging including computed tomography (CT scans), positron emission tomography (PET), and magnetic resonance imaging (MRI) has allowed for better staging, target localization, and treatment planning. Advances in microsurgery and radiation therapy techniques have decreased treatment related morbidity and long term toxicities, while maintaining locoregional control (LRC)^[3].

Historically, in the developed world head and neck squamous cell carcinoma (HNSCC) was associated with tobacco use and alcohol consumption, and the combination of the two produced a synergistic increase in risk^[4]. However, a significant minority of patients (15%-20%) did not smoke or drink, indicating a different etiology existed. In 1983 investigators found immunohistochemical evidence of human papillomavirus (HPV) infection in tissue samples from 6 oral SCC^[5]. Since that time, there has been increased interest in the molecular biology of HPV-positive HNSCC. Multiple studies now show that HPV has clearly shifted the epidemiology and prognosis of head and neck squamous cell carcinoma (HNSCC). These studies provide strong evidence for improved survival outcomes in patients with HPV-positive HNSCC compared to those with HPV-negative HNSCC. In many reports, HPV status is the strongest predictor of locoregional control (LRC), disease specific survival (DSS) and overall survival (OS)^[6-8].

In response to these findings, there has been significant interest in the best management of HPV-positive disease. Discussions within major cooperative groups including the Radiation Therapy Oncology Group (RTOG) and the Eastern Cooperative Oncology Group (ECOG) have considered new trial designs based on HPV-status including: induction chemotherapy with response adapted radiation; alternatives to concurrent cisplatin chemotherapy, de-escalation of radiation dose, and the integration of minimally invasive surgery into the treatment algorithm. The primary objective of these approaches is to maintain the current strong survival outcomes while reducing the long-term treatment toxicities. A detailed analysis and comparison of these techniques and results is beyond the scope of this review, but may be found elsewhere^[8]. There have been a number of reviews that have addressed HPV in HNSCC^[9,10]. Here we review the

discoveries and observations (including clinical retrospective reviews, clinical prospective trials, and basic science research) that showed how HPV dramatically changed the epidemiologic, clinical, pathological and molecular landscape of head and neck cancer. In addition, we present hypotheses for the causes of these changes, and how they may guide future clinical trial design.

EPIDEMIOLOGIC CHANGES

Retrospective analyses have shown a clear change in the epidemiology of HNSCC in the United States over the last 4 decades. A review of 60 published studies using PCR-based methods to detect and genotype HNSCC biopsies demonstrate a higher prevalence of HPV-positivity in oropharyngeal SCC (OPSCC) *vs* oral or laryngeal SCCs (35.6% *vs* 23.5% *vs* 24.0%). HPV-16 was found in 86.7% of the HPV-positive OPSCCs, while the second most prevalent high risk type HPV-18 was found in only 2.8% of HPV-positive OPSCCs^[11]. A Surveillance, Epidemiology, and End Results (SEER) study of HNC cases between 1973-2003 showed a 1.85% annual reduction in smoking-related HNSCC, while HPV-related HNSCC had increased by 0.8% per year^[12]. HPV-related sites were defined as oropharyngeal, while smoking-related sites were defined as oral cavity, nasopharynx and larynx. The decrease in smoking-related HNSCC correlated with a decrease in smoking in the United States during that time^[13]. In addition, the age at diagnosis for HPV-related HNSCC decreased by 1.5 years while the age at diagnosis for HPV-unrelated HNSCC increased. Analysis of tissue from 271 cases in the SEER database between 1984 and 2004 revealed that HPV prevalence in OPSCC tumors increased from 16.3% during the 1980s to 72.7% during the 2000s^[14]. Table 1 shows the estimated number of HPV-positive and HPV-negative HNC cases in the United States in 2011 by site. Similar increases were shown in Swedish, Australian, and Canadian populations^[15-17].

Changes in sexual practices are potential causes for the observed increase in HPV-related HNSCC. HPV transmission occurs primarily through direct sexual contact, most often during vaginal or anal intercourse, but may also occur during oral sex or other forms of mucosal contact. As a general trend, Americans are reporting their first sexual encounter at a younger age, they are having more sexual partners, and are performing more oral sex^[18-20]. In particular, a French study showed the lifetime prevalence of oral sex increased from roughly 50% in 1970 to 90% in 2006^[19]. Two cross-sectional studies conducted in the United States confirmed that increased sexual promiscuity, such as lifetime number oral sex partners, was associated with an increased risk of oral HPV-16 infection^[21,22]. Additional epidemiologic studies have shown that exposure to HPV increases the risk of developing HNSCC, and HPV-16 seropositivity predates cancer development by 9 years^[23,24]. Finally, a case-control study by D'Souza *et al*^[25] revealed that the number of vaginal and oral sexual partners [odds ratios (OR), 3.1 and

Table 1 Human papillomavirus-negative *vs* human papillomavirus-positive tumors in 2011 based upon ACS estimates

Head and neck cancers	Total	HPV-	HPV+
Larynx	12740	10192	2548
OC/P: mouth	11510	9208	2302
OC/P: other	2250	1800	450
OC/P: tongue	12060	4342	7718
OC/P: pharynx	13580	4889	8691
(Oropharyngeal cancer)	(25640)	(9230)	(16410)
Total	52140	31456	20684

HPV-: Human papillomavirus-negative; HPV+: Human papillomavirus-positive.

3.4] and HPV-16 seropositivity (OR, 32.2) correlated with an increased risk of developing OPSCC. Notably, alcohol and tobacco increased the risk of developing OPSCC primarily in those patients without HPV-16 exposure. Similar results have been substantiated in other studies of HNSCC^[26-28].

CLINICAL CHANGES

The changing epidemiology of HNSCC, particularly HPV-positive OPSCC, created a new patient profile in the clinic. Patients began presenting at a much younger age without a strong alcohol or tobacco history and with more advanced disease in the neck^[29]. The natural history of HPV-positive HNSCC began to unfold following several small retrospective studies reported in the late 1990s and early 2000s. In an analysis of 42 patients treated between 1975-1987, HPV-positive SCC of the tonsil showed improved survival compared to HPV-negative SCC^[30]. Furthermore, an incidental finding in a 1998 German study of 208 HNSCC samples (11 of 36 tonsil primaries tested positive for HPV DNA) revealed a better prognosis despite more adverse pathologic features for the HPV-positive samples^[31]. In a Swedish review of 60 patients treated with radiation with or without surgery between 1986-1996, HPV-positive OPSCC cases had better 5-year OS (53.5% *vs* 31.5%) and decreased risk of recurrence (OR, 4.1) regardless of age, stage or gender^[32]. These findings were supported by a Swiss study of 98 patients with OPSCC who received definitive radiation therapy from 1991-1997, which also showed that HPV-positive tumors (14% of the total) had better LRC and OS [risk ratios (RR), 0.33 and 0.35]^[33]. A meta-analysis of 37 studies examining HPV and HNSCC from 2007 showed that HPV-positive OPSCC in particular had a 28% reduced risk of death [meta hazard ratio (HR) 0.72] and a 49% lower risk of disease-failure (HR, 0.51) than HPV-negative OPSCC. The prognostic benefits of HPV-positive tumors was not significant for other sites^[34].

These smaller studies led to larger retrospective analyses of prospective trials. The phase III TAX324 trial comparing two different induction chemotherapy regimens was retrospectively reviewed and included 111 OPSCC patients. HPV-positive patients had OS and locore-

gional failure (LRF) rates of 79% and 13% compared to 31% and 42% for HPV-negative patients^[35]. In the phase II ECOG 2399 trial involving induction chemotherapy followed by chemoradiation, HPV-positive patients (38 of 96) had a better response to induction chemotherapy (82% *vs* 55%) and chemoradiation treatment (84% *vs* 57%)^[8]. Moreover, the HPV-positive OPSCC patients had a 62% lower risk of progression (HR, 0.38) and a 61% lower risk of death (HR, 0.39) when compared to those with HPV-negative OPSCC^[6]. Around the time of ECOG 2399, the Danish DAHANCA 5 phase III clinical trial prospectively collected samples from 156 patients who underwent conventional radiation therapy for OPSCC or supraglottic SCC from 1986-1990. Of the 74 OPSCC samples, 24 were p16-positive (a surrogate marker for HPV-driven disease) and showed a LRC benefit (OR, 5.1) compared to those that were p16-negative^[36]. A phase II prospective trial of OPSCC from 2000-2002 using induction chemotherapy to stratify definitive treatment paths correlated HPV-16 to response and survival. Pre-treatment biopsies of 42 patients were analyzed and 67% were HPV-positive. HPV titer was associated with significantly improved response to induction chemotherapy ($P = 0.001$), improved response to chemoradiation therapy ($P = 0.005$), improved OS ($P = 0.007$), and improved DSS ($P = 0.008$)^[37]. When the Trans-Tasman Radiation Oncology Group (TROG) 20.02 trial comparing cisplatin-based chemoradiation with or without tirapazamine was retrospectively reviewed for HPV and p16 status, 57% of 185 patients with OPSCC were p16-positive. The p16-positive tumors showed better 2-year OS (91% *vs* 74%; HR, 0.36; $P = 0.004$) and failure-free survival (87% *vs* 72%; HR, 0.39; $P = 0.003$). Table 2 summarizes select studies that examined HPV status and prognosis for HNSCC.

It was evident that patients with HPV-positive HNSCC experienced improved OS compared to HPV-negative disease, but whether this was secondary to improved treatment sensitivity, locoregional control or distant metastasis was unclear. Princess Margaret Hospital reviewed the rates of distant metastases (DM) in HPV-positive OPSCC following radiation or chemoradiation therapy. They included 457 HPV-positive and 167 HPV-negative OPSCC cases, and while DM rates were similar at 3 years, 24 of 25 HPV-positive cases of DM occurred within 2 years while 7 of 54 HPV-negative cases were detected 3 years after treatment. The authors found that the post-DM survival rates were 11% *vs* 4% at 2-years in favor of the HPV-positive cases and interestingly, 5 of 6 HPV-positive patients with lung oligo-metastases had stable disease 2-years after salvage procedures, which included chemotherapy, resection, or radiation^[38]. Additional recent retrospective reviews, have shown similar unique patterns of distant metastatic spread including to bone, brain, and multiple organs, and increased post-DM OS for HPV-positive patients^[38-42]. The trend of better post-DM OS was not seen when analyzing data from the SPECTRUM trial of metastatic or recurrent HNSCC.

Table 2 Selected references for association of tumor human papillomavirus status with prognosis

Ref.	Country	N	Site	Detection (PCR, p16, ISH) ^a	Prevalence of HPV-positive disease ^b	Follow-up time ^c (¹ median, ² mean, ³ range)	Significantly improved prognosis for HPV-positive tumor status? (Yes/No)	Prognosis for HPV-positive vs HPV-negative disease ^d	Factors adjusted for
Andl <i>et al</i> ^[31] , 1998	Germany	31	Tonsil	PCR, p16	48%	28 mo ²	Yes	OS: improved ($P = 0.0071$) DFS: median 61.1 mo vs 25.8 mo ($P = 0.028$)	Overall stage
Gillison <i>et al</i> ^[47] , 2000	United States	259	HNSCC	PCR, ISH	25% overall; 57% OP	31 mo ²	Yes	OS: 91 mo vs 76 mo, HR 0.6 (0.35-1.0, $P = 0.07$); DSS: HR 0.41 (0.20-0.88, $P = 0.02$)	Age, LN disease, alcohol
Mellin <i>et al</i> ^[32] , 2000	Sweden	60	Tonsil	PCR	43%	59 mo ¹	Yes	OS: 5-yr 53.5% vs 31.5%; RFS: OR 19.6 ($P = 0.014$); DSS: improved ($P = 0.047$)	RFS: Overall stage DSS: Overall stage, LN metastases, age, gender
Lindel <i>et al</i> ^[33] , 2001	Switzerland	99	OP	PCR	14%		Yes	LFSS: RR 0.31 (0.09-0.99, $P = 0.048$)	T, alcohol, intratumoral microvessel density
Weinberger <i>et al</i> ^[78] , 2006	United States	123	OP	p16	13%	33 mo ²	Yes	OS: 5-yr 60% vs 21%, HR 0.422 (0.2-0.9, $P = 0.021$) DFS: 5-yr 62% vs 19%, HR 0.359 (0.2-0.7, $P = 0.006$)	tumor type (primary vs recurrent), overall stage, grade, treatment
Weinberger <i>et al</i> ^[78] , 2006	United States	79	OP	PCR, p16	61%	22 mo ²	Yes	OS: 5-yr 79% vs 18%-20%, HR 0.19 (0.1-0.7, $P = 0.13$) DFS: 5-yr 75% vs 13%-15%, HR 0.20 (0.1-0.6), $P = 0.005$	primary vs recurrence, treatment, overall stage, grade
Licitra <i>et al</i> ^[82] , 2006	Italy	90	OP	PCR, p16	19%	5.8 yr ¹	Yes	OS: 5-yr 79% vs 46% ($P = 0.0018$), improved when adjusted for stage	Overall stage
Reimers <i>et al</i> ^[72] , 2007	Germany	106	OP	PCR, p16	30%		Yes	DFS: 85% vs 49% ($P = 0.009$), HR for HPV-negative tumors 7.5 (1.22-46.19), $P = 0.030$	EGFR expression status, overall stage
Kumar <i>et al</i> ^[73] , 2008	United States	66	OP	PCR, p16			Yes	OS: improved ($P = 0.006$) DSS: improved ($P = 0.02$)	Smoking, gender
Fakhry <i>et al</i> ^[6] , 2008	United States	96	OP, larynx	PCR, ISH	63% OP, 0% larynx	39.1 mo ¹	Yes	OS: 2-yr 95% vs 62% ($P = 0.005$), HR 0.36, (0.15-0.85) PFS: HR 0.27 (0.10-0.75)	age, overall stage, ECOG performance status
Hafkamp <i>et al</i> ^[71] , 2008	Netherlands	81	Tonsil	ISH, p16	41%	30 mo ²	Yes	DSS: 5-yr 55% vs 29%, unadjusted HR 2.3 (1.1-4.5)	
Worden <i>et al</i> ^[37] , 2008	United States	66	OP	PCR	64%	64 mo ¹	Yes	OS: improved ($P = 0.008$) DSS: improved ($P = 0.004$)	gender, smoking, T, N, age, site
Smith <i>et al</i> ^[28] , 2009	Germany	60	OP	PCR, ISH, p16	47%	27.5 mo ¹	Yes	DFS: 5-yr 71% vs 46% ($P = 0.02$)	
Lassen <i>et al</i> ^[36] , 2009	Denmark	156	Supraglottic larynx, pharynx	p16	22%	> 5 yr	Yes	OS: 5-yr 62% vs 26% ($P = 0.0003$), T, N HR 0.44 (0.28-0.68) DSS: 5-yr 72% vs 34% ($P = 0.0006$), HR 0.36 (0.20-0.64)	
Ang <i>et al</i> ^[7] , 2010	United States	323	OP	ISH, p16	63.80%	4.8 yr ¹	Yes	OS: 3-yr 82.4% vs 57.1% ($P < 0.001$), HR 0.42 (0.27-0.66) PFS: 3-yr 73.7% vs 43.4% ($P < 0.001$), HR 0.49 (0.33-0.74)	age, race, T, N, tobacco exposure, treatment assignment
Rischin <i>et al</i> ^[44] , 2010	United States, Canada, Australia, New Zealand, Europe	184	OP	PCR, ISH, p16	57%	29 mo ²	Yes	OS: 2-yr 91% vs 74% ($P = 0.004$), HR 0.43 (0.20-0.93), $P = 0.031$	ECOG performance status, hemoglobin, T, N
Chaturvedi <i>et al</i> ^[14] , 2011	United States	271	OP	PCR, ISH	44%	112 mo ¹	Yes	OS: median 131 vs 20 mo ($P < 0.001$), HR 0.31 (0.21-0.46)	age, calendar period of diagnosis, overall stage, treatment
Posner <i>et al</i> ^[35] , 2011	United States	111	OP	PCR	50%	82-83 mo ¹	Yes	OS: improved, unadjusted HR 0.2 (0.10-0.38, $P < 0.0001$) PFS: 73% vs 29% ($P < 0.0001$) LRF: 13% vs 42% ($P = 0.0006$) DSS (OP): HR 0.1 (0.02-0.4)	
Liang ^[36] , 2012	United States	488	HNSCC	PCR, p16	62%		No		

^aPCR is polymerase chain reaction to assess for HPV-related DNA; p16 refers to immunohistochemistry for HPV oncoprotein p16; ISH is in-situ hybridization for HPV; ^bHPV tumor status as defined by individual study; ^cFollow-up time included when reported (¹Median, ²Mean, ³Range); ^dIncludes prognosis

tic data when available and as reported. HR are adjusted and for HPV-positive tumors unless indicated otherwise. 95% confidence intervals are included in parentheses. PCR: Polymerase chain reaction to assess for HPV-related DNA; ISH: *In-situ* hybridization; P16: Immunohistochemical assay for p16 expression; OS: Overall survival; DSS: Disease-specific survival; RFS: Recurrence-free survival; EFS: Event-free survival; LR: Local recurrence; LFFS: Local failure free survival; FFS: Failure-free survival; LRC: Locoregional control; LRR: Locoregional recurrence; HR: Hazard ratio; OR: Odds ratio; RR: Relative risk; LN: Lymph node; OC: Oral cavity; OP: Oropharynx; BOT: Base of tongue; DSS: Disease-specific survival; FOM: Floor of mouth; T: Tumor stage; N: Nodal stage.

Of 443 patients, 99 were p16-positive, and no significant difference was observed in OS for these patients when compared to p16-negative patients^[43]. Additionally, when Rischin *et al*^[44] analyzed the TROG 20.02 trial there was no difference in DM rates between HPV-positive and HPV-negative OPSCC patients.

As previously discussed, HPV-positive HNSCC generally presents in younger healthier patients with small primary tumors and advanced lymph node (LN) metastases. The analysis of the TAX324 trial confirmed the association with smaller primaries and improved performance status. The authors found that HPV-positive patients were more likely to have T1 or T2 tumors (49% *vs* 20%) and an ECOG performance status of 0 (77% *vs* 49%)^[35]. Similar results were found when the TROG 02.02 Phase III trial data was retrospectively reviewed; p16-positive tumors were more likely to have a lower T stage ($P = 0.001$), higher N stage ($P = 0.001$), and better performance status ($P = 0.002$)^[44]. The ECOG 2399 data showed that although nodal status and overall AJCC TNM stage did not differ by HPV status, HPV-positive tumors were more likely to have a tumor stage of T2 *vs* T3 or T4, and an ECOG performance status of 0 (66% *vs* 33%)^[6]. An analysis of cystic lymph node metastases (20 of 100) from neck dissections from 2002-2004 showed a strong association with HPV-positive SCC of the tonsil^[45]. Princess Margaret Hospital reviewed data from 493 N2-3 HNSCC patients between 2003-2009 and found that HPV-positive LNs (257) were larger (2.9 *vs* 2.5cm), were more likely to be cystic (38% *vs* 6%), regressed more often post treatment (36% *vs* 41% of initial size), and were more likely to resolve after 36 wk (90% *vs* 70%), when compared to LNs of HPV-negative patients^[46]. The etiology of these differences in clinical presentation is unknown, and do not seem to be prognostic when controlling for HPV status.

Microscopic pathologic differences have also been observed between HPV-driven HNSCC and tobacco and alcohol-related HNSCC. HPV-positive tumors are more likely than HPV-negative tumors to be poorly differentiated and to have basaloid features, though the relevance of this difference is still undetermined^[6,47]. The improved LRC observed in HPV-positive HNSCC may be due to a lack of field cancerization historically observed in smoking-related HNSCC causing a higher risk of both recurrence and development of a second primary tumor^[48-50]. A Germany study of 25 HPV-16 positive HNSCCs in 2001 showed that HPV-16 was more likely to localize to the tonsil and HPV DNA did not appear outside of the tumor, suggesting that HPV-positive patients may not have entire mucosal fields predisposed to tumor development^[51].

MOLECULAR DIFFERENCES

The molecular biology of high risk HPV-driven tumorigenesis has been thoroughly studied in cervical cancer. HPV is a small, nonenveloped double-stranded DNA virus that infects keratinocytes and promotes transformation by altering cell cycle control primarily through expression of the nuclear proteins E6 and E7. Normally, p53 levels increase in response to DNA damage and prevent entry into S phase at the G1 checkpoint. E6 prevents accumulation of p53 and p53-mediated cell cycle arrest by causing the ubiquitination and subsequent degradation of p53^[52-54]. E6 likely promotes immortalization through other mechanisms including increasing telomerase activity^[55].

E2F is a transcription factor necessary for DNA synthesis, and is inhibited by hypophosphorylated retinoblastoma protein (pRb). Mitogenic signals cause an increase in cyclin-dependent kinases (CDKs), which promote phosphorylation of pRb leading to the release of E2F allowing entry into S phase. E7 preferentially binds to hypophosphorylated pRb causing its degradation and uncoupling the G1 checkpoint from CDK control^[56-58]. The CDK inhibitor p16 is normally suppressed by pRb and E7-mediated pRb degradation causes p16 upregulation^[59]. However, E7 stimulates the S-phase cyclins E and A, which bypass the normal p16 cell cycle inhibitory effects^[60]. E7 also binds to another DNA damage checkpoint protein p21, a p53-induced CDK inhibitor, driving the cell cycle into S phase^[61].

Molecular investigations of HNSCC initially did not differentiate between HPV-positive and HPV-negative samples because they were conducted in the era when HPV-negative tumors were more prevalent. Studies showed that the *TP53* gene was mutated in 45% of HNSCC and *TP16* gene inactivation by either mutation, deletion, or promoter hypermethylation occurred in 80% of HNSCC^[62-65]. To determine the prognostic significance of *TP53* mutations, a large scale prospective study of 560 patients with HNSCC who were treated with primary surgery with curative intent was performed. Disruptive *TP53* mutations were found in 53% of patients and predicted for decreased OS (HR, 1.7)^[66]. The epidermal growth factor receptor (EGFR) pathway has been extensively studied in various cancers and several trials have shown clear clinical efficacy of a targeted EGFR blockade. In regards to HNSCC, EGFR is overexpressed in approximately 80% of cases, and EGFR overexpression and copy number both correlate with a poorer prognosis^[67-70].

A German study from 1998 examined the status of the retinoblastoma (Rb) pathway in 208 HNSCC that were treated surgically. The investigators found that 11%

of the tumors had no or dramatically reduced levels of the pRb without genetic disruption of the *Rb1* gene, and these samples localized to the tonsil. They also overexpressed p16 and had wild type *p53*. The authors detected high risk HPV DNA in 11 of the 12 pRb-deficient tumors strongly suggesting that the E7 viral protein inactivated the Rb pathway. These tumors had poorer differentiation and they were all metastatic at the time of resection, yet they had better clinical outcomes post treatment compared to the rest of the cohort^[31]. Although this study had a very small number of HPV-positive patients, several additional studies examining HPV status and cell cycle regulators followed. A study in 2000 including 253 patients with HNSCC tested archived samples for HPV-status and *TP53* mutations. HPV was detected in 25% of the samples, and HPV-positive tumors were less likely to harbor *TP53* mutations (OR, 0.06)^[47]. A Dutch group investigating the relationship between HPV-status and *p53* mutational status examined 47 HNSCC in 2003 and found 10 were HPV-positive with 8 of those being OPSCC. All 10 HPV-positive cases overexpressed p16, while 8 of the 10 overexpressed *p53*, yet none harbored mutations in *p53*. They also noted an inverse relationship between smoking and HPV-positivity^[71]. The interest in EGFR prompted an examination of EGFR and p16 expression in 106 OPSCC patients, which showed a significantly higher 5-year disease-free survival (DFS) and OS for patients who overexpressed p16, but not EGFR when compared to those with tumors overexpressing EGFR, but not p16^[72]. Further analysis of a phase II trial for OPSCC showing HPV copy number correlated with prognosis, revealed that HPV copy number was associated with p16 expression ($P < 0.0001$), and p16 overexpressing tumors had a better response to therapy ($P = 0.009$) and OS ($P = 0.001$). In addition, EGFR expression correlated with worse OS ($P = 0.001$) and DSS ($P = 0.002$), and was inversely associated with HPV copy number and p16 expression^[73].

In 2005 investigators attempted to use gene expression analysis to accurately predict LN metastasis in oral and OPSCC. They included 45 tumors from patients who were N+ postoperatively or who subsequently developed LN metastasis and 37 tumors from individuals who were N0 postoperatively and remained metastasis-free. The 102 predictor genes outperformed the current clinical diagnosis when independently validated^[74]. Gene expression profiles for 8 HPV-positive and 28 HPV-negative HNSCC were generated and published in 2006. Statistical analysis based on HPV status identified 91 differentially expressed genes, which included HPV-related overexpression of *p16*, *p18*, and *CDC7*, and a significant proportion of the HPV-positive genes localized to 3q24^[75]. Recently, multi-institutional groups conducted whole-exome sequencing of 74 HNSCC tumor-normal pairs (14% HPV-positive) and of 32 HNSCC samples. The results identified previously known HNSCC genes (*TP53*, *CDKN2A*, *PTEN*, *PIK3CA*, and *HRAS*), and found mutations in genes that regulate squamous differentiation (*NOTCH1*,

IRF6, and *TP63*). Compared with traditional tobacco-induced HNSCC, HPV-positive samples had one half the mutation rate ($P = 0.004$) and an inverse relationship with *TP53* mutations ($P = 0.001$)^[76,77]. These studies have not resulted in dramatic breakthroughs yet, but form an important foundation for elucidating critical pathways in HNSCC.

Molecular characterization and environmental history has allowed stratification of patients with OPSCC into risk categories. In 2006, the HPV and p16 status of 79 OPSCC patients with long term follow-up were determined leading to a three-class model based on p16 expression and the presence of HPV DNA. The class that was HPV-positive and overexpressed p16 had better OS (79% *vs* 20% and 18%; $P = 0.0095$), DFS (75% *vs* 15% and 13% ($P = 0.0025$), 5-year local recurrence (14% *vs* 45% and 74%; $P = 0.03$), and lower *p53* and pRb expression ($P = 0.017$ and 0.001)^[78]. Ang *et al*^[7] retrospectively reviewed 323 OPSCC patients from the phase III prospective trial RTOG 0129 comparing standard and accelerated fractionation for HPV status. They found that 63.8% of patients had HPV-positive tumors and these patients had better 3-year OS (82.4% *vs* 57.1%, $P < 0.001$). In addition, the risk of death significantly increased with each additional pack-year of tobacco smoking. Using recursive-partitioning analysis for HPV-status, pack-years of tobacco smoking (≤ 10 *vs* > 10), tumor stage (T2-T3 *vs* T4), and nodal stage (N0-N2a *vs* N2b-N3), patients were classified as having a low (3-year OS 93.0%), intermediate (3-year OS 70.8%), or high risk (3-year OS 46.2%) of death^[7].

One explanation for the improved LRC seen in HPV-driven HNSCC is an increased sensitivity to chemoradiation^[79]. E6-related degradation of *p53* in HPV-positive cancers may be functionally inequivalent to HPV-negative *p53* mutations, and therefore, HPV-positive tumors may have an intact apoptotic response to radiation and chemotherapy. However, enforced expression of E6 and/or E7 in cell lines did not cause radiosensitization *in vitro*^[80,81]. Interestingly, *p53* overexpression has been found in HPV-positive tumors, which indicates that another mechanism unrelated to E6 may be involved^[71]. Moreover, survival for patients with HPV-positive oropharyngeal cancers was improved relative to HPV-negative patients both with and without *p53* mutations in their tumors and in patients treated with and without radiation therapy^[82].

Pre-clinical studies show that successful chemoradiation depends on innate and adaptive antitumor immune responses, and the increased immunogenicity of HPV-infected tumor cells may contribute to their robust treatment response^[83,84]. As part of a prospective phase II trial for OPSCC, 47 patients had baseline immune cell counts in addition to assessment of EGFR and HPV status. The authors found that improved survival was associated with an elevated percentage of CD8 cells ($P = 0.04$), a low CD4:CD8 ratio ($P = 0.01$), low EGFR expression ($P = 0.002$), and HPV status ($P = 0.02$). The percentage of CD8 cells was significantly higher ($P = 0.04$) and

the CD4:CD8 ratio was significantly lower ($P = 0.02$) in HPV-positive patients. A higher percentage of CD8 cells was associated with response to induction chemotherapy ($P = 0.02$) and complete tumor response after chemoradiotherapy ($P = 0.045$)^[85]. These associations were studied at a basic level in HPV-positive and HPV-negative OPSCC cell lines. *In vitro*, there was a decreased response to either cisplatin or radiation for the HPV-positive cell line. However, *in vivo*, there was an increased response to both cisplatin and radiation. The authors found this only to be true in immunocompetent mice and immune-deficient mice that had been injected with competent immune cells, suggesting more of an immunologic mechanism for HPV-driven disease response to therapy^[86].

FUTURE DIRECTIONS

There are clear clinical, epidemiologic and molecular differences between tobacco-driven tumorigenesis in the oropharynx and HPV-driven OPSCC. However, these complex interactions are inadequately described, and clinically, it appears that some OPSCCs are caused by a combination of both tobacco and HPV infection. While the relationship between HPV-induced oncogenesis, tumor sensitivity and improved clinical outcomes is still being investigated, we now know that approximately 50%-80% of OPSCCs are associated with HPV-positivity. With these trends, it is estimated that HPV will eventually become the primary etiology for head and neck cancer in the United States.

Induction chemotherapy, alternative systemic regimens to cisplatin and other approaches have been investigated prior to the era of HPV-positive disease. The retrospective analyses of these trials, which we have discussed above, created the overwhelming body of evidence for the better prognosis of HPV-positive OPSCC. In response to the improved survival outcomes, several collaborative groups have considered dose reduction in patients with HPV-positive HNSCC. Patients receiving lower doses of radiation and/or chemotherapy should experience less acute and long-term toxicities. Because patients with HPV-positive HNC are typically younger and demonstrate excellent LRC and OS, the value of limiting long-term toxicities such as lymphedema, swallowing dysfunction, and xerostomia is particularly important. ECOG 1308, a phase II trial, recently reported excellent early outcomes in patients where radiation dose reduction was based on a complete response to chemotherapy^[87]. Several groups have initiated clinical trials investigating other forms of treatment de-intensification such as using less toxic radiosensitizers. For example, the RTOG 1016 phase III trial plans to compare concurrent cisplatin and radiotherapy *vs* concurrent cetuximab and radiotherapy for HPV-positive OPSCC. Three additional randomized trials investigating patients with HPV-positive disease are currently pending enrollment or underway in the United States^[88].

Molecular markers are being collected prospectively in

these trials, and continued research will offer new insight into the oncogenic pathways that influence clinical outcomes. Many questions remain including: why do HPV-driven tumors present with smaller primary tumors and more advanced nodal involvement; why do HPV-driven OPSCCs respond better to locoregional chemoradiation and induction chemotherapy, but have similar distant metastasis rates; and would the molecular analysis of distant metastatic disease show similar differences in HPV- *vs* tobacco-driven OPSCC?

Perhaps HPV-driven tumors are more localized to the tonsillar crypts and the lack of mucosal field changes influences the size of the primary tumor. The lack of field cancerization may also contribute to the improved LRC, although it would not fully explain improvements seen in response to both induction and chemoradiation. Another possibility is that HPV-driven tumors are more immunogenic and are tempered by an active immune response. Perhaps the molecular characteristics of HPV-driven tumors predispose malignant cells to metastasize to LNs, and once HPV-driven disease enters the LNs it creates a more robust immune response. This response may be due to intracellular characteristics or cell surface markers on HPV-positive cells, such as E7.

Since metastasis rates are similar for HPV-positive and -negative tumors, it is possible that once an OPSCC cell becomes metastatic, it dedifferentiates to a particular molecular state regardless of HPV-status. However, recent reports point towards increased time to DM, and improved OS after DM for HPV-positive disease, which appears to contradict previous evidence of a comparable response to salvage/palliative chemotherapy. Both pre-clinical and correlative studies suggest that immunogenicity is an important component to HPV-driven disease response. Perhaps differences in clinical outcomes will be due to differential immune responses. HPV vaccines in adolescents promise to reduce the incidence in HPV-driven OPSCC in decades to come, but to date they have not shown efficacy as therapy for these tumors after they develop. We may discover clinical benefits using immunomodulators in HNSCC, just as we do with current trials in malignant melanoma and non-small cell lung cancer.

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Towards optimal management of the axilla in the context of a positive sentinel node biopsy in early breast cancer

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Abstract

The sentinel lymph node biopsy (SLNB) was initially pioneered for staging melanoma in 1994 and it has been subsequently validated by several trials, and has become the new standard of care for patients with clinically node negative invasive breast cancer. The focussed examination of fewer lymph nodes in addition to improvements in histopathological and molecular analysis has increased the rate at which micrometastases and isolated tumour cells are identified. In this article we review the literature regarding the optimal management of the axilla when the SLNB is positive for metastatic disease based on level 1 evidence derived from randomised clinical trials.

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Key words: Sentinel lymph node biopsy; Early breast cancer; Axillary radiotherapy; Axillary dissection; Evidence-based medicine

Core tip: There has been a shift in the management

of the axilla when the sentinel lymph node biopsy is positive towards less radical surgery thus reducing the incidence of arm morbidity and improving the quality of life of our patients.

Wazir U, Manson A, Mokbel K. Towards optimal management of the axilla in the context of a positive sentinel node biopsy in early breast cancer. *World J Clin Oncol* 2014; 5(5): 792-794 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i5/792.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i5.792>

INTRODUCTION

Sentinel lymph node biopsy (SLNB) was pioneered for the staging of melanoma in 1994^[1]. Shortly thereafter, Giuliano *et al*^[2] demonstrated the feasibility of SLNB for breast cancer. Over the subsequent two decades, the SLNB has been validated by several trials, and has become the new standard of care for patients with clinically node negative invasive breast cancer. Since the SLNB is positive in approximately 30% of patients undergoing surgical treatment of clinically node negative breast cancer, therefore, 70% of women are now able to avoid radical surgery, in the form of complete axillary node dissection (ALND), which is known to be associated with a higher incidence of morbidity and longer hospitalisation, with impairment of quality of life^[3].

The SLNB technique formally utilises a radioactive isotope tracer in addition to a blue dye. When both modalities are used, the procedure has been reported to be 96% accurate when performed by an experienced operator^[4]. It was noted in early studies that there was not any identifiable advantage of lymphoscintigraphy mapping even for surgeons learning the techniques. Intra-operative frozen section analysis of the sentinel node has been shown to be accurate for the evaluation of metastatic disease with high sensitivity and excellent specificity^[5].

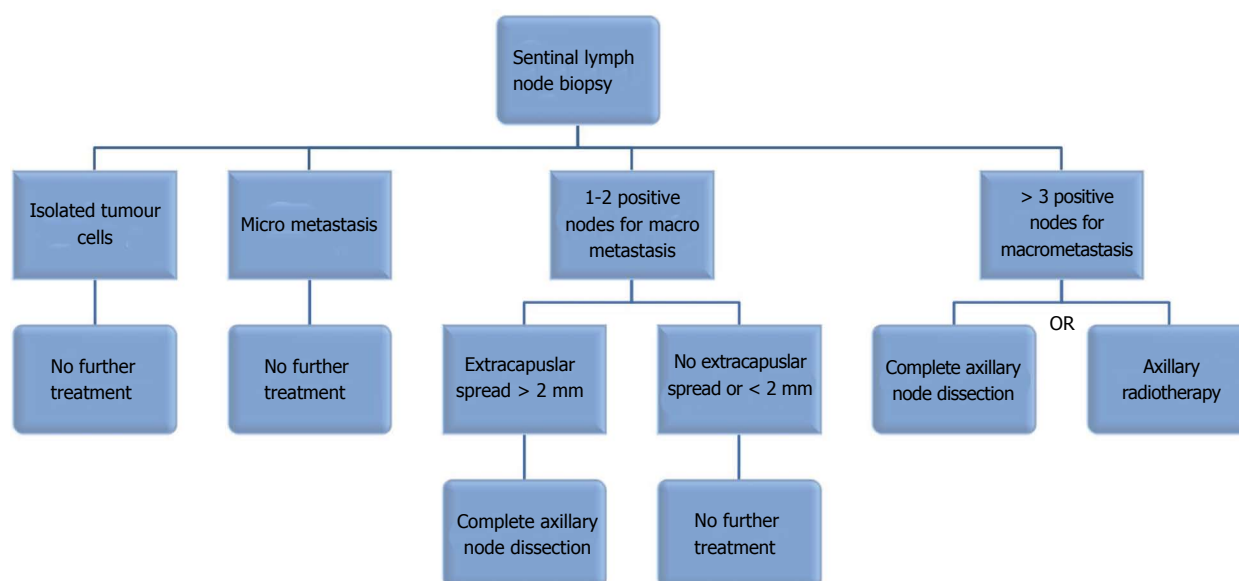


Figure 1 Evidence-based algorithm for the management of the axilla in clinically node negative women with T1-T2 breast cancer undergoing breast conserving.

Since the sixth edition of the AJCC/UICC TNM classification, the extent of metastatic disease in the sentinel node has been classified into three categories: isolated tumour cells (ITCs; < 2 microns); micrometastatic disease (MM; 2 microns-2 mm); and macrometastatic disease (> 2 mm). In addition, the sixth edition also mentions real-time polymerase chain reaction (RT-PCR) as a possible means of detecting cancerous cells in sentinel nodes^[6].

MANAGEMENT OF ITCs AND MM

ITCs and MM have become more common findings due to the increased use of immunohistochemistry (IHC). In the current seventh edition of the TNM staging, the trends noted in the previous edition have become the norm in most major centres^[7]. Similarly, new refinements in RT-PCR, such as One-Stop Nodal Analysis, have been recently introduced for intra-operative evaluation, and have been demonstrated to be even more sensitive for detecting metastatic deposits than IHC^[8].

The clinical implications of these findings was unclear, with little progress towards a consensus until very recently due to non-availability of suitable level evidence^[9-11]. Although the presence of ITCs in the sentinel nodes has a prognostic significance, however, there is a consensus that it does not represent an indication for further treatment of the axilla^[11]. The presence of micrometastatic disease in the sentinel node was, until recently, considered an absolute indication for a complete axillary node dissection. However, more recent evidence is highly suggestive that a more conservative approach to such micro-deposits would be more appropriate despite modest upstaging of the disease^[9]. A recent randomised controlled trial (RCT) confirmed the oncological safety of omitting complete ALND for MM positive SLNB (IBCSG 23-01)^[12].

MANAGEMENT OF MACRO-METASTASES

Until recently the presence of macrometastases in the sentinel nodes has been considered a routine indication of complete ALND, however, a recent randomised controlled trial carried out by the American College of Surgeons Oncology Group (Z0011) showed that in women undergoing breast conserving surgery (BCS) for clinically node negative T1/T2 invasive breast cancer, complete axillary node dissection is not required if only 1-2 sentinel nodes were found to be involved by malignancy. The five year disease-free survival and overall survival were similar in the axillary node dissection group *vs* no axillary node dissection group. There was no significant difference between the two groups in relation to the primary tumour characteristics and the use of adjuvant systemic therapy. All the patients received adjuvant radiotherapy following breast conserving surgery^[13]. Therefore, patients who fulfil the criteria for this mature trial can avoid complete ALND, which is associated with a higher incidence of complications. The presence or absence of extra-capsular extension (ECE) was not analysed in this trial. A recent retrospective study showed that the presence of an ECE greater than 2 mm in the sentinel nodes was associated with significant tumour burden in non-sentinel nodes during ALND and therefore this feature can be added to the eligibility criteria for omitting ALND when the SNB is positive (*i.e.*, ECE < 2 mm)^[14].

Complete axillary node dissection is still indicated in women undergoing breast conserving surgery, who are found to have metastatic disease involving three or more lymph nodes and those undergoing total mastectomy with positive sentinel node biopsy involving any lymph nodes.

In a further shift towards less radical surgery to the axilla, the recent EORTC ARAMOS randomised controlled trial showed that axillary radiotherapy was as effective as complete axillary node dissection in terms of five year overall and disease-free survivals. Furthermore, radiotherapy (50Gy in 25 fractions) was associated with a lower incidence of lymphedema of the arm, in the short and long terms. The authors, however, observed non-significant trend towards impairment in the shoulder movement in women undergoing radiotherapy to the axilla in the short term. Earlier analysis of the trial data showed that the lack of knowledge of the pathological status of the non-sentinel nodes did not influence the treatment decisions in relation to adjuvant systemic therapy^[14]. Moreover, oncologists are increasingly basing their systemic treatment recommendations on the use of multigene molecular signatures of the primary tumour, such as Mammaprint[®], Oncotype DX[®], and Endopredict[®].

The American Society of Clinical Oncology (ASCO) has recently updated its guidelines to reflect the results of these trials^[15]. The adoption of these guidelines worldwide will spare thousands of women radical surgery to the axilla, which is associated with a higher incidence of complications and longer hospitalisation without compromising their clinical outcome. Needless to say that the final treatment recommendations are made in the context of multidisciplinary discussion. The findings of these trials are consistent with the biological behaviour of breast cancer and the longstanding perception that axillary surgery aims to provide staging and prognostic information to guide systemic treatment and radiotherapy recommendations rather than achieving mechanical eradication of the disease. Evidence-based medicine means that we should embrace the results of these RCTs and introduce the new ASCO recommendations into our clinical practice in order to improve the quality of life of our patients. The proposed management of a positive SLNB in patients undergoing BCS for T1-T2 breast cancer has been summarised in the algorithm shown in Figure 1.

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Emerging gene-based prognostic tools in early breast cancer: First steps to personalised medicine

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Abstract

Breast cancer remains a major cause of neoplastic disease in much of the developed world. The majority of cases are diagnosed with oestrogen receptor (ER)-positive and human epidermal growth factor receptor-2 negative invasive ductal carcinoma and are treated predominantly by surgery which includes sentinel node biopsy and adjuvant endocrine therapy \pm adjuvant radiotherapy. It is believed that an indeterminate subset of the patient population is needlessly incurring chemotherapy related morbidity without attaining any increase in survival due to therapy. Furthermore in the era of extended adjuvant endocrine therapy it is important to identify those patients who can be safely treated with 5 years rather than 10 years of endocrine therapy thus optimising the benefit-risk balance. This perception has propelled the development of more personalised prognostic tools for newly diagnosed cases of ER-positive breast cancer. In this article, we shall review the evidence regarding the currently available gene assays for human breast cancer.

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Key words: Personalised medicine; Breast cancer; Prognosis; Polymerase chain reaction

Core tip: Recurrence score, Prosigna and EndoPredict (EP) currently have the most convincing evidence available, of which Prosigna and EP have a significant degree of external validation. In terms of cost and turnover, EP has an advantage over its competitors, being designed to be performed at a local laboratory rather than at a central facility. The results of the MINDACT and TailorX trials are awaited.

Wazir U, Mokbel K. Emerging gene-based prognostic tools in early breast cancer: First steps to personalised medicine. *World J Clin Oncol* 2014; 5(5): 795-799 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i5/795.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i5.795>

INTRODUCTION

Breast cancer remains a major cause of neoplastic disease in much of the developed world, comprising of 30.7% of cancers diagnosed in 2011. The 41523 cases were registered during that year^[1]. The majority of cases are diagnosed with oestrogen receptor (ER) positive and human epidermal growth factor receptor-2 (HER2) negative invasive ductal carcinoma, which predominantly undergo surgery and staging, including sentinel node biopsy (SNB)^[2]. Subsequent decisions by the multi-disciplinary team regarding the use of chemotherapy, radiotherapy and endocrine therapy are determined by the perceived risk of recurrence. Conventionally, the risk of recurrence is estimated based on histology, receptor status and the result of the SNB, or by composite prognostic tools, such as the Nottingham Prognostic Index^[3], and Adjuvant! Online (Adjuvant, Inc., San Antonio, TX)^[4].

However, it is believed that an indeterminate subset of the patient population is needlessly incurring chemotherapy related morbidity without attaining any increase

in survival due to therapy. This perception has propelled the development of more personalised prognostic tools for newly diagnosed cases of breast cancer^[3]. Furthermore, the results of the ATLAS randomised trial suggests that the survival benefit of continuing adjuvant tamoxifen for 10 years may be superior to stopping at 5 years after diagnosis of ER positive breast cancer. This finding has necessitated the development of new tools that could identify the subset of women who would not benefit from extended adjuvant endocrine therapy beyond 5 years^[5].

Central to these developments was the identification of sub-types of breast cancer based on so-called “molecular patterns” or “signatures”. These classifications are now referred to as intrinsic sub-types, and have a broad if imperfect concordance with breast cancer classifications based on histology and receptor status. Initially, breast cancers were typed as luminal, HER2 enriched and basal. Luminal are further sub-typed into luminal-A and -B^[6]. The intrinsic typing of breast cancers continues to be an area of continuous research. As of the time of writing, 7 intrinsic types have been identified thus far. Luminal-A is characterised as strongly ER positive, while luminal-B is less so, with a greater preponderance of proliferative genes. Broadly speaking, luminal-A corresponds with ER positive and HER2 negative tumours, which are characterised as relatively low risk for recurrence^[7].

With a suitable prognostic test, it may be possible to treat a portion of luminal-A patient with post-resection endocrine therapy rather than chemotherapy with endocrine therapy. This subset of patient has been the target of the majority of the extant prognostic and predictive assays.

The major gene-based prognostic assays for breast cancer have been discussed below (Table 1).

MAMMAPRINT

This the oldest test available, developed by Agendia BV (Netherlands). This is a 70 gene DNA microarray test performed on frozen or formalin-fixed tissue by a central reference laboratory, which returns a score which stratify patients into a high and low risk categories^[8].

The assay was developed in a non-randomised cohort of 78 patients treated at the Netherlands Cancer Institute, in which the median age was 55^[9]. Subsequent internal studies characterised this assay to be an independent prognostic assay in the primary target group, outperforming clinical parameters^[10].

However, it is yet to be validated externally. The studies pertaining to this assay were performed in only one market. Furthermore, owing to the lack of randomisation, the cited studies do not qualify as level 1 evidence. In addition, the population in which it was used is considerably younger than that seen in many countries where this assay may potentially be utilised^[11]. Furthermore, the test seems to be a reliable predictor of recurrence occurring in the early follow up period. In meta-analysis of

published studies, a high MammaPrint score was found to predict a 12% distant disease-free survival benefit from the addition of chemotherapy^[12]. Prospective validation is awaited with the results of the ongoing MINDACT (Microarray In Node negative and 1-3 positive lymph node Disease may Avoid ChemoTherapy) trial^[13].

ONCOTYPE-DX

Oncotype-DX (Genomic Health Inc., CA, United States) is currently the most widely available prognostic assay for breast cancer. It is a 21-gene assay in which quantitative real-time polymerase chain reaction (qRT-PCR) is performed on formalin-fixed breast cancer tissue samples taken during initial surgical resection and processed in a central laboratory, which returns a recurrence score (RS) out of a maximum score of 100. It is quoted to have a turnaround time of 7-10 d. It is primarily advised in early ER positive and HER2 negative disease with negative SLN^[14]. In addition, it is also recommended in ER positive and HER2 negative disease in elderly patients with positive SNB^[15].

The RS score was formulated and validated in patients enrolled in the National Surgical Adjuvant Breast and Bowel Project (NSABB) trials. Specifically, the predictive value of the score was initially validated by the NSABB trial B14, in which patients were randomly allocated into placebo and tamoxifen groups^[16]. This was followed up by the NSABB trial B20, in which patients on tamoxifen alone were compared with patients receiving tamoxifen with chemotherapy^[17]. A subsequent retrospective study is cited for validation of Oncotype-DX for predicting prognosis in relatively elderly patients treated with tamoxifen with SNB positive disease^[18].

These initial studies stratified RS scores into low, intermediate and high risk groups, with RS score below 18 being labelled as low risk of recurrence (< 5% risk), and 31 and above as high risk (39.5%)^[16]. Since the beginning, the clinical implications of an intermediate score has been ambiguous. Furthermore, the thresholds have been revised downwards to 11 and 25. Validation for the new thresholds is less clear^[8]. The results of the Trial Assigning Individualized Options for Treatment (TAILORx) are awaited to help clarify the recommendations for the intermediate group^[19].

There have been several studies suggesting that Oncotype-DX is cost effective as a prognostic test^[20]. Furthermore, this assay has been recommended by a number of regulatory bodies^[11,21]. However, the cost and turnover time of the test are not insignificant primarily due to test centralization. Although the Oncotype-DX was validated by randomised controlled trials, it must be emphasised that these studies were supported by funding from industry, and are regarded as internal trials by regulatory bodies^[11]. It should be also highlighted that only 26% and 29% of patients (some of whom had HER2 positive tumours) in the B-14 and B-20 trials respectively were available for analysis thus reducing the effect of randomi-

Table 1 Comparison of gene-based prognostic assays for early oestrogen receptor + breast cancer

Prognostic assay	Manufacturer	Underlying technology	No. of genes	Test induction	Output/score	Comments
MammaPrint	Agendia BV, The Netherlands	DNA microarrays	70	Reference lab	Risk category for recurrence (low risk vs high risk)	Prospective validation is awaited with the results of the ongoing MINDACT trial
Oncotype-DX	Genomic Health Inc., CA, United States	qRT-PCR	21	Reference lab	RS scores (1-100) stratified into low, intermediate and high-risk groups for recurrence	Oncotype-DX has been included in several guidelines, and has been validated by internal industrial studies (NSABB trial B14). The characterisation of intermediate risk group awaits the results of the TAILORx trial
PAM50/ Prosigna	NanoString Technologies, Inc., WA, United States	DNA microarrays and qRT-PCR using nCounter technology	50	Reference lab	ROR scores (1-100) stratified into low, intermediate and high risk groups	The assay was been validated in studies based on the ATAC and ABCSG-8 trials
EndoPredict	Sividon Diagnostics GmbH, Köln, Germany	qRT-PCR	8	Local lab	Low or high risk groups on the basis of EP or EPclin scores	EndoPredict has been validated in ABCSG-6 and ABCSG-8 trials, and has been included in German guidelines. Potentially shorter turnover at lower cost, as there is no need for dispatching samples to a reference laboratory

qRT-PCR: Quantitative real-time polymerase chain reaction; ROR: Risk of recurrence.

sation. This significantly weakens the evidence regarding the predictive role of Oncotype-DX in adjuvant chemotherapy, so much so that the evidence does not reach level 1 as per the Marker Utility Grading System^[22]. Moreover, the Oncotype-DX is not specific to HER2 negative disease and does not incorporate any clinicopathological features which could improve its prognostic ability of longer term clinical outcome. Although the test has not been validated externally for reproducibility and reliability due to industrial centralization, the internal industry reports suggest that the test is reliable.

PAM50

Parker *et al.*^[23] developed a risk of recurrence (ROR) score (also called Prosigna) which is applicable to all tumour types including those that are ER positive. The score is derived by analysing of the expression levels of a set of 50 genes using qRT-PCR and DNA microarrays. The ROR score was developed as a prognostic tool in a cohort of 761 patients^[23]. The DNA microarray cluster partitioning and analysis was done using the partitioning around medoid or microarray (PAM) methodology^[24]. In addition, a related test was developed primarily as an intrinsic sub-type classifier for breast cancer. This test was termed PAM50 (NanoString Technologies, Inc., WA, United States)^[25].

Currently, the ROR score and PAM50 test are performed on formalin fixed samples by a central laboratory utilising proprietary nCounter technology^[26,27]. Like Oncotype-DX, ROR scores (1-100) are stratified into low, intermediate and high risk groups. The ROR score has predictive value in the neoadjuvant setting, as well as in the case of newly diagnosed patients with node negative disease^[23]. The assay was validated in studies based on the ATAC^[28] and ABCSG-8 trials^[29]. In addition, a recent study validated the Prosigna assay for use at local labora-

tories^[26]. Dowsett *et al.*^[28] found Prosigna to be superior to immunohistochemistry and RS in ER positive node negative patients receiving endocrine therapy.

ENDOPREDICT

EndoPredict (EP) is a relatively new assay developed by Sividon Diagnostics GmbH (Köln, Germany), which until recently was largely limited to German-speaking markets. It is an 8-gene qRT-PCR assay performed on formalin fixed breast tissue, design in the first instance to be performed at a local laboratory. Remarkably, whilst these genes are related to proliferation and hormone receptor activity, the assay does not include ER, PR, or HER2 status^[30]. It was validated on 1702 samples taken from two randomised control trials, ABCSG-6 and ABCSG-8^[31].

There is a level Ib evidence showing that EP is an independent prognostic parameter in patients with ER-positive, HER2 negative breast cancer. Patients with a low EP score can be safely treated with endocrine therapy as the only adjuvant systemic treatment, therefore, they can be spared chemotherapy^[32]. The level of evidence regarding its independent prognostic role is similar to that of Oncotype-DX^[33]. Furthermore, a hybrid score incorporating clinical parameters (EpClin) has been shown to be superior to purely clinical assessment tools^[32]. In addition, Muller *et al.*^[34] found that use of EP resulted in change in clinical decision in 37.7% of patients when applied to a cohort of 167 patients. The effects of the change in therapy are to be assessed.

A further consideration is the inherent costs and logistics such a test may incur. In this regard, EP has an advantage over other similar test, being designed to be performed at a local laboratory rather than at a central facility. Proponents of this assay cite the fact that EndoPredict can be performed on-site resulting in a faster result at a lower cost. In addition, it also has the advantage of

dividing tumours into two categories: low and high thus avoiding the immediate group or grey zone of characterisation, which can create anxiety and dilemma to both the oncologist and the patient. EP has achieved CE certification, and has been included in German guidelines^[35]. In addition to reliably identifying patients who can be safely treated with adjuvant endocrine therapy only, EP has other potential applications including further stratification of tumours with intermediate RS (18-31) in order to make final recommendations regarding the need for chemotherapy and selection of patients for 5 years *vs* 10 of adjuvant endocrine therapy. Finally, the hybrid score EpClin is applicable to patients with node positive ER-positive breast cancer.

However, owing to its relative novelty, other regulatory bodies are yet to consider EP in their recommendations.

CONCLUSION

The recent developments in our understanding of intrinsic sub-types within breast cancer, and the explosion in the use of PCR and DNA microarrays have resulted in a growing number of promising prognostic tools for human breast cancer. OncoType-DX, Prosigna and EP currently have the most convincing evidence available, of which Prosigna and EP have a significant degree of external validation. EpClin is the only tool available that combines molecular signature with important clinico-pathological parameters with the potential advantage of superior prognostication regarding the longer term clinical outcome. The RS is the only assay that has been investigated in a randomised trial population as a predictive tool of chemotherapy benefit. However the evidence in this context is considered to be of low quality^[22].

Whilst some products are more mature than others, the results of several ongoing trials, such as MINDACT and TailorX, can be expected to have profound implications for the selection of the optimal test.

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Endometriosis and ovarian cancer

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Abstract

Endometriosis is the leading cause of morbidity among premenopausal women and the complex pathogenesis of this disease remains controversial despite extensive research. This disease represents one of the most common gynecological problems. It is generally believed that this disease is due primarily to retrograde menstruation or transplantation of shed endometrium. Based on overwhelming data, ovarian endometrioma is considered a neoplastic process, since most endometriosis-associated ovarian carcinoma occur in the presence of atypical ovarian endometriosis. A study comparing patients with typical epithelial ovarian cancer with endometriosis-associated ovarian cancer demonstrated that the patients with the latter disease strongly differ in both biological and histological characteristics. The prevalence of this disease is not completely established, but approximately 15 percent of women suffer from this disease. In addition, we know about the possible links between endometriosis and cancer for almost 100 years. Despite clear evidence revealing that endometriosis increases ovarian

cancer risks, it is possible that it may not affect disease progression after the appearance of ovarian cancer. However, despite clear evidence revealing that endometriosis increases ovarian cancer risk, our knowledge of the risk factors is far from established. In our review, we focused on the most recent approaches including possible biomarkers and genetic approaches.

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Key words: Endometriosis; Ovarian cancer; Biomarkers; Cancer; Histology

Core tip: Endometriosis is a multifactorial disease, which, despite intensive research in the last decades, is still not fully explained. In addition, many questions remain to be answered as to the exact events leading from cysts to endometriosis-associated ovarian cancer. Surprisingly, having endometriosis might be less risky than undergoing *in vitro* fertilization, which can increase the risk of ovarian cancer. Our review summarizes current hypothesis on probable mechanisms and attributing factors such as longstanding estrogen stimulation, repeated heavy menstruation and early events on the molecular level. Thus far, however, no single one can be used for diagnosis or treatment.

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INTRODUCTION

Endometriosis is a common gynecologic disorder usually defined as presence and/or growth of endometrial tissue outside the uterine cavity. Although the prevalence of this disease is not clear, approximately 7%-15% of women of reproductive age^[1] have endometriosis, but significantly increases in cases of infertility^[2]. Despite the fact that this

disorder was first described in 1860^[3], the definite cause of endometriosis is still unclear and this disease remains to be one of the most enigmatic female diseases. Probably the most accepted mechanism is the adhesion and subsequent growth of endometrial fragments occurring in the peritoneum by retrograde menstruation. Another possibility is the occurrence of anatomical abnormalities. The repeated events of hemorrhage can also contribute to carcinogenesis and further cancer progression^[4]. Lately, the hereditary aspect gained traction, with *CYP11A1* m1 polymorphism (particularly *CYP10* variable number of tandem repeats allele) and *GSTM1* null deletion playing an important role^[5]. A detailed summary of possible theories is reviewed by Baldi *et al.*^[6] and Yuan *et al.*^[7]. In addition, at least three theories supporting the concept of *in situ* development exist, including a Mullerian and Wolffian rest theory, coelomic metaplasia theory and metaplasia following endometrial stimulation theory (for review see Thomas *et al.*^[8]). However, none of these theories explain all the different types of endometriosis^[9]. Based on overwhelming data, ovarian endometrioma can be viewed as a neoplastic process.

RELATION BETWEEN ENDOMETRIOSIS AND OVARIAN CANCER

The possibility of transformation of endometriotic tissue into malignant ovarian cancer has been recognized for almost 100 years^[10]. In 1925, Sampson^[10] first described malignant changes in endometriosis and proposed the following criteria for diagnosing the carcinomatous development in endometriosis: (1) coexistence of carcinoma and endometriosis within the same ovary, (2) a similar histological pattern and (3) exclusion of a second malignant tumor elsewhere. Later, in 1953, Scott^[11] postulated that in addition to the criteria by Sampson, morphological changes demonstrated by benign endometriosis that are contiguous with malignant tissue is a prerequisite for adjudication of a malignancy originating from endometriosis. Histopathological studies have assumed atypical endometriosis to be a transition between benign endometriosis and cancer^[12] suggesting the possibility that endometriosis is in fact a premalignant condition. This is supported by the fact that between 60% to 80% of endometriosis-associated ovarian carcinoma occur in the presence of atypical ovarian endometriosis^[13].

A summary of more recent cohort studies showed a standardized incidence ratio around 2^[14]. Tumors arising from the transformed endometriomas are predominantly of the clear cell and endometrioid types^[15]. A study of operated ovarian cancer patients showed that full 10% of patients had coexisting ovarian endometriosis. This number increased to 36.8% in patients with clear cell ovarian cancer^[16]. These types of studies led to the definition of “endometriosis-associated ovarian carcinoma” (EAOC), which describes ovarian cancer having both cancer cells and endometriosis in the same ovary, presence of cancer in one ovary and endometriosis in second ovary or the

presence of ovarian cancer and pelvic endometriosis. Histological evaluations showed significant clinical differences between patients with EAOC and non-endometriosis-associated ovarian cancer^[17]. Some studies found that the risk is increased up to 8-fold^[18], but the actual increases fluctuate widely among individual studies^[19]. For a review of ovarian cancer etiology and risk factors see Hunn *et al.*^[20].

However, despite clear evidence revealing that endometriosis increases ovarian cancer risk, the results from meta-analysis suggested that it may not affect disease progression after the appearance of ovarian cancer^[21]. In addition, some studies even suggested that the association with endometriosis might have a survival benefit^[22]. No associations were observed for common benign gynecologic disorders such as uterine myoma, adenomyosis or endometrial polyps^[23]. On the other hand, Brinton *et al.*^[24] showed an increased risk of extra-pelvic carcinomas (including breast cancer) in patients with endometriosis. The newest results are less clear since the outcome of studies examining the association between endometriosis and breast cancer are often contradictory^[14] and the newest population-based cohort studies show no overall association^[25]. A study evaluating possible risk factors revealed only one-ovarian endometriomas of 9 cm or more in diameter was a strong predictor of development of ovarian cancer^[26].

An interesting observation was the measurement of the incidence of ovarian cancer confined to endometrial polyps, which can reach between 0.5% to almost 5% depending on the diagnostic methods^[27]. These polyps can be found with increased frequency in women with endometriosis, but the retrospective analysis did not reach clear conclusions^[28].

Despite the extensive research, the exact mechanisms leading to malignant transformation of endometrial tissue are not established. In endometriosis-associated cancer, the benign-appearing ovarian masses can be detected several years before cancer diagnosis^[29].

Ovarian cancer resulting from endometriosis has some special characteristics such as having endometrioid or clear cell histology, being diagnosed earlier than other types of ovarian cancer and have a better prognosis^[30]. However, the question of endometriosis being a prognostic factor for cancer survival is not clear. On one hand, some studies found no definitive association between the presence of endometriosis and survival^[31]. On the other hand, a large Swedish study found significantly better survival in women with endometriosis than for all malignancies combined. In the cases of breast and ovarian cancer, this survival was even more pronounced^[32]. The protective effect for both one-sided oophorectomy and radical excision of all visible endometriosis was described^[33]. The most recent meta-analysis of risks and prognosis of ovarian cancer confirmed that endometriosis is strongly associated with the increased risk of ovarian cancer and these cancers show favorable characteristics including low grades and specific histology^[21].

Both diseases are progressive and estrogen dependent, often associated with late menopause and infertility. In addition, tubal ligation, hysterectomy and progesterone treatment can decrease the incidence of both diseases^[34].

HORMONE THERAPY

Despite the fact that postmenopausal endometriosis is rare, there is a risk in patients taking hormone therapy^[35]. Hormone therapy, particularly estrogen therapy, is often supposed to stimulate the growth of endometriosis, with additional increase in the risk of ovarian cancer development, which is particularly dangerous in postmenopausal endometriosis, where estrogen therapy should be used only in combination with progesterone^[35]. Estrogen-induced triggering is the same in endometriosis and estrogen-dependent cancer. The quantification of estrogen derivatives was even suggested to be a prognostic factor in ovarian cancer development^[36]. *In vitro* experiments using ovarian cancer cell lines showed similarly increased expression of ER- α receptors as in active endometriosis^[37] which was connected with the pathogenesis of endometriosis. Implants of endometriosis contain estrogen, progesterone and androgen receptors, but the effects of these hormones are opposite-estrogen stimulates proliferation and androgens cause atrophy and regression. The findings show that the basalis has approximately five times as many lymphatic vessels as the functionalis, but it cannot be currently explained^[38].

STEM CELLS

Since several theories about the causes of endometriosis failed to explain all types of lesions and suggest the possibility of combined mechanisms, recent hypothesis include the likelihood that endometriotic lesions arise from ectopic endometrial stem cell progenitors (for review see Maruyama *et al.*^[39]). The origin of oocytes in adult ovaries has been disputed for a long time. Some of the recent studies suggested the possibility that newly implantation of persistent fetal stem cells might play role in tissue regeneration and/or growth. A finding that several *HOX* genes controlling lineage infidelity in ovarian cancer^[40] are expressed in primitive hematopoietic cells suggested a role in early hematopoietic differentiation. Some studies even suggested that *HOX* genes expressed in ovarian epithelial cells might regulate cancer stem cells. At the same time, stem cell transformation can be underlying cause of ovarian cancer^[41,42].

BIOMARKERS

The main risk factor is clearly the age at endometrioma diagnosis. As independent prognostic factors, endometrioma of a diameter above 9 cm and postmenopausal status were established^[26]. There is some evidence that drugs employed in *in vitro* fertilization might increase the risk of ovarian cancer^[43]. However, some studies found

opposite results, so at present, no clear conclusion can be reached.

An effort to determine the potential association between endometriosis and ovarian cancer led to the study evaluating the expression of possible biomarkers which have been previously shown to participate in the pathogenesis of these diseases. The results revealed that endometriosis and epithelial ovarian cancer cells manifested significantly higher levels of mRNAs of transforming growth factor- β 1, cyclooxygenase-2, vascular endothelial growth factor, ER-1 α , aromatase and androgen receptors, whereas the mRNA levels of progesterone receptors were much lower^[44]. The clinical importance of this report is somewhat limited due to the low number of patients; however, its conclusion deserves further investigation.

An additional possible biochemical marker might be cancer antigen CA125^[13], which is commonly used for the monitoring of epithelial ovarian cancer. Since endometriosis is also often associated with a high level of this antigen, it cannot be used for differential diagnosis. A detailed study suggested that a CA72-4 antigen can serve in this role as a biomarker useful to confirm the benign nature of endometriomas in patients with high CA125 levels^[45].

An immunohistochemistry study of ovarian cancers arising from endometriosis confirmed that the estrogen-dependent cancers are substantially more associated with endometrioid adenocarcinoma than clear cell carcinomas. The positive hepatocyte nuclear factor-1 beta was common in clear cell carcinomas, but rare in endometrioid adenocarcinoma, which correlated with *p53* staining, but reversely correlated with estrogen receptor presence^[46].

Suryawanshi *et al.*^[47] focused on plasma microRNAs as a novel biomarker. Using a reverse transcriptase quantitative polymerase chain reaction, the authors identified 23 microRNAs which are differentially expressed in healthy people and patients with either endometriosis or EAO. These microRNAs were subsequently further evaluated in a larger cohort. The results showed that plasma microRNA expressing patterns might serve as specific and reliable diagnostic biomarkers^[47] resulting in some authors suggesting that microRNA studies will lead to changes in current treatment of both endometriosis and ovarian cancer^[48].

GENETIC APPROACHES

A wide variety of molecular alterations have been reported to be involved in the malignant transformation of endometriosis, some of which are common in both endometriosis and ovarian cancer, whereas the others are universal among various tumors. Only a very few were suggested to specifically refer to the malignancies of endometriosis.

Many hypothesize that changes in the expression of tumor suppressor genes and oncogenes occurring in the eutopic endometrium might lead to overgrowth of endometrial foci outside the uterus^[49,50].

Microsatellite analysis showed that loss of heterozygosity on p16 (Ink4), gut-associated lymphatic tissue (GALT), and *p53* occurs in endometriosis. Similarly, activation of mutated *K-ras* gene is an important step in both genesis and progression of ovarian cancer. Alteration of *p53* gene caused aberrant regulation of the H-ras protooncogene^[51]. Using a murine model of mutationally activated *K-ras* gene, it was demonstrated that these animals develop both endometrial lesions and the ovarian tissue. The following mutation blocking the expression of *PTEN* caused ovarian cancer^[52]. *K-ras* mutation may promote carcinogenesis of endometriosis leading to ovarian clear cell carcinoma^[53].

Mutations in *ARID1A* and *PIK3CA* were first described in numerous cases of ovarian clear cell carcinoma, but later also in precursor endometriosis tissues. In addition, *PTEN-PIK3CA-mTOR* pathway was strongly implicated by finding *PIK3CA* mutation in up to 46% clear cell ovarian cancer^[54]. *PIK3CA* mutation is considered to be an early event in the development of endometriosis-associated ovarian clear cell adenocarcinoma. For a better understanding of the effects of these two mutations, functional studies evaluating effects of individual mutations separately and in both normal endometrium and endometriotic epithelium are necessary. The sequence of events leading from normal eutopic endometrium to endometriosis and subsequent ovarian cancer is still hypothetical^[55].

Some studies suggested that a histologically normal endometrium may bear genetic damage caused by iron-dependent oxidative stress^[56]. Some authors suggested that suppression of pre-apoptotic gene Bax and/or up-regulation of anti-apoptotic gene Bcl-2 can be involved in endometriosis and malignancies^[57].

Genetic instability might lead to both endometriosis and ovarian cancer. It can include deactivation of some tumor-suppressing genes, changes in activity of enzymes involved in DNA repairs or mutations in genes such as GALT and GSTM. Similarly, mutations in tumor suppressive gene *PTEN* was often found both in endometrial and cancer tissues^[58]. In addition, *c-erbB-2* and *p53* genes have been found to associate with endometriosis-related ovarian cancer^[59]. The recent study showed that endometriotic lesions have mutations in cancer-related genes such as *PTEN*, *KRAS*, *p53*, and *ARID1A*^[48,60].

In addition, both endometriosis and EAOCs share some of the mediators implicated in inflammatory angiogenesis. They exhibit genetic polymorphisms of several genes including intercellular cell adhesion molecule-1, interleukin (IL)-6 and IL-10 promoters, tumor necrosis factor- α , and nuclear transcription factor- κ B^[61]. Further genetic factors such as loss of heterozygosity, *K-ras*, *P53*, and *PTEN* mutations or hepatocyte nuclear factor-1 β were suggested and tested (for review see Kobayashi *et al.*^[4]). Genome-wide association studies and as well as transcriptome sequencing have shown that genes from the 1p36 region might be important in both endometriosis and endometriosis-associated cancer development^[62].

In a recent study, the endometriosis-associated ovarian carcinogenesis has been linked to oxidative stress-induced increased genomic instability, aberrant methylation, and aberrant chromatin remodeling, as well as mutations of tumor suppressor genes^[63]. For a summary of the molecular biology aspects of ovarian cancer in endometriosis, see Mandai *et al.*^[64].

CONCLUSION

Endometriosis is a multifactorial disease and despite intensive research in the last several decades, many questions remain to be answered as to the exact events leading from endometriotic cysts to endometriosis-associated ovarian cancer. The probable mechanisms and attributing factors include longstanding estrogen stimulation, repeated heavy menstruation resulting in tissue damage and early events on the molecular level were repeatedly shown. Numerous markers have been proven to correspond with this type of cancer. Thus far, however, no single one can be used for diagnosis, not to mention treatment. Despite the fact that the links between endometriosis and ovarian cancer appear to be clear, we have to keep in mind that having endometriosis might be less risky than undergoing *in vitro* fertilization, which can increase the risk of ovarian cancer three times^[65]. The exact mechanisms of the endometriosis-ovarian cancer conversion are still not fully established and the need for new approaches in the understanding and treatment of endometriosis is urgent.

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WJCO 5th Anniversary Special Issues (1): Lung cancer**Nexus of signaling and endocytosis in oncogenesis driven by non-small cell lung cancer-associated epidermal growth factor receptor mutants**

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Abstract

Epidermal growth factor receptor (EGFR) controls a wide range of cellular processes, and aberrant EGFR signaling as a result of receptor overexpression and/or mutation occurs in many types of cancer. Tumor cells in non-small cell lung cancer (NSCLC) patients that harbor EGFR kinase domain mutations exhibit oncogene addiction to mutant EGFR, which confers high sensitivity to tyrosine kinase inhibitors (TKIs). As patients invariably develop resistance to TKIs, it is important to delineate the cell biological basis of mutant EGFR-induced cellular transformation since components of these pathways can serve as alternate therapeutic targets to preempt or overcome resistance. NSCLC-associated EGFR mutants are constitutively-active and induce ligand-independent transformation in nonmalignant cell lines. Emerging data suggest that a number of factors are critical for the mutant EGFR-dependent tumorigenicity, and bypassing the effects of TKIs on these pathways promotes drug resistance. For example, activation of downstream pathways such as Akt, Erk, STAT3 and Src is critical for mutant EGFR-mediated biological processes. It is now well-established that the potency and spatiotemporal features of cellular signaling by receptor tyrosine kinases such as EGFR, as well as the specific pathways activated, is determined by the nature of endocytic traffic pathways through which the active receptors traverse. Recent evidence indicates that NSCLC-associated mutant EGFRs exhibit altered endocytic trafficking and they exhibit reduced Cbl ubiquitin ligase-mediated lysosomal downregulation. More recent work has shown that mutant EGFRs undergo ligand-independent traffic into the endocytic recycling compartment, a behavior that plays a key role in Src pathway activation and oncogenesis. These studies are beginning to delineate the close nexus between signaling and endocytic traffic of EGFR mutants as a key driver of oncogenic

processes. Therefore, in this review, we will discuss the links between mutant EGFR signaling and endocytic properties, and introduce potential mechanisms by which altered endocytic properties of mutant EGFRs may alter signaling and vice versa as well as their implications for NSCLC therapy.

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Key words: Non-small cell lung cancer; Epidermal growth factor receptor; Signaling; Endocytosis; Src; Cbl; Ubiquitination

Core tip: Since their discovery ten years ago, much work has revealed the signaling properties of non-small cell lung cancer-associated mutant epidermal growth factor receptors (EGFRs). While therapeutic options for patients harboring mutants have emerged, these are beset with rapid development of resistance, making it critical that mutant EGFR biology be better understood to design more effective therapies. Emerging data suggests that mutant EGFRs exhibit altered endocytic trafficking, a process critical for the regulation of EGFR signaling. Deregulated endocytic traffic appears to enable mutant EGFRs to activate oncogenic signaling pathways. This review highlights the signaling and endocytic trafficking of mutant EGFR and the intimate link between the two processes.

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INTRODUCTION

Epidermal growth factor receptor (EGFR) is a member of the ErbB (avian erythroblastic leukemia virus oncogene homolog) or human EGF receptor (HER) family of receptor tyrosine kinases (RTKs). The ErbB family comprises of EGFR (also known as ErbB-1/HER1), ErbB-2 (neu, HER2), ErbB-3 (HER3) and ErbB-4 (HER4). Studies of EGFR as a model RTK and prototypic oncogene have provided much of our understanding of cellular and molecular mechanisms of RTK function and regulation^[1-4]. EGFR is a transmembrane glycoprotein with extracellular domains that bind to ligands such as EGF and transforming growth factor α (TGF α) to promote receptor dimerization and activation of a cytoplasmic tyrosine kinase domain. The resulting phosphorylation of the receptor and downstream signaling proteins mediate the various biological responses downstream^[1,5]. In particular, EGFR is known to play crucial roles in cellular proliferation, survival, migration, and differentiation. Indeed, impaired epithelial development in several organs

as well as perinatal lethality among *EGFR* knockout animal models illustrates the essential nature of EGFR in cellular functions^[6,7]. Furthermore, oncogenic viruses exploit the EGFR signaling network in many different ways, altering both receptor tyrosine kinase activity and gene expression^[8].

The role of aberrant EGFR signaling in oncogenesis has been investigated for many years. A major mechanism for aberrant signaling involves the overexpression of EGFR, found in various epithelial tumors^[3]. The cancers where overexpression of EGFR is found include breast cancer, glioblastomas, head-and-neck cancer, non-small cell lung cancer (NSCLC), renal cancer, ovarian cancer, and colon cancer^[1,9]. Transgenic studies^[10] and *in vitro* studies, using NIH 3T3 mouse fibroblasts^[11], demonstrate that high-level expression of EGFR and EGF ligands can transform cells. Recent studies using genetic deletion of *EGFR* illustrate the essential role of this receptor in oncogenesis in a pancreatic cancer model^[12]. In addition, EGFR activation initiates cytoprotective signaling, enabling tumor cells to become resistant to radiation and chemotherapy^[13,14]. Increased expression of EGFR is associated with poorer survival, and EGFR serves as a strong prognostic indicator in many cancer types^[15].

In addition to overexpression, recent studies have demonstrated a key oncogenic role of mutant forms of EGFR in driving oncogenesis. EGFR overexpression in glioblastomas is associated with an alternatively-spliced variant, EGFRvIII, lacking the extracellular sequences encoded by exon 2-7 as a result of an 804 base pair in-frame deletion that corresponds to the removal of N-terminal amino acid residues from 6-273^[16]. EGFRvIII is expressed in approximately 25% of glioblastomas^[17] and in a higher percentage of patients with *EGFR* amplification^[18,19]. This mutant initiates ligand-independent signaling and is transforming in animal models of glioblastoma^[20]. Missense point mutations or small in-frame deletions in the kinase domain have been identified in NSCLC and shown to be constitutively active and oncogenic^[21-23]. Notably, NSCLC-associated somatic *EGFR* mutations impart a higher sensitivity to EGFR-directed TKIs such as gefitinib (Iressa) or erlotinib (Tarceva)^[3,22]. Because the NSCLC-associated EGFR mutants are constitutively-active and capable of transforming cells, they present fascinating models to study signaling pathways and defects in negative regulatory mechanisms. Therefore, this review will discuss our current understanding of NSCLC-associated mutant EGF receptors and their signaling properties, and the critical links between the endocytic and signaling pathways of mutant EGF receptors.

EGFR MUTATIONS IN NSCLC

Lung cancer is the leading cause of cancer deaths in both men and women in the United States, and NSCLC accounts for about 85% of lung cancers^[24]. Studies in gastrointestinal stromal tumors showed that activating

mutations of *c-KIT* gene were associated with clinical responses to small molecule TKI imatinib, and fueled interest to search for similar mutations in *EGFR* in NSCLC patients that responded favorably to gefitinib and erlotinib^[25]. Indeed, in 2004, *EGFR* mutations associated with gefitinib sensitivity were identified in NSCLC^[21,23].

Somatic mutations in the *EGFR* kinase domain are found in about 10% of NSCLC patients from the United States and about 25% of those from East Asia^[26,27]. In-frame deletions in exon 19 (*EGFR* Δ746-750) and an arginine to leucine mutation at position 858 (*EGFR* L858R) account for about 90% of these mutations^[26]. The mutations confer constitutive activity by disrupting the inactive conformation of the kinase domain of *EGFR*, and a 20-fold increased TKI binding accounts for their hypersensitivity to TKIs^[28]. *EGFR* mutations in NSCLC have been correlated with gene amplification^[29]. Somatic mutations of the *ErbB2* kinase domain in NSCLC (in-frame insertions in exon 20) have also been identified in a subset (1.6%) of patients with a similar profile as those that harbor *EGFR* mutations: never smoker, East Asian ethnicity, and female gender^[30]. More recent studies of breast cancer patients identified nine additional somatic mutations among *EGFR* family members that represent potential TKI therapeutic targets^[31].

Multiple factors modify the sensitivity of NSCLC patients with *EGFR* mutations to *EGFR* TKIs. For instance, in-frame deletion in exon 19 is more sensitive to erlotinib inhibition than the L858R mutant^[32]. Similarly, patients with an in-frame deletion mutant showed better response and longer overall survival with gefitinib or erlotinib treatment than did patients with the L858R mutant^[33]. High *EGFR* gene copy number identified by fluorescence *in situ* hybridization (FISH) was proposed to be an effective molecular predictor of gefitinib efficacy in advanced NSCLC^[34]. However, a meta-analysis has found that *EGFR* overexpression is not associated with overall survival in NSCLC patients^[35]. Increased *ErbB2* expression is also associated with increased sensitivity to gefitinib both in the presence^[36] and absence of *EGFR* mutations^[37], although phosphorylated-*ErbB2* along with total *ErbB3* levels have been associated with resistance to gefitinib in head and neck squamous cell carcinoma^[38].

Despite their success in a subset of patients, the overall effectiveness of *EGFR* inhibitor treatment for cancer therapy remains elusive. While erlotinib can be effective for the initial treatment of those with sensitizing *EGFR* mutations, overall median survival of patients treated with erlotinib *vs* placebo is only 6.7 mo *vs* 4.7 mo^[24]. Together with the fact that patients with erlotinib reported severe side effects including rash and diarrhea^[39], it is vital that alternative and improved regimens be developed to better treat NSCLC.

Further complicating TKI treatment efficacy, patients with drug-sensitive *EGFR* mutations develop acquired resistance after about 12 mo, and up to 50% of resistant cases can be attributed to a secondary mutation at position 790 (*EGFR* T790M)^[26,40,41]. It is thought that the

T790M mutation leads to steric hindrance for erlotinib binding due to the bulky methionine side chain in the ATP-binding pocket^[42]. However, another study revealed that the T790M mutation causes drug resistance simply by increasing the affinity for ATP^[43].

Amplification of the hepatocyte growth factor receptor tyrosine kinase (MET) has been implicated in drug resistance, presumably by driving *ErbB3*-dependent activation of phosphoinositide-3 kinase (PI3-K)^[44]. MET amplification was reported in about 20% of TKI-resistant patients, sometimes concomitantly with *EGFR* T790M^[44]. In addition, insulin-like growth factor I receptor reportedly interferes with anti-*EGFR* directed therapies^[45], and a block in apoptosis is implicated as one of the mechanisms of TKI resistance^[46].

A multigene signature indicative of an epithelial to mesenchymal transition (EMT) was also identified as a determinant of insensitivity to erlotinib^[47]. Cells expressing the epithelial cell junction protein E-cadherin show greater sensitivity to *EGFR* inhibition, whereas cells that have undergone EMT, expressing vimentin or fibronectin, are insensitive^[48]. Src-mediated cell signaling has been proposed to be another mechanism for anti-*EGFR* directed therapy resistance, by bypassing the dependency on *EGFR* for cell growth and survival^[49]. *KRAS* mutational status also predicts resistance to anti-*EGFR* directed therapies apparently as cancer cells may no longer need *EGFR* for survival^[50-52].

To circumvent the problem of TKI resistance and to enhance the efficacy of *EGFR*-directed therapies, alternative strategies are being utilized to enhance the survival rate of cancer patients, albeit with mixed results. Because erlotinib has a lower IC₅₀ than gefitinib against wild-type *EGFR*, it has been suggested that gefitinib-resistant patients be treated with erlotinib, but such studies have not had much success as erlotinib could not overcome the resistance conferred by the T790M mutation^[53]. Phase II clinical trial data also showed that patients with activating *EGFR* mutations do not respond to monoclonal antibody-based therapy with cetuximab even though they respond to a TKI^[54]. However, a different study showed cetuximab to be effective against cells expressing either TKI-sensitive or resistant NSCLC *EGFR* mutations^[55].

Promising results were observed in an animal model using a combination of cetuximab and TKI, which yielded enhanced tumor regression^[56]. Cells expressing mutant *EGFR* also show sensitivity towards an inhibitor of vascular endothelial growth factor receptor-2 (VEGFR-2), ZD6474, indicating that VEGFR-2 may be critically involved in mutant *EGFR*-mediated cell survival^[57]. An irreversible pan-*ErbB* inhibitor PF00299804 is also a potent inhibitor of gefitinib-resistant *EGFR* and *ErbB2* mutations^[58].

It is evident from the current directions of the anti-*EGFR* directed therapy that it is critical to identify key partners and pathways of *EGFR*-mediated tumorigenicity and co-target these factors simultaneously. This approach will not only benefit NSCLC, but other cancer

types in which EGFR may play a critical role.

MUTANT EGFR SIGNALING

Early studies using NSCLC tumor cell lines indicated that mutant EGFRs are constitutively-active^[22,28,59], transform nonmalignant cell lines in a ligand-independent manner^[60], and enhance tumor growth in xenograft models^[60]. Furthermore, cells harboring mutant EGFRs undergo “oncogene addiction” and require the mutant receptor activity for survival^[61]. In transgenic mouse models, reduction in the expression of mutant EGFR or inhibition of its kinase activity caused rapid tumor regression, demonstrating that mutant EGFR is required for tumor maintenance^[62].

It is becoming increasingly evident that in addition to oncogene addiction, cells also depend on non-driver oncogenic pathways for survival^[63]. It has been found that downstream signaling pathways that are key to mutant EGFR function include Akt, Erk1/2, and STAT3^[23,34,64-67]. Sensitivity to growth inhibition by gefitinib is associated with signaling molecules downstream of activated EGFR such as Akt and Erk^[68,69], and gefitinib effectively blocked Akt and Erk phosphorylation in gefitinib-sensitive NSCLC cell lines^[66].

Transfection studies using the EGFR exon 19 in-frame deletion mutant revealed highly phosphorylated Akt and STAT3 compared to transfection of wild-type EGFR (wtEGFR)^[70]. In fact, continued activation of PI3-Kinase signaling by a *PIK3CA* (PI3-K, catalytic, alpha polypeptide) oncogenic mutant is sufficient to abrogate gefitinib-induced apoptosis in NSCLC-associated EGFR mutant-expressing cells^[71]. PI3-K was found to exclusively associate with ErbB3 in gefitinib-sensitive NSCLC cell lines, and, interestingly, the gefitinib-sensitive wtEGFR-expressing NSCLC cell lines showed greater ErbB3 expression than gefitinib-insensitive cell lines^[72]. In addition, the expression of NSCLC-associated EGFR mutants correlates with constitutive activation of mammalian target of rapamycin (mTOR) and Erk5 as well as enhanced expression of cyclin D1 and EGR1^[73-76].

Subsequent studies have also identified Src to be critical for cell proliferation, survival and migration of mutant EGFR-expressing cells. In contrast to mutant EGFRs, overexpression of wtEGFR in primary cells is not oncogenic. High levels of exogenous ligands^[77] and/or cooperating oncogenic partners are required for the wtEGFR to transform cells. In this regard, Src has been established to cooperate with EGFR and to be an important determinant of EGFR-mediated oncogenesis^[78,79]. EGFR and Src overexpression in fibroblast systems led to synergistic increases in EGF-induced DNA synthesis, soft agar colony formation, and tumor formation in nude mice^[80]. This cooperativity has also been demonstrated in a model of epithelial cell transformation: loss of polarity in three-dimensional cultures of nonmalignant human mammary epithelial cells as well as their anchorage-independent growth were only seen when both EGFR

and Src were co-overexpressed^[81]. Consistent with these studies, EGFR and Src are often co-overexpressed in human cancers^[82], and enhanced Src activity was observed in NSCLC tissue compared to normal lung tissues^[83].

Importantly, mutant EGFR-expressing NSCLC cell lines exhibit increased Src phosphorylation^[64], and more Src is associated with mutant EGFR compared to wtEGFR^[84,85]. Mutant EGFR-expressing cells were sensitive to Src inhibitors such as Dasatinib, PP1, or SKI-606^[64,65], and Src-dependent phosphorylation on EGFR Y845 was required for full phosphorylation of mutant EGFR and downstream signaling molecules such as Akt, Erk and STAT3^[85,86]. Interfering with Src-dependent phosphorylation on EGFR Y845 resulted in decreased mutant EGFR-mediated biological processes such as cell transformation and migration^[85]. Further studies are needed to identify additional factors and processes critical to mutant EGFR-mediated oncogenic signaling and biological outcomes.

ENDOCYTIC TRAFFIC OF EGFR

In normal cells, EGFR signaling is tightly regulated by endocytic traffic. The ligand-induced EGFR dimerization and activation are associated with rapid internalization from the cell surface into endosomes followed by further traffic into lysosomes, or alternatively into an endocytic recycling compartment from which the receptor returns back to the cell surface (Figure 1). The pathways of endocytic traffic to the lysosome and their functional impact have been substantially elucidated in recent years. Activated EGFR undergoes ubiquitination, which provides a signal for sorting of internalized EGFR into lysosomes for degradation^[2,87], a mechanism for signal termination^[88,89]. In this context, endocytosed EGFR migrates down a system of heterogeneous compartments that have generally been characterized as “early” or “late” endosomes based on their morphology, kinetics of labeling with endocytic cargo as well as compartment-specific markers^[90]. While punctate early endosomes are primarily located towards the cell periphery, the late endosomes are larger and more spherical, and often positioned closer to the nucleus^[90]. Furthermore, some late endosomes have a multivesicular appearance by ultrastructure and are referred to as multivesicular bodies (MVBs). The ubiquitinated cargo, such as EGFR, at the outer limiting membrane of MVBs is recognized by a series of protein complexes called the endosomal complex required for transport (ESCRT) 1 to 3, and selectively sorted into invaginating vesicles that bud off to form the internal vesicles of MVB^[91]. The MVB subsequently matures and/or fuses into the lysosome where the receptor is degraded by lysosomal hydrolases^[91]. Under conditions where EGFR does not undergo ubiquitination, or if ubiquitin chains are cleaved by deubiquitinases^[92], the receptor is alternatively sorted into vesicles that traffic along the endocytic recycling pathway back to the cell surface for additional rounds of ligand binding and signaling^[93]. Endocytic recycling can

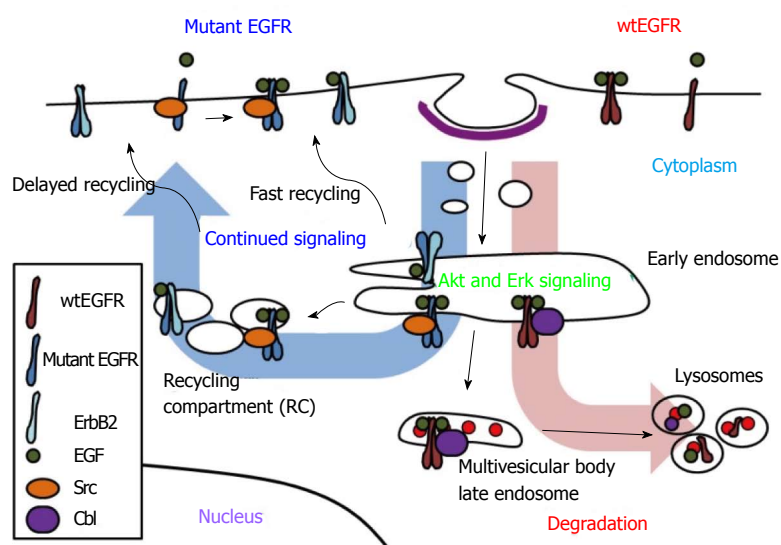


Figure 1 Model of mutant epidermal growth factor receptor endocytic trafficking. Upon ligand binding, activated wtEGFR becomes internalized and localized to endosomes. Internalized EGFR has been linked to Erk and Akt activation. Depending on type of ligand bound, ligand concentration, dimerization partner, mutational statuses and/or availability of other regulators, wtEGFR may recycle to cell surface or be sorted to lysosomes. EGFR bound to EGF is mostly targeted for lysosomes, where it becomes degraded. Sorting of ligand-induced wtEGFR to lysosome is mediated by E3 ubiquitin ligase Cbl which remains attached to receptor throughout endocytosis. Mutant EGFR, however, escapes ligand-induced downregulation through decreased interaction with Cbl, enhanced dimerization with ErbB2, which prefers recycling pathway, and/or constitutive interaction with Src, which antagonizes Cbl. EGFR: Epidermal growth factor receptor.

take place *via* the fast/direct recycling from the early endosomes back to the plasma membrane, or *via* a delayed recycling pathway that involves the perinuclear endocytic recycling compartments (Figure 1)^[93,94]. Members of Rab-family of small GTPases play key roles in facilitating alternate sorting itineraries. The delayed recycling pathway utilizes Rab11 as well as Arf6 small GTPase^[94]. Notably, members of a family of dynamin-like ATP-binding and EPS15-homology domain-containing (EHD) proteins have been identified as key regulators of the delayed endocytic recycling compartments^[95,96].

The endocytic trafficking fates of EGFR are regulated by a complex, and still incompletely understood, molecular machinery. First, the nature of the ligand bound to EGFR can dictate alternate fates of the ligand-receptor complex. In particular, EGF, which forms a more stable complex with EGFR in the low pH environment of endosomes, primarily targets the receptor for ubiquitination and lysosomal degradation; in contrast, other ligands such as TGF α and amphiregulin form less stable complexes with EGFR, induce lower levels of ubiquitination, and promote recycling of the receptor accompanied by more sustained signaling responses^[88,97]. The concentration or the availability of ligands is another factor that determines the fate of EGFR degradation as low concentrations of EGF induce clathrin-mediated endocytosis of EGFR for recycling while higher concentrations induce internalization *via* lipid rafts for degradation^[98,99]. The dimerization partner may also affect the regulation of EGFR; for example, overexpression of ErbB2 has been shown to reduce EGFR downregulation by increasing its recycling or decreasing internalization^[100,101].

The Cbl (Casitas B-lineage Lymphoma proto-oncogene) family of ubiquitin ligases plays an essential role in promoting ubiquitination and lysosomal degradation of EGFR^[102]. Cbl proteins selectively associate with activated EGFR, *via* phosphorylated tyrosine 1045 (number corresponding to human EGFR) and facilitate the juxtaposition of Cbl-bound ubiquitin conjugating

enzymes to facilitate EGFR ubiquitination^[102,103]. Once bound, Cbl remains associated with the activated EGFR throughout its endosomal transport to lysosomes^[103]. Ubiquitination mediated by the association of Cbl proteins is essential for EGFR sorting into lysosomes (Figure 1). It has also been suggested that Cbl can function as an adaptor protein to recruit Cbl-interacting protein of 85 kDa (CIN85) together with its partner Endophilin A to promote initial internalization of EGFR^[104]. However, a more recent study suggested that CIN85 is dispensable for EGFR internalization^[105]. Grb2, which interacts with the proline-rich region of Cbl and phosphotyrosine-containing motifs on EGFR, may also mediate EGFR-Cbl complex formation and initiate EGFR internalization by promoting delivery into clathrin-coated pits^[104,106,107]. Disruption of Grb2 interactions with EGFR or Grb2 knockdown using a small interfering RNA significantly inhibited the receptor internalization^[108,109]. A recent study also demonstrated that the cooperative recruitment of Cbl, in complex with Grb2, to EGFR determines the threshold of ubiquitination of EGFR^[110]. Other factors are also known to affect EGFR endocytosis and down-regulation. For example, Sprouty2 or activated Cdc42^[111], which appear to block Cbl function, as well as suppressor of T-cell receptor signaling (Sts-1/Sts-2), and Cortactin, inhibit efficient EGFR trafficking to the lysosome and block receptor downregulation^[107,112,113]. In addition, SNX1, stimulatory G protein subunit (G α s), and factors important in mediating or regulating ESCRT complex function, such as Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate), signal-transducing adaptor molecule (STAM), tumor susceptibility gene product 101 (TSG101), and other components of ESCRT complexes, are required for the MVB sorting of EGFR and efficient receptor degradation^[7,114]. Interfering with Cbl-dependent negative regulatory process prolongs the EGFR activity and enhances the EGFR-mediated cell transformation^[87]. Thus, it is clear that endocytic traffic of EGFR plays a critical role in controlling its signaling and regulating its

oncogenicity.

ENDOCYTIC TRAFFIC OF MUTANT EGFRS

Mutant EGFRs function as oncogenic drivers in NSCLC and other cancers including glioblastomas. To understand the biological basis of how mutant receptors drive oncogenesis, it is important to gain insights into how the regulatory mechanisms that control EGFR operate in the context of mutant receptors. A key component of EGFR regulation involves the ligand-induced receptor endocytosis which leads to degradation of the receptor and termination of signaling, or to receptor recycling for continued signaling. Because of the radically different outcomes of the alternate endocytic fates, elucidating mechanisms of mutant EGFR endocytic trafficking is fundamentally important to understanding mutant EGFR-driven signaling and oncogenesis, with a potential to improve the EGFR-directed therapies.

Since mutant EGFR exhibits constitutive signaling, it is likely that this is associated with altered endocytic trafficking. Indeed, multiple lines of evidence suggest that mutant EGFRs undergo altered endocytic trafficking compared to the wild-type receptor^[115-118]. In this section, we will describe mutant EGFR endocytic trafficking in terms of basal receptor localization, as well as ligand-induced internalization and degradation.

MUTANT EGFR LOCALIZATION AND LIGAND-INDUCED INTERNALIZATION

Mature wtEGFR is primarily localized at the cell surface prior to ligand binding, but becomes internalized upon ligand binding. There are conflicting reports in regards to ligand-induced mutant EGFR internalization compared to that of wtEGFR. It has been reported that EGF-induced internalization of gefitinib-sensitive mutant EGFR expressed on PC9 cell line was faster than that of wtEGFR on gefitinib-insensitive cell lines A549 and QG56^[68,119]. Another study, however, reported that mutant EGFR-expressing NSCLC cell lines H1975 and H1650 showed delayed internalization of labeled EGF in comparison to a wtEGFR-expressing cell line H358^[116]. Yet another study found that rhodamine-conjugated EGF uptake was comparable between H1299 cell lines permanently transfected with mutant EGFRs or wtEGFR, suggesting that NSCLC EGFR mutation did not affect ligand-induced receptor internalization^[120]. Differences in EGF-induced mutant EGFR internalization may be attributed to cell lines used to compare wtEGFR and mutant EGFRs, and underscore the need for more comprehensive and concurrent studies using multiple assays to fully understand if and how the NSCLC-associated mutations of EGFR affect its ligand-induced EGFR internalization.

Compared to the uncertainty of the impact of NSCLC-

associated EGFR mutations on ligand-induced internalization, emerging evidence suggests that mutant EGFRs are constitutively internalized. Mutant EGFR ectopically overexpressed in a murine pro-B cell line model was shown to undergo EGF-independent internalization, whereas wtEGFR was primarily localized to the cell surface in the absence of ligand^[121]. Another study showed that mutant EGFR in PC9 cell line, but not the wtEGFR, in QG56 cell line was distributed inside the cell^[119]. These data suggest that mutant EGFRs may undergo enhanced constitutive internalization compared to wtEGFR. Indeed, unlike wtEGFR, mutants EGFRs showed constitutively intracellular localization and colocalized with endosomal markers^[118]. Inhibition of endocytic recycling pathway using monensin resulted in the accumulation of mutant EGFRs in perinuclear vesicles, and mutant EGFRs showed colocalization with various recycling endosomal markers, suggesting that mutant EGFRs undergo altered endocytic trafficking through recycling pathway^[118]. Importantly, recycling inhibition delayed the ligand-mediated mutant EGFR degradation and enhanced the mutant EGFR association and colocalization with Src (Figure 1)^[118]. These findings support the notion that enhanced endocytic trafficking of mutant EGFRs *via* the recycling pathway provides a potential compartment where mutant EGFR may engage in preferential interaction with Src and sustained oncogenic signaling.

As for the cellular localization and internalization of EGFRvIII, there remains much confusion. Earlier work has suggested that EGFRvIII expressed in a glioma cell line remains on the plasma membrane even after EGF stimulation, whereas wtEGFR was removed from the cell surface and appeared in perinuclear vesicles corresponding to endosomes and lysosomes^[122]. Similarly, EGFRvIII, when transfected into a small cell lung cancer cell line, localizes predominantly at the cell surface^[123]. However, confocal microscopic analyses on biopsy samples of human gliomas showed that the subcellular localization of EGFRvIII was identical to that described for wtEGFR; predominant cell membrane expression, with some perinuclear distribution^[124]. Future studies will need to delineate the exact subcellular localization and endocytosis of EGFRvIII.

The mechanisms underlying the tendency of NSCLC-associated EGFR mutants to remain constitutively internalized are currently unclear. As mutant EGFRs are constitutively-active, a role for kinase activity in promoting ligand-independent internalization appears plausible. Published studies on the role of kinase activity for internalization of wtEGFR have arrived at opposite conclusions, suggesting either a requirement for or dispensability of the kinase activity for internalization^[125-127]. In fact, ligand-induced internalization of EGFR in the presence of TKIs was previously employed by investigators to initiate signaling directly from the endosomes^[128]. Rather than the kinase activity *per se*, it may be the conformational changes associated with activation that expose endocytic motifs in EGFR and permit its internalization^[43,60]. Fur-

thermore, activated EGFR recruits adapter proteins such as Epsin and Grb2 that have been shown to promote internalization^[129,130]. Given the constitutive activity of mutant EGFRs, these mechanisms may mediate enhanced internalization of mutant compared to wtEGFR. Notably, gefitinib inhibits the ligand-induced internalization of mutant EGFR in gefitinib-sensitive PC9 cell line but does not affect internalization of wtEGFR in gefitinib-insensitive QG56 cell line^[119]. Therefore, kinase activity might play a more critical role in the internalization of mutant EGFR compared to wtEGFR. Other studies have shown that EGFR dimerization is critical for wtEGFR internalization^[125]. Given that mutant EGFR has been found to be constitutively dimerized^[131], dimerization may indeed be critically involved in the constitutive internalization of mutant EGFRs.

Some studies also suggest sensitivity to TKI to play a role in ligand-induced EGFR internalization. For example, it has been reported that a H1650 NSCLC cell line rendered gefitinib-resistant showed increased ligand-induced mutant EGFR internalization when compared to the parental gefitinib-sensitive cell line^[132]. In contrast, the reverse was true for wtEGFR, as others showed that ligand-induced internalization of wtEGFR in erlotinib-sensitive H292 cells was greater than that in erlotinib-insensitive H1703 cells^[133]. Quantification also showed that inhibition of EGF-induced EGFR internalization by erlotinib was greater in sensitive cell line compared to that in the insensitive cells^[133]. Further studies are needed to more clearly delineate key determinants of ligand-induced and constitutive mutant EGFR internalization as well as the relationship of these processes with TKI sensitivity *vs* resistance.

ALTERED LIGAND-INDUCED DEGRADATION OF MUTANT EGFRS

As mentioned in the introduction, lysosomal degradation of EGFR is critically dependent on ubiquitination promoted by Cbl-family ubiquitin ligases. Upon ligand activation and phosphorylation of EGFR, Cbl associates with the phosphorylated (active) receptor and facilitates its ubiquitination^[102,134-137]. The Cbl-EGFR association has been shown to persist throughout the endosomal pathway and Cbl-family proteins are essential for the lysosomal sorting step of activated EGFR downregulation^[103,134,138]; accordingly, ubiquitin ligase activity-defective Cbl mutants enhance the EGFR recycling^[135]. Ubiquitin ligase activity-deficient Cbl itself can become oncogenic due to loss of negative regulatory control on receptor signals^[135,139-141]. Depletion of Cbl proteins or expression of mutant forms has clearly shown that lack of Cbl function deregulates EGFR traffic, elevates downstream signaling and promotes epithelial cell migration^[134,137,142]. As NSCLC mutant EGFRs appear defective in Cbl-dependent downregulation, it is quite likely that the ensuing recycling and endosomal signaling contribute to the

oncogenicity of mutant EGFRs^[115-117] (Figure 2).

Several studies have examined the association of NSCLC EGFR mutants with Cbl, but have provided conflicting results. Reduced ligand-induced association of mutant EGFR with Cbl, as compared to that of wtEGFR, was reported in NSCLC cell lines H1975 and PC-9 expressing EGFR L858R/T790M or Δ 746-750 mutants respectively, as well as in human embryonic kidney and normal lung bronchial epithelial cells made to overexpress EGFR L858R or Δ 746-750^[116,117,143,144]. However, another study using TGF α as a ligand showed intact and constitutive mutant EGFR-Cbl association in NSCLC cell lines^[115].

Similar to conflicting reports on mutant EGFR-Cbl association, the phosphorylation status of the Cbl binding site, EGFR-Y1045, on mutant EGFRs remains unclear^[87]. Reverse-phase protein microarray was used to quantify levels of phosphorylation of various EGFR phosphorylation sites on pure tumor cell populations isolated by laser capture microdissection from human lung tumor biopsy specimens^[145]. The group found that phosphorylation of EGFR-Y1045 was reduced across patient samples that expressed all classes of mutant EGFRs (inframe deletion mutant, EGFR L858R and H773L/V774M) compared with wtEGFR^[145]. Similarly, EGFR L858R and EGFR Δ 747-753 mutants expressed in a mouse fibroblast cell line or COS-7 cells showed lower levels of EGFR-Y1045 phosphorylation when compared to wtEGFR, while EGFR Δ 746-750 showed hypoubiquitination, delayed downregulation, and increased surface retention upon ligand stimulation^[146]. Another study, however, showed that when mutant EGFRs were stably expressed in a NSCLC cell line H1299, the mutant EGFRs showed higher basal phosphorylation levels at all tyrosine residues, including Y1045^[120]. Intact ligand-induced Y1045 phosphorylation has been observed in other mutant EGFR cell systems, including L858R mutant-expressing and in-frame deletion mutant-expressing non-transformed mouse mammary epithelial cells^[23], SF9 insect cells^[147], and murine hematopoietic cells^[121]. Similarly, endogenous EGFR Δ 746-750 or L858R/T790M expressed in NSCLC cell lines H1650 or H1975, respectively showed robust phosphorylation on EGFR Y1045 compared to that in wtEGFR-expressing NSCLC cell line H358^[116]. Interestingly, human embryonic kidney 293 cells transfected with EGFR L858R showed intact ligand-induced Y1045 phosphorylation and association with Cbl, whereas EGFR Δ 747-753 showed decreased Y1045 phosphorylation and EGFR-Cbl association^[55]. The discrepancies among reported results are likely to be due to the difference in EGFR mutation type, different cell type used, and/or the types of cells used as controls to make comparisons. Nonetheless, a consistent picture on Cbl-mutant EGFR association has not emerged, and the role of other Cbl family members remains unclear.

In contrast to a lack of consensus on Y1045 phosphorylation of mutant EGFRs and their association with Cbl, different studies have consistently noted an impair-



Figure 2 Mutant epidermal growth factor receptor vs wt-epidermal growth factor receptor signaling. While wtEGFR signaling to Akt and Erk is subjected to Cbl-mediated degradation, mutant EGFR cooperates with Src to exaggerate signaling through downstream effectors. EGFR: Epidermal growth factor receptor.

ment of ligand-induced ubiquitination and downregulation of mutant EGFRs. It was reported that mutant EGFRs undergo reduced ubiquitination and delayed downregulation upon ligand stimulation in NSCLC cell lines H1650 and H1975, expressing endogenous mutant EGFRs, and in human embryonic kidney cells ectopically overexpressing mutant EGFR^[116,143]. Decreased ligand-induced ubiquitination and delayed downregulation were also observed in various NSCLC cell lines expressing endogenous EGFR Δ 746-750 or L858R (HCC827 and H3255, respectively), and in normal human bronchial epithelial cells stably expressing EGFR Δ 746-750 or L858R^[117].

Interestingly, even under conditions that permitted mutant EGFR-Cbl association, mutant EGFR showed decreased ligand-induced ubiquitination and impaired degradation; this correlated with constitutive association of mutant EGFR with the molecular chaperone Hsp90^[115]. Constitutive association of mutant EGFR with Hsp90^[115,148] may provide a mechanism to impair Cbl-dependent mutant EGFR downregulation. However, Cbl overexpression in HCC827 cell line resulted in enhanced mutant EGFR downregulation, suggesting that mutant EGFRs retain the ability to undergo Cbl-dependent downregulation but the process is less efficient^[117]. Among the ErbB family receptors, ErbB2 is known to be stably associated with Hsp90 while EGFR-Hsp90 interaction is transient^[149]. It is therefore noteworthy, that heterodimerization with ErbB2 has been identified as a mechanism for the ability of EGFR L858R or EGFR L858R/T790M to avoid ligand-induced downregulation^[116] (Figure 1). Previous studies in breast cancer and other models have established that ErbB2 is impaired in downregulation, and its co-overexpression with EGFR inhibits the downregulation of EGFR by increasing the recycling rate of EGFR and/or inhibiting internalization^[100,101,150,151]. Indeed, treatment of gefitinib-resistant EGFR L858R/T790M-expressing NSCLC cells with a EGFR/ErbB2 dual TKI, lapatinib, decreased STAT3 activation and this was associated with reduced mutant EGFR-ErbB2 heterodimerization^[56]. Combining lapatinib and cetuximab treatment resulted in enhanced cytotoxicity against gefitinib-resistant EGFR L858R/T790M-expressing cells *in vitro* and in xenograft models *in vivo*^[56].

However, EGFR mutants expressed in Chinese hamster ovary cells were less sensitive to lapatinib, indicated by the levels of autophosphorylation, than wtEGFR^[152]. Therefore, the impact of ErbB2 co-expression based on the effects of lapatinib must be considered in the context of the genetic makeup of the cell system used, including the levels of ErbB2 expression. It has also been shown that certain stimuli activate EGFR and promote its internalization but do not induce efficient downregulation. Such stimuli include specific EGFR ligands, such as amphiregulin and TGF α , and exposure to certain chemicals including H₂O₂ or cigarette smoke^[97,153] and the role of such factors in inefficient downregulation of mutant EGFRs in NSCLC needs to be considered.

A number of interacting proteins are thought to affect Cbl-dependent lysosomal trafficking of EGFR. Cbl interacting proteins CD2AP and CIN85 are thought to cooperate with Cbl to promote EGFR endocytosis^[113,154], whereas Cool-1, Sprouty and Sts-1/Sts-2 interfere with EGFR downregulation^[107,112,113,155]. Therefore, alterations of these components may account for defective Cbl-dependent downregulation of mutant EGFRs even though EGFR Y1045 phosphorylation and association with Cbl remain intact^[23,115,116,120].

In addition, there are other factors to be considered for the altered endocytic trafficking of mutant EGFRs. Cdc42-associated tyrosine kinase 1 associates with activated EGFR and is involved in ligand-induced clathrin-coated vesicle-mediated EGFR endocytosis and degradation^[156-158]. Rab5 controls endosome fusion and enhances lysosomal degradation of EGFR^[159,160], and Rab5 exchange factor GAPex-5 mediates EGFR ubiquitination, lysosomal trafficking and degradation^[161]. Alternatively, TBC1D3 enhances EGFR internalization but suppresses Cbl-dependent EGFR ubiquitination and degradation^[162]. In addition, STAM1/2, Hrs, Rin1 and ESCRT proteins also regulate EGFR endocytic traffic at various stages^[91,163]. Verifying expression levels of proteins critical to Cbl-dependent EGFR downregulation, and/or RNAi-mediated knock-down of proteins implicated in interfering with EGFR downregulation may identify those critical to mutant EGFR endocytic trafficking and provide mechanism of altered endocytic traffic of mutant EGFRs. Given the biological consequences of inefficient mutant

EGFR downregulation, elucidation of cell biological and biochemical mechanisms responsible represent a fertile area of future research.

ENDOSOMAL SIGNALING BY EGFR

Aside from the importance of endocytosis as a necessary step in lysosomal downregulation of ligand-activated EGFR, endocytosis has emerged as a requirement for efficient activation of specific downstream signaling pathways. For example, inhibition of the internalization machinery demonstrated that activation of PLC γ 1 upon EGF stimulation occurs primarily at the cell surface while activation of Erk and Akt signaling occurs primarily post-internalization^[164]. Therefore, oncogenic signaling from mutant EGFRs may result from or be sustained by altered receptor endocytic trafficking. It is now well-established that internalized receptor tyrosine kinases, including EGFR, continue to be active unless degraded, providing a mechanism for persistent signaling as well as activation of distinct pathways through the formation of spatially-distinct signaling complexes^[165]. Many studies have shown that Erk activation upon EGF stimulation critically depends on endosomal localization of EGFR; activated EGFR in endosomal compartments participate in the activation of Ras, the upstream activator of Erk signaling^[166]. A relationship between accumulation of EGFR in endosomes and enhanced Erk activation is provided by studies in which overexpression of SEF (for similar expression to FGF) enhances EGF-induced EGFR internalization but delays its targeting to lysosome, and results in sustained Erk activation and differentiation in the PC12 rat pheochromocytoma cell model^[167]. Knockdown of Cbl in 293 cells^[168], or lack of Cbl in Cbl-knockout mouse embryonic fibroblasts^[134], delayed exit of ligand-stimulated EGFR out of early endosomes towards lysosomes, and resulted in prolonged Erk activation. Combined knockdown of Cbl and Cbl-b had a similar impact on EGFR traffic and Erk signaling in human mammary epithelial cells^[132].

Endosomal localization of not just EGFR but also of Ras signaling cascade proteins is required for Erk activation. Dynamin-regulated endocytosis of activated MEK is required for Erk activation^[169]. Analysis of endosomal localization of MEK2-GFP suggests that endosomal localization of MEK2 requires clathrin-dependent endocytosis, the presence of an upstream kinase, RAF, and the catalytic activity of MEK^[170]. Erk activation mediated by beta 2-adrenergic receptor transactivation of EGFR is sensitive to clathrin-dependent endocytosis in transfected COS-7 cells^[171], although it has been recently reported that clathrin-dependent endocytosis is not required for Erk activation in HeLa cells^[170]. MAP kinase signaling may itself affect EGFR endocytic traffic, as activation of p38 MAP kinase induced the internalization of EGFR, suggesting that p38 may provide an important feedback regulatory loop in the regulation of EGFR trafficking and signaling^[172]. Recently, it was shown that activated

Akt promotes early endosome to lysosome transition and degradation of EGFR by activating PIKfyve (FYVE-containing phosphatidylinositol 3-phosphate 5-kinase)^[173]. By utilizing reversible kinase inhibitors to promote ligand-induced internalization into endosomes followed by inhibitor removal, it was established that endosomal EGFR signaling is sufficient to activate major signaling pathways leading to cell survival and proliferation^[128,174]. Thus, it is easy to visualize how constitutive internalization into endosomes together with impaired lysosomal downregulation of NSCLC-associated EGFRs can promote endosomal signaling-dependent oncogenic cascades, as further discussed below.

MUTANT EGFR TRAFFIC AND SIGNALING

As discussed above, studies of mutant EGFR signaling pathways have identified Akt, Erk, STATs and Src as critical downstream molecular players. Our studies established that preferential trafficking of mutant EGFRs *vs* wtEGFR into the endocytic recycling compartment promotes association of mutant EGFRs with their oncogenic partner Src (Figure 1)^[118]. The requirement of the major Src phosphorylation site of the mutant EGFRs (Y845) for their ability to transform NIH 3T3 cells supports a role for Src signaling within the endosome in mutant EGFR oncogenic activity^[85] (Figure 2). This is consistent with the impact of Src inhibitors on NSCLC *in vitro* and in animal models^[85,175]. Further studies are needed to directly assess if Src-dependent signaling by mutant EGFRs takes place primarily within the endosomal compartments or if endocytic recycling is required to traffic Src to its site of action. In this regard it is notable that inactive Src is primarily localized on lysosomal membranes and an endocytic traffic pathway orchestrated by the ESCRT machinery is required for its traffic to focal adhesions/invadopodia, where Src activity is essential for cell migration and invasion^[176,177]. Notably, lysosomal regulatory small GTPase Rab7 and ESCRT1 component TSG101 were shown to be required for unoccupied wtEGFR to enter into the endocytic recycling compartment and to recycle back to the cell surface^[178]. It will be of considerable interest in the future to determine if NSCLC-associated mutant EGFRs and c-Src co-traffic through a lysosomal/MVB compartment to enter the endocytic recycling compartment.

Notably, endocytic traffic-dependent EGFR signaling is also critical for cell migration. The developmentally regulated border cell migration in drosophila requires localized EGFR signaling, and deletion of proteins involved in endocytic traffic of EGFR (Cbl and Rab5 guanine nucleotide exchange factor) disrupted border cell migration^[142]. In this system, receptor endocytosis is necessary to help establish ligand gradients and localized RTK signaling, preserving spatial information that is critical to guide migrating cells in a directional manner^[142]. Recently, it was shown that TGF α -induced cell migration in mammalian corneal epithelial cells required endocytic

recycling of EGFR^[1179]. Thus, it is plausible that endocytic recycling and endosomal signaling may contribute to cell migration, invasion and metastatic behavior of mutant EGFR-expressing NSCLCs.

NSCLC-associated EGFR mutants exhibit constitutive internalization^[118,119,121]. Since hyperactive Erk signaling is a prominent feature of mutant EGFRs in NSCLC^[23,66], enhanced endosomal localization of mutant EGFR may provide one of the mechanisms contributing to enhanced Erk activation. Indeed, the upstream activators of Erk signaling pathway have been shown to be associated with endosomal EGFR. Analysis of rat livers following administration of EGF, internalization of EGFR coincided with recruitment of the adaptor protein Shc, and its association with GRB2 and the Ras guanine nucleotide exchange factor, mSOS, and the complex of tyrosine phosphorylated Shc, Grb2 and mSOS was largely present in the endosomal fraction^[180]. FRET measurements also indicated that activated EGFR-CFP interacted with YFP-Shc and Grb2-YFP in endosomes^[181]. Similarly, inhibition of EGFR internalization through knockdown of Grb2 by RNA interference or use of a dynamin mutant resulted in inhibition of EGF-induced MAPK and Erk activities^[106,164].

Akt activation has also been linked to endosome-localized EGFR. Inhibiting internalization through clathrin heavy chain or AP-2 knockdown reduced EGFR-mediated Akt as well as MAPK activation^[99]. Initiation of EGFR signaling directly from the endosomes, by allowing EGFR endocytosis in the presence of a washable inhibitor, demonstrated that endosomal signaling of EGFR is sufficient for activating signaling pathways including Erk and Akt, as well as cell survival and proliferation^[128,174]. Likewise, ligand-induced trafficking of internalized EGFR from early endosomes to lysosomes was severely delayed in cells lacking presenilin 1 and resulted in prolonged EGFR, Akt and Erk activation^[182]. A similar impact of depletion of Cbl proteins has been demonstrated on EGFR-induced Erk and Akt activation^[134,137]. Therefore, similar to Erk, Akt activation may also be dependent on EGFR localization in endosomes. Interestingly, some studies indicate that endosomal EGFR does not recruit and activate PI3-K^[183,184], suggesting that EGFR-dependent Akt activation may involve additional factors and/or mechanisms. Clathrin-dependent EGFR internalization has also been shown to be essential for STAT3 nuclear translocation and Stat3-dependent gene regulation, and Stat3 co-localizes with labeled EGF in endocytic vesicles^[185]. These studies, done on wtEGFR, support the likelihood that constitutively endosome localized mutant EGFRs promote enhanced Map kinase and potentially PI3-kinase and STAT signaling through endosomal signaling. Future investigations targeting this key question are urgently needed.

ROLE OF SRC IN EGFR ENDOCYTOSIS

In addition to its critical involvement in EGFR-mediated oncogenesis^[85], Src plays a role in EGFR endocytosis.

Active Src has been shown to phosphorylate clathrin heavy chain^[186], and overexpression of Src was shown to accelerate clathrin-mediated internalization of EGFR without increasing EGFR degradation in fibroblasts. Interestingly, Src co-overexpression with EGFR in human mammary epithelial cells led to reduction in surface EGFR levels without a decrease in total EGFR levels, together with EGFR hyperactivation, suggesting that overexpressed Src promotes the traffic of surface EGFR into a non-degradative and likely signaling endosomal compartment^[187]. Src-mediated tyrosine phosphorylation has been shown to be required for dynamin function in ligand-induced EGFR internalization^[188]. Src is also critical for proper ubiquitination and degradation of EGFR; Src activity antagonizes the function of Cbl by mediating its phosphorylation and degradation^[189]. In the presence of a Src inhibitor, EGF-induced phosphorylation of Cbl and ubiquitination of EGFR were blocked^[190]. Similarly, mouse embryonic fibroblasts with deletion of Src-negative regulator C-terminal Src kinase results in hyperactive Src; suppression of Src family kinases in these cells resulted in delayed EGFR degradation and prolonged EGF-induced activation of Erk1/2^[191]. Src-mediated phosphorylation was also shown to antagonize the function ALG-2 interacting protein X (Alix) in receptor tyrosine kinase internalization^[192]. A study using Src-GFP fusion protein showed that upon EGF stimulation, Src traffics into endosomal compartments with activated EGFR, and that Src expression and kinase activity prolong the EGFR activation^[193]. Src overexpression also induced activation of EGFR and of EGFR-mediated downstream signaling targets Erk and Shc^[193]. At a biological level, Src overexpression was found to promote the ability of EGFR overexpression to transform rodent fibroblasts^[194], and promote a transformed phenotype in three-dimensional cultures of human mammary epithelial cells^[81]. Therefore, constitutive interaction between mutant EGFR and Src^[84,85,118] suggests that, in addition to activating EGFR signaling, the hyperactivity of Src in NSCLC may provide a mechanism for delayed ligand-induced association of Cbl with EGFR, and reduced EGFR ubiquitination and/or downregulation^[116,117,143,146] (Figure 2). In this scenario, the preferential trafficking of mutant EGFRs to the endocytic recycling compartment^[118] may be due, in part, to their constitutive interaction with Src. Future studies are needed to establish if this mechanism can indeed explain the altered trafficking and signaling of mutant EGFRs.

CONCLUSION

Much insight has been gained on naturally occurring NSCLC-associated mutant EGFRs, thanks in large part to studies instigated by their clinical relevance which resulted in targeted therapies with TKIs. However, acquired resistance and other drug interfering mechanisms limit the efficacy of EGFR-directed therapies in NSCLC patients with EGFR mutations. Mutant EGFR biology remains poorly understood, and as yet their biologically-

linked and essential traits of signaling and endocytic traffic have not been integrated for mutant EGFRs. The current review summarizes our current understanding of mutant EGFR signaling and traffic and areas where we lack a clear picture, and points to a need for further understanding and integration. It is clear that mutant EGFRs, in addition to attaining constitutive activity, exhibit deregulated endocytic traffic that appears to promote the ability of mutant receptors to signal into oncogenic pathways. Increased understanding of mechanisms that underlie defects in mutant EGFR endocytic traffic could help define novel approaches to refine EGFR-directed therapies by intercepting at key endocytic traffic nodes.

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WJCO 5th Anniversary Special Issues (1): Lung cancer

Positron emission tomography to assess hypoxia and perfusion in lung cancer

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Abstract

In lung cancer, tumor hypoxia is a characteristic feature, which is associated with a poor prognosis and resistance to both radiation therapy and chemotherapy. As the development of tumor hypoxia is associated with decreased perfusion, perfusion measurements provide more insight into the relation between hypoxia and perfusion in malignant tumors. Positron emission tomography (PET) is a highly sensitive nuclear imaging technique that is suited for non-invasive *in vivo* monitoring of dynamic processes including hypoxia and its associated parameter perfusion. The PET technique enables quantitative assessment of hypoxia and perfusion in tumors. To this end, consecutive PET scans can be performed in one scan session. Using different hypoxia tracers, PET imaging may provide insight into the prognostic significance of hypoxia and perfusion in lung cancer. In addition, PET studies may play an important role in various stages of personalized medicine, as these may help to select patients for specific

treatments including radiation therapy, hypoxia modifying therapies, and antiangiogenic strategies. In addition, specific PET tracers can be applied for monitoring therapy. The present review provides an overview of the clinical applications of PET to measure hypoxia and perfusion in lung cancer. Available PET tracers and their characteristics as well as the applications of combined hypoxia and perfusion PET imaging are discussed.

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Key words: Molecular imaging; Positron emission tomography; Hypoxia; Perfusion; Quantification; Lung cancer

Core tip: This review provides an overview of the current applications of positron emission tomography for hypoxia and perfusion imaging in lung cancer. Available PET tracers are discussed and the benefits of combined hypoxia and perfusion PET imaging are clarified. Hypoxia imaging could aid in selecting patients for hypoxia-specific treatment strategies. To achieve this, consensus about the optimal imaging protocol and quantification method is essential. Large clinical trials are needed to confirm the value of hypoxia imaging for improving patient care.

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INTRODUCTION

Worldwide, lung cancer is the most common cause of cancer related death among men and women^[1]. Every year, approximately 1.2 million new cases of lung cancer

are diagnosed globally and 1.1 million patients die of this disease^[2]. Non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) are the main histological types and represent approximately 85% and 15% of the lung cancer cases, respectively^[3,4]. The prognosis of both NSCLC and SCLC is poor and depends on the stage of the disease^[5,6]. For example, the 5-year overall survival is approximately 1% and 2% for stage IV NSCLC and extensive stage SCLC, respectively. Treatment of lung cancer depends on histological type, stage and performance status. The available treatment options include surgery, radiation therapy and chemotherapy, or a combination of these modalities. Systemic therapy of lung cancer consists mainly of a platinum-based doublet, such as cisplatin or carboplatin, in combination with a third generation cytotoxic drug such as gemcitabine, pemetrexed, paclitaxel or docetaxel^[7,8]. In addition, targeted agents, including gefitinib, erlotinib, bevacizumab and crizotinib, have been introduced for the treatment of advanced NSCLC^[9-16]. For the last decades, several tumor characteristics have been under investigation in order to further understand the biology of lung cancer and enhance the efficacy of the several treatment modalities.

In lung cancer, tumor hypoxia is a characteristic feature^[17], which is associated with a poor prognosis^[18-20] and resistance to both radiation therapy^[21] and chemotherapy^[22]. Hypoxia is a reduced O₂ tension in tissue and is defined between normoxia (pO₂ levels of 40-60 mmHg) and anoxia (0 mmHg)^[23]. In clinical practice, no consensus has been achieved for hypoxic thresholds in tumors, but tumors with pO₂ values below 10 mmHg are usually considered hypoxic^[23]. Tumor hypoxia is the result of an imbalance between oxygen supply and consumption and can be caused by the following mechanisms^[23]: (1) the structurally and functionally abnormal tumor vasculature leads to a perfusion-limited delivery of oxygen^[24], thereby inducing “acute” hypoxia; (2) tumor proliferation increases the distance between tumor cells and blood vessels that provide nutrients and oxygen to tumor cells. Consequently, the distances to blood vessels can become larger than the diffusion distance of oxygen (> 70 µm), locally causing diffusion-limited hypoxia (referred to as “chronic” hypoxia); (3) tumor hypoxia is also associated with a systemic decrease in oxygen supply, *i.e.*, anemia, which can be caused by tumor-related factors as well as anticancer therapy.

To promote cell survival in hypoxic conditions hypoxia inducible factor-1 (HIF-1) is upregulated, which in turn activates a number of processes including growth factor signaling, angiogenesis, proliferation, glycolysis, tissue invasion, and finally metastasis^[25]. As a result, markers of the HIF signaling cascade such as HIF-1α, glucose transporter-1, and vascular endothelial growth factor (VEGF), have been investigated as surrogate markers for tumor hypoxia in lung cancer^[18,19,26,27]. Alternatively, immunohistochemical staining using injectable exogenous bioreductive markers like pimonidazole and 2-(2-nitro-1-[H]-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)-

acetamide (EF5) can be applied^[28]. However, immunohistochemistry requires tissue samples and represents an indirect measurement of tumor hypoxia. Alternatively, pO₂ levels in tumors can be directly assessed using Eppendorf polarographic electrodes. This an invasive technique that can be applied in tumors that are easily accessible^[29]. In lung cancer, this technique is not feasible^[17], as these tumors are usually deeply seated within in de body. Positron emission tomography (PET) may be useful, as PET enables direct assessment of tumor hypoxia in patients non-invasively^[30].

As the development of tumor hypoxia is associated with decreased perfusion, perfusion PET imaging may provide more insight into the relation between hypoxia and perfusion in malignant tumors. PET scans may not only reveal the prognostic significance of hypoxia and perfusion in lung cancer, but may also help to select patients for specific treatments including radiation therapy, hypoxia modifying therapies, and antiangiogenic drugs^[31,32]. This review provides an overview of the clinical applications of PET to measure hypoxia and perfusion in lung cancer.

PET PRINCIPLES

PET enables non-invasive 3D imaging of dynamic processes *in vivo*. To this end, molecules of interest are radiolabeled with positron emitting radionuclides. For PET imaging, commonly used radionuclides are oxygen-15 (¹⁵O), carbon-11 (¹¹C) and fluorine-18 (¹⁸F). These radionuclides are isotopes of elements that are often naturally present in organic molecules as well as in chemically produced molecules, *e.g.*, anticancer drugs. After replacing one of the molecules' atoms by its radioactive isotope, the molecular structure is unchanged, leaving chemical properties unaffected. After intravenous injection of a PET tracer, the radiolabeled molecules can be located within the body by detecting the emitted photons. Since only a small amount of radiotracer is required for PET imaging, it is assumed that the radiotracer does not affect the dynamic process under study.

PET is based on the detection of positron emission. During radioactive decay, the radionuclide, *e.g.*, ¹⁸F, emits a positron which, after traveling a short distance (few mm) in tissue, annihilates with a nearby electron to emit two 511 keV photons in opposite directions. These two “annihilation” photons are registered by the PET scanner using a coincidence detection circuitry, providing 3D information of the tracer distribution with high sensitivity and resolution. To achieve quantitative accuracy, imaging data needs to be corrected for attenuation: when emitted from tissues deeper in the body, photons are more likely to be absorbed than from superficial structures. As a result, 3D images would falsely show low tracer concentrations in deeper structures compared to superficial structures. In PET, the attenuation perceived by the annihilation photon pairs, traveling in opposite directions over a line through the body, is mathematically equivalent

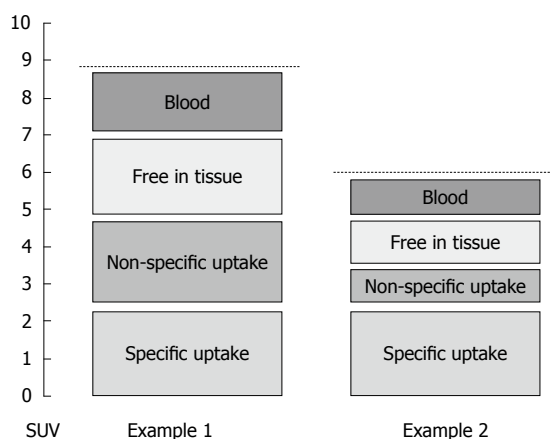


Figure 1 Graphical representation of the different components that determine the total positron emission tomography signal. Examples 1 and 2 can represent either different patients, different lesions in one patient or different scans of one patient, for example before and after therapy. In both examples the contributions of specific uptake (the uptake of interest) are equal, but the total signal is different due to differences in contribution of other (non-relevant) signals. Measured standardized uptake value (SUV) values are reflected by the dashed lines. As SUV does not only reflect the specific signal, its use should be validated before it is used in a clinical setting, *i.e.*, it is required to assess if contributions from non-specific signals affect SUV values in a non-predictable way. For the purpose of illustration, the Y-axis represents SUV values on an arbitrarily chosen scale.

to the attenuation perceived by one photon transmitted through the body over that same line. Therefore, accurate attenuation correction can be achieved using a transmission source, *e.g.*, computed tomography (CT). In addition, PET/CT systems can correct for false detections due to random coincidence detection or scattered annihilation photons. As a result, PET provides radioactivity measurements with high quantitative accuracy^[33].

Quantification of tracer uptake, however, remains challenging. First, the measured radioactivity concentration in tissue depends on the tracer concentration in blood over time, which, in turn, depends on the injected dose and distribution volume. The standardized uptake value (SUV) takes this variability into account, as the radioactivity concentration in tissue is normalized by the ratio of the injected dose to patient weight. Second, the PET signal does not necessarily reflect specific uptake, *e.g.*, trapping of the tracer by the process of interest. A tracer could also be free in tissue, trapped by a different process or reside in blood vessels within the region of interest, *e.g.*, tumor (Figure 1). Pharmacokinetic modeling can be applied to distinguish between the various kinetic processes and separates the total signal into these components^[34].

In addition to spatial information, temporal information of the tracers' distribution is used in pharmacokinetic modeling. To obtain information on the changes in tracer activity concentrations over time (time activity curves or TAC), sequential PET images are acquired over the same body area. In addition, accurate temporal data on tracer concentration in plasma is obtained from arterial blood sampling and dedicated lab analysis. Mathematical models ("compartment models") are then used

to extract measures of the relevant components of the tracers' kinetics, such as specific uptake or binding. As absolute quantification by kinetic modeling can be challenging and cumbersome in the clinic, alternatives have been introduced to measure tracer uptake. Before clinical implementation, these "simplified parameters" (such as SUV) should be validated and correlated with parameters from pharmacokinetic modeling.

To date, 2-deoxy-2-[¹⁸F]fluoro-D-glucose ([¹⁸F]FDG) is the most commonly used PET tracer. As [¹⁸F]FDG is a glucose analogue, it accumulates in malignant tumors with high glucose consumption. As a result, [¹⁸F]FDG PET is extensively used for diagnosis, staging and response monitoring of cancer. Currently, [¹⁸F]FDG PET is routinely performed for initial staging^[35] and pre-operative staging^[36,37] of patients with NSCLC. As tumor hypoxia is associated with increased glycolysis, it is conceivable that hypoxia is associated with increased [¹⁸F]FDG uptake. However, results on [¹⁸F]FDG to assess tumor hypoxia have been conflicting^[38], indicating that [¹⁸F]FDG is not specific enough to identify hypoxia. Therefore, other PET tracers have been developed to measure hypoxia and perfusion in tumors more specifically. In the following paragraphs, these PET tracers will be discussed.

TUMOR HYPOXIA IMAGING

Clinical relevance

Tumor hypoxia is associated with resistance to both radiation therapy^[21] and chemotherapy^[22]. Radiation therapy requires oxygen to induce DNA damage and hypoxic cancer cells are three times less sensitive to radiation therapy than normoxic cancer cells^[39,40]. In addition, the resistance to anticancer drugs is attributed to the lack of O₂ available for drug activation, the increased genetic instability, the antiproliferative effects of hypoxia, and the increased gene transcription induced by HIF-1^[41,42]. Currently, drugs that selectively target tumor hypoxia and its increased gene transcription are still under study and have entered the first clinical trials^[43-45]. Since tumor hypoxia may affect clinical outcome, hypoxia imaging may be useful to determine prognosis and tumor response in lung cancer patients. Furthermore, hypoxia assessment may help to optimize treatment strategies in individual patients.

In particular, the efficacy of radiation therapy may be increased by several interventions. First, the systemic oxygenation level can be increased by hyperbaric chamber treatment^[46], carbogen breathing^[47] and improved oxygen transport by hemoglobin. For the latter, blood transfusions and erythropoietin injections are available^[48]. Oxygen transport can be further improved by agents that improve perfusion and affect vascular permeability^[49]. Second, the apparent oxygenation level in tumors can be increased using radiosensitizers, which are usually based on a nitroimidazole-group and specifically target hypoxic tumor cells (pO₂ < 10 mmHg). Once incorporated in hypoxic tumor cells, radiosensitizers mimic oxygen, thereby

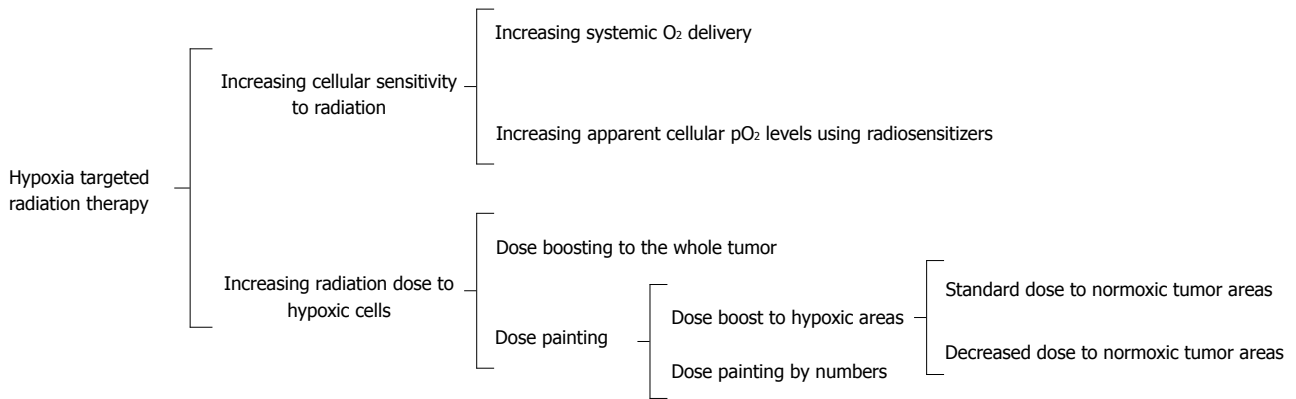


Figure 2 Radiation therapy treatment strategies for tumor hypoxia.

increasing the efficacy of radiation therapy^[50]. Third, the radiation therapy plan can be adjusted to increase the dose administered to hypoxic tumor tissue. This can be achieved by dose boosting to the whole tumor, dose painting, or dose painting by numbers^[51]. For dose boosting, an increased dose is administered to hypoxic areas, thereby increasing the radiation dose to normal tissue and, potentially, its associated side effects. For dose painting, the dose to a specific area (*e.g.*, hypoxic area) is increased, whereas the radiation to the remaining part of the tumor can be either maintained or decreased. In the latter case the total dose level can be maintained. Dose painting can be further refined when it is directly based on the voxel-by-voxel values of a PET image (referred to as “dose painting by numbers”). For successful implementation of the previous mentioned radiation therapy strategies, hypoxia imaging may help to identify hypoxic tumors, prevent unnecessary side effects in patients with normoxic tumors, and reveal heterogeneous distribution of hypoxia within tumors. Figure 2 summarizes the potential applications of hypoxia imaging for radiation therapy.

Characteristics of a hypoxia PET tracer

The ideal hypoxia tracer would freely and rapidly diffuse to tissue, including remote areas. For optimal contrast of the PET image, accumulation of the tracer should be high in hypoxic cells, whereas no binding should occur in normoxic cells. To achieve the best image quality, an optimal balance between tracer half-life, accumulation rates and clearance rates is required: the tracers’ half-life should be long enough to obtain a high signal-to-noise ratio whilst allowing the tracer enough time to diffuse and bind to hypoxic cells and clear from normoxic tissues and blood. Accumulation and clearance rates are influenced by the tracers’ octanol/water partition coefficient. More lipophilic compounds may more readily pass through the cell membrane. On the other hand, more hydrophilic compounds may more easily diffuse across tissues and show faster clearance from blood and normoxic tumors through the urinary pathway^[30,52]. Besides these hypoxia specific characteristics, the tracer should be metabolically

inert, since the formation of radiolabeled metabolites results in a decreased amount of the original tracer available for hypoxia specific uptake, poor image contrast and inaccurate tracer quantification.

For clinical implementation, hypoxia tracers require fast kinetics, allowing for rapid accumulation in hypoxic tissues, thereby limiting the time between tracer injection and imaging. In addition, simplified and reproducible methods (*e.g.*, SUV) are needed to quantify tracer uptake.

Hypoxia tracers for PET

Over the last decades, several PET tracers have been developed to measure tumor hypoxia. To identify all relevant hypoxia tracers in lung cancer, a literature search was conducted in PubMed to identify studies published before 1 January 2014. To this end, PET specific search terms (PET, positron emission tomography) were combined with hypoxia specific search terms (hypoxia, anoxia), and/or lung cancer specific search terms (lung cancer, lung neoplasms, non-small cell lung cancer, small cell lung cancer), and/or kinetic modeling specific search terms (kinetic modeling, modeling), and/or radiation therapy specific search terms (radiation therapy, radiation). For these search terms, the corresponding Mesh terms were included. Thereafter, the obtained English abstracts were evaluated for relevance. Based on the obtained publications, a specific search strategy was subsequently performed for each identified hypoxia PET tracer. Additional publications were identified by cross-referencing. Brain studies were excluded since the blood-brain barrier may affect tracer kinetics. Figure 3 and Table 1 give an overview of the identified hypoxia tracers that have been evaluated in oncology. The tracer names and abbreviations are displayed in Table 2. These hypoxia tracers can be subdivided in nitroimidazole-based and thiosemicarbazone-based tracers. In the following paragraphs, these tracers and their potential applications in lung cancer patients will be discussed.

Nitroimidazole-based tracers: Originally, nitroimidazoles have been developed as radiosensitizers. Already in 1984, Chapman^[53] have proposed nitroimidazoles for

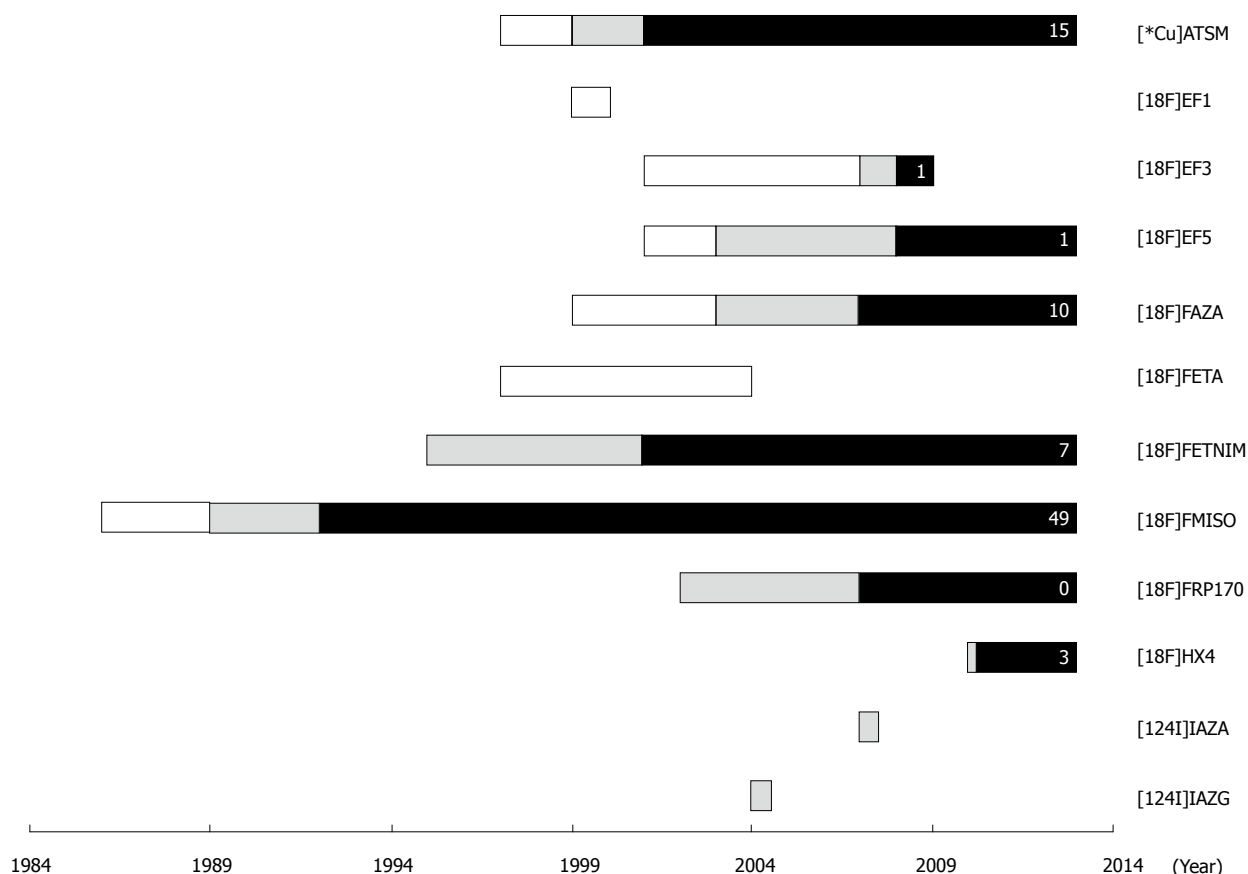


Figure 3 Timeline for development and evaluation of hypoxia specific tracers that have been evaluated by preclinical or clinical positron emission tomography. Development and *in-vitro* analysis (blank), preclinical positron emission tomography (PET) evaluation (grey), and clinical PET evaluation (black). The number of published clinical studies in oncology is indicated (excluding brain studies). See Table 2 for full names.

hypoxia imaging. Upon entering the cell, nitroimidazole undergoes electron reduction, thereby becoming a radical. In normoxic cells, this reaction is reversed by O_2 . In hypoxic cells, the radical can react with an intracellular macromolecule instead and remains trapped. As the latter process occurs at $pO_2 < 10$ mmHg, an oxygenation level associated with increased radiation therapy resistance, nitroimidazoles are able to detect clinically relevant hypoxia^[54].

Among the developed hypoxia tracers for PET (see Figure 3), [^{18}F]FMISO has been investigated most extensively. Although [^{18}F]FMISO showed rapid metabolism in mice studies, it appeared to be a robust hypoxia tracer in humans, with parent fractions up to 96% at 90 min after injection^[55]. Since [^{18}F]FMISO is rather lipophilic with a partition coefficient ($\log P$) of 0.4, clearance from blood and normoxic tissues is slow. Therefore, the required time intervals between injection and imaging are long, at least 3 h^[56]. Efforts have been made to develop hypoxia tracers with more favorable characteristics. Being the most evaluated and validated hypoxia tracer to date, the performance of new hypoxia tracers is often compared with [^{18}F]FMISO (see Table 1). Among these tracers, [^{18}F]FAZA has been introduced in the clinic. [^{18}F]FAZA ($\log P = 0.04$) is more hydrophilic than [^{18}F]FMISO and shows faster clearance from blood and normoxic tis-

sues^[57]. This allows for a shorter time interval between injection and imaging^[58]. In addition, [^{18}F]FAZA has a high parent fraction during imaging, accounting for a parent fraction of 90% at 70 min after injection^[59]. Other more hydrophilic nitroimidazole tracers include [^{18}F]FETNIM and [^{18}F]HX4, which have a partition coefficient ($\log P$) of 0.17^[60] and -0.69^[61], respectively. An example of a more lipophilic tracer is [^{18}F]EF5, which is the ^{18}F -labelled version of exogenous hypoxia marker EF5, with a partition coefficient ($\log P$) of 0.6.

Thiosemicarbazone-based tracers: Thiosemicarbazone-based tracers represent another subgroup of hypoxia tracers for PET. Thiosemicarbazones possess a strong antitumor activity, particularly when coupled with a metal ion like copper (Cu)^[62]. [Cu]ATSM is a therapeutic agent which, by replacing the Cu atom with a suitable radioactive Cu isotope, can be used for hypoxia PET imaging^[63]. In nuclear medicine, Cu is of particular interest for its favorable radiochemical properties. First, Cu is relatively easy to incorporate in molecules and has multiple radioactive isotopes suitable for PET imaging. Second, with half lives ranging from 24 min to 13h for ^{60}Cu and ^{64}Cu , respectively, Cu has several potential applications. The short-lived radionuclides can be used for sequential measurements, whereas radionuclides with longer half lives do

Table 1 Evaluated hypoxia tracers in oncology

Tracer ¹	Half-life	Validation studies ^{2,3}				Evaluated in clinical oncology ³			
		BC	Probe	Ex-M	En-M	FMISO	Lung cancer	Other cancer types	
[*Cu]ATSM	[60Cu]: 23.7 min	Lewis <i>et al</i> ^[133]	Ballegeer <i>et al</i> ^[137]	Ballegeer <i>et al</i> ^[137]	Grigsby <i>et al</i> ^[141]	Dence <i>et al</i> ^[145]	Dehdashti <i>et al</i> ^[146]	Chao <i>et al</i> ^[147] , Dehdashti <i>et al</i> ^[148] , Dehdashti <i>et al</i> ^[149] , Dietz <i>et al</i> ^[150] , Grassi <i>et al</i> ^[151] , Grigsby <i>et al</i> ^[151] , Kositwattanarek <i>et al</i> ^[152]	
	[61Cu]: 3.3 h	Yuan <i>et al</i> ^[134]	Bowen <i>et al</i> ^[138]	Hansen <i>et al</i> ^[140]	Tateishi <i>et al</i> ^[142]	Kerseman <i>et al</i> ^[135]	al ^[86] , Lohith <i>et al</i> ^[86]		
	[62Cu]: 9.7 min	Kerseman <i>et al</i> ^[135]	<i>et al</i> ^[133] , Myerson <i>et al</i> ^[139]	Matsumoto <i>et al</i> ^[136]	Valtorta <i>et al</i> ^[143]	Lewis <i>et al</i> ^[146]	al ^[67] , Wong <i>et al</i> ^[67]	Laforest <i>et al</i> ^[153] , Lewis <i>et al</i> ^[154] , Minagawa <i>et al</i> ^[155] , Nyflot <i>et al</i> ^[156]	
	[64Cu]: 12.7 h	Matsumoto <i>et al</i> ^[136]	O'Donoghue <i>et al</i> ^[172]	McCall <i>et al</i> ^[166]	Weeks <i>et al</i> ^[144]	Matsumoto <i>et al</i> ^[136]	al ^[80] , Zhang <i>et al</i> ^[81]		
[¹⁸ F]JEF1	110 min	NA	NA	O'Donoghue <i>et al</i> ^[172] , Oh <i>et al</i> ^[174] , Yuan <i>et al</i> ^[134]	NA	O'Donoghue <i>et al</i> ^[172]	NA	NA	
		Mahy <i>et al</i> ^[158]	NA	Evans <i>et al</i> ^[157]	NA	NA	NA	Mahy <i>et al</i> ^[161]	
		Mahy <i>et al</i> ^[159]	NA	Mahy <i>et al</i> ^[159]	NA	Dubois <i>et al</i> ^[160]	NA	Komar <i>et al</i> ^[164]	
[¹⁸ F]JEF5	[¹⁸ F]FAZA	NA	NA	Yapp <i>et al</i> ^[163] , Ziemer <i>et al</i> ^[163]	NA	NA	NA	Grosu <i>et al</i> ^[182] , Souvatzoglou <i>et al</i> ^[183] , Schuetz <i>et al</i> ^[184] , Shi <i>et al</i> ^[186]	
		Reischl <i>et al</i> ^[165]	Busk <i>et al</i> ^[170]	Busk <i>et al</i> ^[170] , Busk <i>et al</i> ^[172]	Belloli <i>et al</i> ^[178]	Sorger <i>et al</i> ^[157] , Piert <i>et al</i> ^[166] , Reischl <i>et al</i> ^[165]	Postema <i>et al</i> ^[180]	Mortensen <i>et al</i> ^[185] , Havelund <i>et al</i> ^[186]	
		Piert <i>et al</i> ^[166]	Mortensen <i>et al</i> ^[171]	<i>et al</i> ^[174] , Busk <i>et al</i> ^[173]	Picchio <i>et al</i> ^[167]	al ^[165]	Bollinini <i>et al</i> ^[181]	NA	
[¹⁸ F]JETA	[¹⁸ F]FETA	Picchio <i>et al</i> ^[167]	Piert <i>et al</i> ^[166] , Tran <i>et al</i> ^[169]	Troost <i>et al</i> ^[179]	Trinka <i>et al</i> ^[187]	Rasey <i>et al</i> ^[188]	Verwer <i>et al</i> ^[189]	NA	
		Maier <i>et al</i> ^[168] , Tran <i>et al</i> ^[169]	Barthel <i>et al</i> ^[187]	Graves <i>et al</i> ^[176] , Maier <i>et al</i> ^[177]	Valtorta <i>et al</i> ^[143]	NA	NA	NA	
		al ^[169]	Barthel <i>et al</i> ^[187]	NA	NA	Grönroos <i>et al</i> ^[189]	Hu <i>et al</i> ^[185]	Lehtiö <i>et al</i> ^[191] , Lehtiö <i>et al</i> ^[192] , Vercellino <i>et al</i> ^[193]	
[¹⁸ F]JFETNIM	[¹⁸ F]FETNIM	Barthel <i>et al</i> ^[187]	Yong <i>et al</i> ^[190]	NA	Hu <i>et al</i> ^[185]	Grönroos <i>et al</i> ^[189]	Hu <i>et al</i> ^[185] , Li <i>et al</i> ^[84]	Lehtiö <i>et al</i> ^[191] , Lehtiö <i>et al</i> ^[192] , Vercellino <i>et al</i> ^[193]	
		Grönroos <i>et al</i> ^[189]	Yong <i>et al</i> ^[190]	NA	NA	Tolvonen <i>et al</i> ^[190]	Yong <i>et al</i> ^[190]	Yue <i>et al</i> ^[194]	
		NA	NA	NA	NA	Yong <i>et al</i> ^[190]	NA	NA	
[¹⁸ F]FMISO	[¹⁸ F]FMISO	Bentzen <i>et al</i> ^[195]	Bentzen <i>et al</i> ^[125] , Gagel <i>et al</i> ^[125]	Hatano <i>et al</i> ^[205] , Huang <i>et al</i> ^[206]	Dubois <i>et al</i> ^[211]	Cherk <i>et al</i> ^[212]	Koh <i>et al</i> ^[221] , Liu <i>et al</i> ^[222] , Yeh <i>et al</i> ^[223] , Rischin <i>et al</i> ^[224] , Bentzen <i>et al</i> ^[225] , Rajendran <i>et al</i> ^[226] , Rajendran <i>et al</i> ^[227] , Lawrentschuk <i>et al</i> ^[228] , Loi <i>et al</i> ^[229] , Thorwarth <i>et al</i> ^[230]	Koh <i>et al</i> ^[221] , Liu <i>et al</i> ^[222] , Yeh <i>et al</i> ^[223] , Rischin <i>et al</i> ^[224] , Bentzen <i>et al</i> ^[225] , Rajendran <i>et al</i> ^[226] , Rajendran <i>et al</i> ^[227] , Lawrentschuk <i>et al</i> ^[228] , Loi <i>et al</i> ^[229] , Thorwarth <i>et al</i> ^[230]	
		Rasey <i>et al</i> ^[196]	Bruehlmeier <i>et al</i> ^[199] , Lawrentschuk <i>et al</i> ^[200] , O'Donoghue <i>et al</i> ^[201]	Oehler <i>et al</i> ^[208] , Cho <i>et al</i> ^[209] , Troost <i>et al</i> ^[210]	Riesterer <i>et al</i> ^[213]	Cherk <i>et al</i> ^[212]	Eschmann <i>et al</i> ^[271] , Gagel <i>et al</i> ^[272] , Koh <i>et al</i> ^[273]	Eschmann <i>et al</i> ^[271] , Gagel <i>et al</i> ^[272] , Koh <i>et al</i> ^[273]	
		Bentzen <i>et al</i> ^[196]	Bruehlmeier <i>et al</i> ^[199] , Lawrentschuk <i>et al</i> ^[200] , O'Donoghue <i>et al</i> ^[201]	Oehler <i>et al</i> ^[208] , Cho <i>et al</i> ^[209] , Troost <i>et al</i> ^[210]	Riesterer <i>et al</i> ^[213]	Cherk <i>et al</i> ^[212]	Eschmann <i>et al</i> ^[271] , Gagel <i>et al</i> ^[272] , Koh <i>et al</i> ^[273]	Eschmann <i>et al</i> ^[271] , Gagel <i>et al</i> ^[272] , Koh <i>et al</i> ^[273]	
[¹⁸ F]FRP170	[¹⁸ F]FIMO	Troost <i>et al</i> ^[198]	O'Donoghue <i>et al</i> ^[201] , Piert <i>et al</i> ^[202] , Sørensen <i>et al</i> ^[203] , Carlin <i>et al</i> ^[204] , Chang <i>et al</i> ^[204] , Mortensen <i>et al</i> ^[207]	Matsumoto <i>et al</i> ^[136] , Troost <i>et al</i> ^[138] , Dubois <i>et al</i> ^[211]	Lehmann <i>et al</i> ^[214] , Chen <i>et al</i> ^[215] , Campanile <i>et al</i> ^[216] , Cheng <i>et al</i> ^[217] , Sato <i>et al</i> ^[218] , Norikane <i>et al</i> ^[219]	Rasey <i>et al</i> ^[200] , Vera <i>et al</i> ^[183]	Rajendran <i>et al</i> ^[230] , Rischin <i>et al</i> ^[231] , Thorwarth <i>et al</i> ^[232] , Zimny <i>et al</i> ^[233] , Eschmann <i>et al</i> ^[233] , Gagel <i>et al</i> ^[232] , Thorwarth <i>et al</i> ^[234]	Rajendran <i>et al</i> ^[230] , Rischin <i>et al</i> ^[231] , Thorwarth <i>et al</i> ^[232] , Zimny <i>et al</i> ^[233] , Eschmann <i>et al</i> ^[233] , Gagel <i>et al</i> ^[232] , Thorwarth <i>et al</i> ^[234]	
		NA	NA	NA	NA	NA	Cherk <i>et al</i> ^[212]	Thorwarth <i>et al</i> ^[235] , Lee <i>et al</i> ^[236] , Lin <i>et al</i> ^[237] , Nehmeh <i>et al</i> ^[238] , Roels <i>et al</i> ^[239] , Dirix <i>et al</i> ^[240] , Mortensen <i>et al</i> ^[241] , Wang <i>et al</i> ^[242]	Thorwarth <i>et al</i> ^[235] , Lee <i>et al</i> ^[236] , Lin <i>et al</i> ^[237] , Nehmeh <i>et al</i> ^[238] , Roels <i>et al</i> ^[239] , Dirix <i>et al</i> ^[240] , Mortensen <i>et al</i> ^[241] , Wang <i>et al</i> ^[242]
		NA	NA	Busk <i>et al</i> ^[174]	NA	NA	Abolmaali <i>et al</i> ^[242] , Bowen <i>et al</i> ^[243] , Eary <i>et al</i> ^[243] , Kikuchi <i>et al</i> ^[244]	Abolmaali <i>et al</i> ^[242] , Bowen <i>et al</i> ^[243] , Eary <i>et al</i> ^[243] , Kikuchi <i>et al</i> ^[244]	Abolmaali <i>et al</i> ^[242] , Bowen <i>et al</i> ^[243] , Eary <i>et al</i> ^[243] , Kikuchi <i>et al</i> ^[244]
[¹⁸ F]JH4	[¹⁸ F]JH4	Dubois <i>et al</i> ^[61]	NA	Dubois <i>et al</i> ^[61]	Chen <i>et al</i> ^[215]	Chen <i>et al</i> ^[215]	van Loon <i>et al</i> ^[253]	Hugonnet <i>et al</i> ^[151] , Yamane <i>et al</i> ^[151] , Mammari <i>et al</i> ^[246] , Zips <i>et al</i> ^[247]	Hugonnet <i>et al</i> ^[151] , Yamane <i>et al</i> ^[151] , Mammari <i>et al</i> ^[246] , Zips <i>et al</i> ^[247]
		NA	NA	NA	NA	NA	Chen <i>et al</i> ^[215]	Chen <i>et al</i> ^[215]	Chen <i>et al</i> ^[215]
		NA	NA	NA	NA	NA	Chen <i>et al</i> ^[215]	Chen <i>et al</i> ^[215]	Chen <i>et al</i> ^[215]
[¹²⁴ I]IAZA	[¹²⁴ I]IAZG	Reischl <i>et al</i> ^[165]	NA	NA	NA	Reischl <i>et al</i> ^[165]	Zegers <i>et al</i> ^[248]	Cheng <i>et al</i> ^[247] , Henriques de Figueiredo <i>et al</i> ^[248] , Okamoto <i>et al</i> ^[249]	Cheng <i>et al</i> ^[247] , Henriques de Figueiredo <i>et al</i> ^[248] , Okamoto <i>et al</i> ^[249]
		NA	Zanzonico <i>et al</i> ^[254]	NA	NA	Riedl <i>et al</i> ^[255]	van Loon <i>et al</i> ^[253]	Sato <i>et al</i> ^[218] , Segard <i>et al</i> ^[250] , Tachibana <i>et al</i> ^[251] , Norikane <i>et al</i> ^[250]	Sato <i>et al</i> ^[218] , Segard <i>et al</i> ^[250] , Tachibana <i>et al</i> ^[251] , Norikane <i>et al</i> ^[250]
		NA	NA	NA	NA	NA	NA	NA	NA

¹Refer to Table 2 for full tracer names; ²Preclinical and clinical studies comparing the uptake of the hypoxia tracer under study with other hypoxia markers; ³Excluding brain studies. BC: Induced hypoxia by breathing conditions; Probe: Polarographic electrode; Ex-M: Exogenous hypoxia marker (pimonidazole, EF3 or EF5); En-M: Endogenous hypoxia marker (HIF-1, CA IX); FMISO: [18F]FMISO PET; NA: Not available.

Table 2 Hypoxia positron emission tomography tracer abbreviations

Abbreviation	Full name or chemical name
[*Cu]ATSM	[*Cu]-diacetyl-bis(N4-methylthiosemicarbazone)
[¹⁸ F]EF1	2-(2-Nitroimidazol-1H-yl)-N-(3-[¹⁸ F]fluoropropyl)acetamide
[¹⁸ F]EF3	2-(2-Nitroimidazol-1H-yl)-N-(3,3,3-[¹⁸ F]trifluoropropyl)acetamide
[¹⁸ F]EF5	2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-[¹⁸ F]-pentafluoropropyl)-acetamide
[¹⁸ F]FAZA	[¹⁸ F]fluoroazomycin arabinoside
[¹⁸ F]FETA	[¹⁸ F]fluoroetanidazole
[¹⁸ F]FETNIM	[¹⁸ F]fluoroerythronitroimidazole
[¹⁸ F]FMISO	[¹⁸ F]fluoromisonidazole
[¹⁸ F]FRP170	1-(2-[¹⁸ F]fluoro-1-[hydroxymethyl]ethoxy)methyl-2-nitroimidazole
[¹⁸ F]FPIMO	[¹⁸ F]pimonidazole
[¹⁸ F]HX4	3-[¹⁸ F]fluoro-2-(4-((2-nitro-1H-imidazol-1-yl)methyl)-1H-1,2,3,-triazol-1-yl)-propan-1-ol
[¹²⁴ I]IAZA	[¹²⁴ I]iodazomycin arabinoside
[¹²⁴ I]IAZG	[¹²⁴ I]iodazomycin galactoside

not require a cyclotron on-site and are more suitable for the clinical setting. Remarkably, ⁶⁴Cu can also be applied as radiation therapy agent, since it also emits a β⁻ particle (40% yield)^[64,65]. In oncology, [Cu]ATSM has been evaluated both preclinically and clinically. This tracer shows favorable kinetics with rapid uptake in hypoxic tissue and fast clearance from normoxic tissues, enabling imaging within 30 min after injection^[66,67]. However, the exact uptake mechanism of [Cu]ATSM is still under debate^[63,68,69] and several preclinical studies have shown that [Cu]ATSM uptake depends on tumor type and other characteristics than hypoxia alone^[70-76].

Clinical evaluation of hypoxia PET tracers in lung cancer

Hypoxia PET imaging is in development and most clinical studies have been focused on notoriously hypoxic cancer types such as cervical cancer and head and neck cancer. Nevertheless, several clinical PET studies have evaluated hypoxia imaging in lung cancer (Table 3). In the following paragraphs, data acquisition, quantification and clinical observations of these hypoxia tracers will be discussed.

Data acquisition and analysis: Nitroimidazole based tracers require relatively long time intervals for accumulation in hypoxic cells and clearance from normoxic cells. In concordance, most studies used images > 2 h after injection for hypoxia assessment. The length of the time interval between injection and imaging may affect the tracers' distribution pattern in tumors. For example, it has been shown that the distribution of [¹⁸F]FMISO at 2 h is significantly different from the distribution at 4 h, whereas only the 4 h data are predictive of tumor recurrence^[77]. In contrast, the distribution of [¹⁸F]HX4 was similar at 2 h and 4 h^[78]. Compared to nitroimidazole based tracers, [Cu]ATSM shows fast kinetics and images were acquired after time intervals as short as 10 min after injection^[67,79-81].

Quantification of hypoxia: To identify hypoxia in tumor tissue, several simplified parameters have been used, including tumor-to-blood ratio, tumor-to-background ratio, tumor-to-muscle ratio, tumor-to-mediastinum ratio, and SUV. In addition, several studies have used dynamic PET scans to investigate the tracers' kinetics in more

detail, for example by using pharmacokinetic modeling for quantification^[59]. Furthermore, consecutive imaging using multiple tracers has been performed to facilitate the identification and quantification of hypoxia. For example, consecutive PET scans have been performed with hypoxia tracer [Cu]ATSM and perfusion tracer copper-pyruvaldehyde-bis(N4-methylthiosemicarbazone ([Cu]PTSM). Here, the ratio of [Cu]ATSM SUV to [Cu]PTSM SUV has been used as a measure of hypoxia^[81].

To date, it is not known which measure and threshold accurately reflects pO₂ levels in tumors. As repeated measurements with a polarographic electrode are not feasible in lung cancer, a more pragmatic approach is required. The clinical relevance of a threshold can be determined by clinical parameters like tumor response, progression-free survival and overall survival.

Determination of clinically relevant hypoxia: Among the clinical studies on hypoxia PET tracers in lung cancer, most studies have only evaluated tumor hypoxia prior to treatment. For [¹⁸F]FMISO, a pretreatment threshold of > 2 for tumor-to-mediastinum ratio was associated with poor outcome after radiation therapy. However, the shape of the time activity curve appeared to be a better predictor of response^[77]. In contrast to these results, other authors did not find a predictive value for [¹⁸F]FMISO after chemoradiation^[82,83]. In other patients treated with chemoradiation, a threshold of tumor-to-blood ratio > 1.9 for [¹⁸F]FETNIM^[84,85] and > 3.0 for [Cu]ATSM^[86] was associated with poor overall survival and tumor response, respectively. In addition, a number of studies have evaluated the changes in hypoxia tracer uptake during therapy. While hypoxic cells are considered to be more resistant to radiation therapy, most studies in lung cancer reported a decrease in hypoxia tracer uptake after radiation therapy^[58,82,87].

PET FOR TUMOR PERFUSION MEASUREMENTS

Tumor angiogenesis

Blood flow is not only required for the delivery of PET tracers and anticancer drugs to tumors, but also for the

Table 3 Hypoxia tracer studies in lung cancer

Tracer ¹	Year	Authors	N ²	Stage	Time ³	Duration ⁴	Measure ⁵	Therapy ⁶
[⁶⁰ Cu]ATSM	2003	Dehdashti <i>et al</i> ^[86]	18	I -IV	30 min	30 min	T/M	Radiation Chemoradiation, chemotherapy
[⁶² Cu]ATSM	2000	Takahashi <i>et al</i> ^[79]	6	NA	10 min	10 min	T/B	
	2008	Wong <i>et al</i> ^[80]	2	NA	15 min	5 min	SUV	
	2009	Lohith <i>et al</i> ^[67]	13	I -IV	10 min	10 min	SUV _{mean}	
	2013	Zhang <i>et al</i> ^[81]	5	I -IV	15 min	5 min	SUV _{hypoxia/perfusion} ⁷	
[¹⁸ F]FAZA	2009	Postema <i>et al</i> ^[180]	13	NA	2-3 h	3-4 min	T/Bg	Chemoradiation
	2013	Trinkaues <i>et al</i> ^[58]	11	III	4 h	30 min	T/Bg	
	2013	Bollineni <i>et al</i> ^[181]	11	III-IV	2 h	NA	T/Bg	
	2013	Verwer <i>et al</i> ^[59]	9	NA	0 h	70 min ⁸	V _t ⁹	
[¹⁸ F]FETNIM	2010	Li <i>et al</i> ^[84]	26	III	2 h	20 min	T/B _{max}	Radiation Chemotherapy
	2013	Hu <i>et al</i> ^[85]	25	II	2 h	NA	T/Me	Chemotherapy
[¹⁸ F]FMISO	1995	Koh <i>et al</i> ^[87]	14	III	2 h	40 min	T/B	Radiation
	1996	Rasey <i>et al</i> ^[220]	21	III-IV	2 h	40 min	T/B	Radiation
	2005	Eschmann <i>et al</i> ^[77]	8	III-IV	4 h	NA	T/Me	
	2006	Cherk <i>et al</i> ^[212]	21	I -II	2 h	NA	SUV _{max}	
	2006	Gagel <i>et al</i> ^[82]	8	III-IV	3 h	30 min	SUV, T/M	Chemoradiation
	2011	Vera <i>et al</i> ^[83]	7	III	3 h	NA	SUV _{max}	Chemoradiation Chemotherapy
[¹⁸ F]FRP170	2007	Kaneta <i>et al</i> ^[252]	3	NA	0 h	60 min ⁸	SUV TAC	
[¹⁸ F]HX4	2010	van Loon <i>et al</i> ^[253]	4	IV	2 h	NA	T/B	
	2013	Zegers <i>et al</i> ^[78]	15	II-IV	4 h	30 min	T/B	

¹Refer to Table 2 for full tracer names; ²Number of lung cancer patients (evaluable scans); ³Start time after injection of the positron emission tomography (PET) frame that was used for quantification; ⁴Duration of the PET frame that was used for quantification; ⁵(Semi)quantitative measure used for evaluation; ⁶Evaluated therapy; ⁷Hypoxia marker uptake normalized to perfusion marker uptake: mean (SUV[Cu]ATSM/SUV[Cu]PTSM); ⁸Dynamic PET data used for quantification; ⁹Volume of distribution derived from full pharmacokinetic modeling. NA: Data not available; SUV: Standardized uptake value; T/M: Tumor-to-muscle ratio; T/Me: Tumor-to-mediastinum ratio; T/B: Tumor-to-blood ratio; T/Bg: Tumor-to-background ratio; VT: Volume of distribution; TAC: Time activity curve.

transport of nutrients, *e.g.*, glucose, and oxygen. Under hypoxic conditions in tumors, the HIF protein is usually up-regulated. Activated HIF translocates to the nucleus of tumor cells and results in transcription of a large repertoire of genes including VEGF^[88,89]. VEGF is a potent protein and plays a key role in tumor angiogenesis, which is the formation of new blood vessels. This tumor angiogenesis is essential for tumor growth, metastatic spread and survival of tumor cells. As a result, VEGF signaling has become an important therapeutic target for the treatment of malignant tumors. To date, several antiangiogenic drugs have been developed including monoclonal antibodies that bind circulating VEGF (*e.g.*, bevacizumab^[90]) and tyrosine kinase inhibitors that target the intracellular domain of the VEGF receptors (*e.g.*, sunitinib and sorafenib^[91]). Among the currently available antiangiogenic drugs, bevacizumab has been registered for the treatment of patients with NSCLC. In combination with paclitaxel and carboplatin, bevacizumab has been approved for first-line treatment of non-squamous NSCLC^[15]. As tumor vascularization is an important factor in the biology of malignant tumors, and antiangiogenic strategies have been introduced for the treatment of lung cancer, imaging techniques are increasingly used for perfusion measurements in lung cancer.

Imaging of tumor perfusion

PET is a sensitive technique to quantify tumor perfusion^[92]. To this end, perfusion tracers like rubidium-82 (⁸²Rb^[93]), radioactive ammonia ([¹³N]NH₃^[94]), radioactive water ([¹⁵O]H₂O^[95-101]) can be administered. Other PET tracers such as [⁶⁸Ga]transferrin^[102] and [¹¹C]methylalbumin^[103] are available to assess vascular permeability. Currently, experience with perfusion PET tracers is rather limited in oncology, except for [¹⁵O]H₂O. In particular, previous PET studies have shown that quantification of tumor perfusion using [¹⁵O]H₂O is feasible in patients with lung cancer^[97,104,105].

[¹⁵O]H₂O PET

As [¹⁵O]H₂O is a freely diffusible tracer with near 100% extraction over a wide perfusion range (0-6 mL/min per mL), its kinetics directly reflect tumor perfusion. As a result, [¹⁵O]H₂O is an ideal tracer for quantitative perfusion imaging. The short half-life of ¹⁵O, which is 2.03 min, enables sequential PET scans using both [¹⁵O]H₂O and another tracer, *e.g.*, [¹⁸F]FDG^[97] or a hypoxia tracer^[106]. However, it requires the presence of a nearby cyclotron. Because [¹⁵O]H₂O is metabolically inert and is not retained in cells, quantification using SUV, which is a parameter for quantification of irreversible uptake, is not

possible. Instead, pharmacokinetic modeling, using short (< 10 min) dynamic PET scans, is required to quantify tumor perfusion.

Monitoring tumor perfusion during treatment

Currently, [^{15}O]H $_2\text{O}$ PET scans are increasingly used to assess response of the tumor vasculature to antiangiogenic therapy^[107-110]. As [^{15}O]H $_2\text{O}$ PET has shown high reproducibility in lung cancer^[105], it can be applied for response monitoring during treatment. de Langen *et al.*^[111] have investigated changes in tumor perfusion in 44 NSCLC patients who were treated with bevacizumab and erlotinib. Three weeks after the start of treatment, a mean decrease of 11% in tumor perfusion was measured using [^{15}O]H $_2\text{O}$ PET^[111]. A significant reduction in tumor perfusion was measured in patients with a partial response according to the response evaluation criteria in solid tumors (RECIST^[112]). More importantly, patients with > 20% reduction in tumor perfusion had an improved progression-free survival as compared to other patients (12.5 mo *vs* 2.9 mo). The latter findings indicate that [^{15}O]H $_2\text{O}$ PET may have predictive value in lung cancer patients who are treated with antiangiogenic drugs. For early prediction of tumor response, early perfusion measurements may be useful, as the effects of antiangiogenic can be very rapid^[113].

Tumor perfusion and drug delivery

As the short half-life of ^{15}O enables sequential PET scans using both [^{15}O]H $_2\text{O}$ and an additional tracer, [^{15}O]H $_2\text{O}$ PET is a useful tool to investigate drug delivery of radiolabeled anticancer agents by correlating uptake of radiolabeled drugs with [^{15}O]H $_2\text{O}$ perfusion data^[114,115]. Apparently, it has been shown that tumor perfusion is an important determinant of drug tumor exposure, as indicated by several PET studies on [^{18}F]5-fluorouracil(FU)^[116-118], [^{11}C]DACA^[119], and [^{11}C]docetaxel^[120,121]. Consequently, tumor perfusion may be predictive of tumor response to the above mentioned anticancer drugs. These findings advocate further studies investigating the predictive value of tumor perfusion for tumor response to chemotherapy. As tumor perfusion is the key factor for the uptake of several anticancer drugs in tumors^[122], antiangiogenic drugs may affect drug exposure in tumors. To investigate this concept, a PET study has been performed in NSCLC patients using both [^{15}O]H $_2\text{O}$ and the radiolabeled taxane [^{11}C]docetaxel^[113]. In that study, bevacizumab reduced both perfusion and net influx rate of [^{11}C]docetaxel within 5 h. These rapid effects persisted after 4 d and were not associated with significant changes in tumor heterogeneity. The mentioned studies indicate that [^{15}O]H $_2\text{O}$ PET may reveal the role of perfusion in drug delivery and antiangiogenic therapy in malignant tumors^[123].

IMAGING HYPOXIA AND PERFUSION

It is conceivable that the development of tumor hypoxia is associated with a decrease in tumor perfusion. This may complicate PET imaging, as tracer delivery will be

reduced in these areas. Although the uptake of the ideal hypoxia tracer is not directly related to perfusion, lack of perfusion will limit tracer delivery.

Diffusion-limited hypoxia is present in tumor cells located away from capillaries, *i.e.*, further than the diffusion distance of oxygen. As perfusion is relatively low in these areas, tracer delivery may be limited and this may, in turn, affect uptake of hypoxia tracers. In addition, low perfused areas can become necrotic. The PET signal will be decreased in areas containing necrosis even though these areas may also contain highly hypoxic cells. In Figure 4, these hypothetical considerations are summarized. The figure also illustrates the limitations of using a predefined threshold to delineate hypoxic areas on a PET image, as areas likely to contain the most severely hypoxic cells will be missed. The mentioned considerations may explain the conflicting results between the uptake of hypoxia tracers and the direct assessment of tissue oxygenation using polarographic electrodes^[124-127]. [^{15}O]H $_2\text{O}$ PET may help to understand these conflicting results and may identify the remote, low perfused areas. An example of images obtained from consecutive perfusion and hypoxia PET imaging is displayed in Figure 5.

Acute hypoxia is directly caused by a (temporary) lack of tumor perfusion. Since acute hypoxia is presumed to be transient or even cycling^[128], hypoxia tracer uptake may not accurately reflect this type of hypoxia. [^{15}O]H $_2\text{O}$ PET may help to study the effect of acute hypoxia and its relation with hypoxia tracer uptake.

Besides the previous considerations for combining [^{15}O]H $_2\text{O}$ perfusion PET imaging with hypoxia tracer PET imaging, the combination may provide further insight into the effects of treatment. Jain has previously proposed that antiangiogenic therapy may normalize the abnormal tumor vasculature, thereby decreasing tumor hypoxia and improving drug delivery of cytotoxic agents^[129,130]. This is underscored by the fact that a decrease in [^{18}F]FMISO uptake has been measured in renal cell cancer after treatment with sunitinib^[131]. On the other hand, an increase in [^{18}F]FMISO uptake after sorafenib^[132] and a rapid decrease in tumor perfusion after bevacizumab have been reported as well^[113]. The latter findings suggest that antiangiogenic therapy may decrease tumor perfusion and subsequently the delivery of hypoxia tracers to tumors. To further clarify these findings, future PET studies need to combine hypoxia tracers with [^{15}O]H $_2\text{O}$ at different time points after drug administration.

FUTURE PERSPECTIVES

In the present review, the currently available tracers for PET imaging of hypoxia and perfusion in lung cancer patients were discussed. Considering the currently available studies, PET seems feasible to assess hypoxia and perfusion in lung cancer. In contrast to traditional probe measurements, PET hypoxia imaging is non-invasive and provides information on the heterogeneous distribution

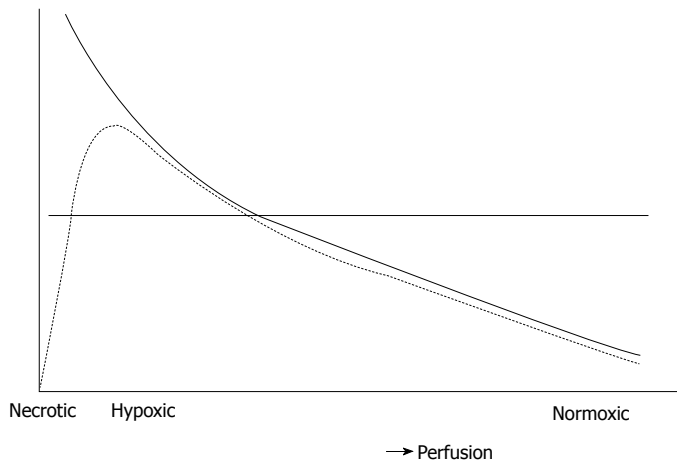


Figure 4 Representation of hypothetical considerations on the link between perfusion and the hypoxia signal as measured by imaging positron emission tomography. The continuous curve represents hypothetical level of hypoxia in tissue for increasing levels of perfusion (i.e., closer to the capillaries). The dotted line represents the positron emission tomography signal obtained from hypoxia imaging using an optimal imaging protocol. The horizontal line represents a threshold used for delineation of hypoxic areas.

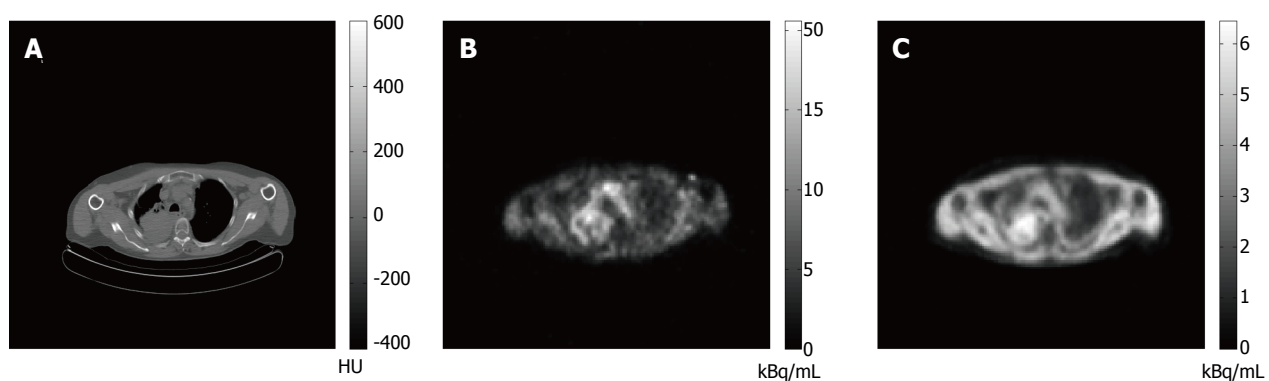


Figure 5 Example of consecutive perfusion and hypoxia positron emission tomography in a patient with non-small cell lung cancer. A: Low dose computed tomography; B: Perfusion image (averaged image acquired over time interval 30-120 s after injection of 370 MBq [^{15}O]H $_2$ O); C: Hypoxia image (averaged image over time interval 40-70 min after injection of 185 MBq [^{18}F]FAZA).

of hypoxia in tumors. In addition, whole body PET scans using a hypoxia tracer can reveal hypoxic areas not only in primary tumors, but in metastases as well. To date, several tracers have been developed to measure tumor hypoxia, whereas tumor perfusion has been mostly quantified using [^{15}O]H $_2$ O. While acquisition and quantification of [^{15}O]H $_2$ O data is rather straightforward, several challenges remain for PET hypoxia tracers.

Although several hypoxia tracers have been developed and evaluated in the clinical setting, no consensus has yet been reached on the most feasible tracer, the optimal timing of acquisition, and the most accurate quantification method. In lung cancer, the studies on hypoxia PET tracers are preliminary and include a limited number of patients. Ideally, the clinical impact of hypoxia imaging would be evaluated in large clinical trials validating hypoxia tracers for prediction of tumor response and survival. In addition, clinical trials are needed to reveal the clinical value of hypoxia tracers for advanced radiation therapy strategies such as dose painting. In NSCLC, several trials are currently recruiting patients for [^{18}F]FMISO based (NCT01576796) and [^{18}F]FDG based (NCT01024829) dose boosting.

In patients with lung cancer, quantification of tracer uptake can be challenging due to tumor movement during respiration. As PET acquisition usually takes 10min

to 1 h, patient motion during PET imaging is unavoidable and the acquired image of the lung tumor will be blurred, which complicates accurate delineation of hypoxic areas. As a result, these images are less suitable for dose painting techniques, especially for dose painting by numbers. For PET imaging, respiratory gated imaging (4D imaging) is currently under study. In respiratory gated imaging, patient motion is continuously monitored during acquisition. As a result, PET data can either be corrected for the registered motion or PET data from a specific interval of the respiratory cycle can be used for reconstruction. As similar techniques are also under study for radiation therapy, dose painting strategies may be further improved by combining 4D PET hypoxia imaging with 4D radiation therapy.

Since the introduction of antiangiogenic drugs, perfusion measurements have been increasingly applied in the clinic. [^{15}O]H $_2$ O PET provides quantification of tumor perfusion and may be useful for response monitoring during antiangiogenic therapy. Further studies are needed to evaluate the predictive value of tumor perfusion for tumor response to anti-cancer drugs. In addition, tumor perfusion may not only affect the delivery of drugs to tumors, but also the delivery of PET tracers such as hypoxia tracers.

In conclusion, PET using both [^{15}O]H $_2$ O and a hy-

poxia tracer is a promising method to further understand the development of hypoxia in lung cancer. As previously mentioned, these PET scans are promising for response monitoring of radiation therapy and antiangiogenic drugs. In addition, hypoxia tracers may be useful to select patients for treatment with radiosensitizers (*e.g.*, nimorazole, NCT01733823) and realize a more precise radiation plan including dose boosting and dose painting. As the available PET studies on hypoxia and perfusion are rather preliminary in patients with lung cancer, further studies are needed for validation and clinical implementation in this patient population.

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WJCO 5th Anniversary Special Issues (1): Lung cancer**Palliative radiotherapy for bone metastases from lung cancer: Evidence-based medicine?**

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Abstract

To review current recommendations for palliative radiotherapy for bone metastases secondary to lung cancer, and to analyze surveys to examine whether global practice is evidence-based, English language publications related to best practice palliative external beam radiotherapy (EBRT) for bone metastases (BM) from lung cancer were sought *via* literature search (2003-2013). Additional clinical practice guidelines and consensus documents were obtained from the online Standards and Guidelines Evidence Directory. Eligible survey studies contained hypothetical case scenarios which required participants to declare whether or not they would administer palliative EBRT and if so, to specify what dose fractionation schedule they would use. There is no convincing evidence of differential outcomes based on histology or for spine *vs* non-spine uncomplicated BM. For uncomplicated BM, 8Gy/1 is widely recommended as current best practice; this schedule would be used by up to 39.6% of respondents to treat a painful spinal lesion. Either 8Gy/1 or 20Gy/5 could be considered standard palliative RT for BM-related neuropathic pain; 0%-13.2% would use the former and 5.8%-52.8% of respondents the latter (range 3Gy/1-45Gy/18). A multifraction schedule is the approach of choice for irradiation of impending

pathologic fracture or spinal cord compression and 54% would use either 20Gy/5 or 30Gy/10. Survey results regarding management of complicated and uncomplicated BM secondary to lung cancer continue to show a large discrepancy between published literature and patterns of practice.

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Key words: Bone metastases; Lung cancer; Survey; Evidence-based practice; Radiotherapy

Core tip: Palliative radiotherapy (PRT) remains the gold standard for treatment of painful bone metastases from lung cancer. While PRT should be appropriately customized to patients, prescription should also be based on robust evidence. Depending on the clinical scenario, between 4%-66% of survey respondents would use dose-fractionation schedules considered congruent with best available current evidence. These results show a large discrepancy between treatment guidelines and international patterns of practice. It is not completely clear why level 1 data supporting specific dose schedules continues to be overlooked, although reasons for reticence in following these recommendations are reviewed.

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INTRODUCTION

Lung cancer causes bone metastases (BM) in a large proportion of patients, up to 40%-80%^[1]. With improvements

in systemic therapy, median survival of patients with advanced lung cancer is now approximately 12 mo; as such, the prevalence of BM has significantly increased. BM can be described as complicated or uncomplicated, where uncomplicated generally refers to the absence of: impending or established pathological fracture (PF), surgical fixation, impending or established spinal cord compression (SCC), impending or established cauda equina or nerve root compression, neuropathic pain, previous RT, or associated soft tissue mass.

By definition, all patients with lung cancer and BM have stage IV disease and treatment is palliative. Goals for the treatment of BM are pain relief, preservation of mobility and function, prevention of future complications, optimized quality of life (QoL), maintenance of skeletal integrity, and minimization of hospitalization. About half of all patients with stage IV non-small cell lung cancer (NSCLC) receive at least one course of palliative external beam radiotherapy (EBRT) within 15 mo of diagnosis^[2].

A prospective observational multicenter study recently documented treatment costs of BM in patients with pathologically-proven lung cancer during the first year after BM diagnosis^[3]. Five hundred and fifty-four patients with radiologically-proven BM were enrolled. A mean monthly cost was calculated using a Markov approach, taking into account direct costs only (hospitalization, drug purchases, medical and transport costs). Indirect costs such as lost income and intangible costs such as pain and suffering were not assessed. 76.5% were male, with a mean age of 62 years, 9% with small cell lung cancer, 69.3% with PS 0-1, and 64.6% had metastases other than to bone. At some point during follow-up, 89.9% had analgesic treatment (77.7% with opioids) and 42.1% had RT. Median survival was 5.8 mo and 1 year OS was 22%. Factors predictive of better OS were adenocarcinoma, performance status (PS) 0-1, and female. Monthly costs for asymptomatic and symptomatic BM were €190 and €374 respectively. Mean disease management costs during the first year after BM diagnosis were €3999 +/- €4135 (95% confidence interval €374-15886)^[3].

EBRT remains the gold standard for treatment of BM^[4], and comprises the largest single component of palliative RT practice^[5], approximately 40%^[6]. The number of lung cancer patients requiring palliative EBRT for BM is continuing to grow, increasing pressure on clinicians, simulators, and treatment units, in addition to health care costs.

Both the limited survival of most patients with advanced lung cancer and a requirement for the judicious use of resources argue for use of the shortest dose fractionation schedule which is effective^[7]. Single fraction (SF) EBRT for palliation of painful BM has many advantages: the risk of acute side effects is minimized which increases patient QoL and acceptance of treatment; RT can be delivered over as little as one day, which decreases transportation and hospital admission requirements; it is more convenient; decreased discomfort with positioning

and travel increases tolerance for those with poor PS; and it frees resources for others. SFRT is cost-effective, and is easier to schedule amongst systemic therapy and other appointments, resulting in increased accessibility and decreased waiting times^[7-17].

Previous patterns of practice surveys focusing on EBRT for BM, including a large international study from 2009, have suggested that considerable controversy over the optimal treatment schedule still exists^[9]. The objectives of this review were to (1) examine the literature supporting current treatment recommendations for BM secondary to lung cancer, to determine for which clinical scenarios SFRT is appropriate; and (2) analyze published surveys to examine evolution in practice and degree to which it can be considered evidence-based.

LITERATURE SEARCH

Publications related to best practice palliative EBRT for complicated and uncomplicated BM from lung cancer were sought *via* literature search (Table 1). Medline and Embase were searched for English language articles published in full between 2003 and 2013. Eligible studies were also identified from reference lists of retrieved papers and review articles. Additional clinical practice guidelines and consensus documents were obtained from searching the online SAGE (Standards and Guidelines Evidence) Directory compiled by the Canadian Partnership Against Cancer (www.cancerview.ca). Eligible survey studies contained hypothetical case scenarios which required participants to declare whether or not they would administer palliative EBRT and if so, to specify what dose fractionation schedule they would use. Retrospective reviews and practice audits which did not include hypothetical patient cases^[18-25] as well as surveys which did not include lung cancer cases^[26-31] were excluded.

RESEARCH

Evidence summary-uncomplicated bone metastases

Four meta-analyses of more than 20 randomized controlled trials [level I evidence, according to the Oxford Centre for Evidence-Based Medicine (www.cebm.net)] have shown that for uncomplicated bone lesions, there is no advantage in degree of pain relief, analgesic use, time to first improvement in pain, time to complete pain relief, time to pain progression, duration of response, rates of PF or SCC, acute toxicity, QoL, or overall survival from protracted fractionation *vs* SFRT above 4Gy^[8,12,13,15]. The randomized trials accrued patients with various histologies; selected studies published since 2000 included up to 26% of patients with lung cancer. The most recent meta-analysis evaluated 25 studies comprising 2818 randomizations to SF and 2799 to MF arms, respectively^[8]. On an intent-to-treat basis, the overall response rate to SF RT was 60%, and complete response (CR) rate was 23%, which was not significantly different from the 61% and 24% rates of patients randomized to MF RT. The overall

Table 1 Search strategy

Step	String	Results
1	Exp Bone Neoplasms/sc OR Bone Neoplasms/rt OR [(boneCarcinoma/sc OR exp Neoplasm Metastasis/rt) AND exp 26956 "Bone and Bones"/] OR [(osseous OR skeletal) ADJ3 metastasis\$] OR (bone ADJ3 metastasis\$)	
2	Exp *Lung Neoplasms/OR exp Lung Neoplasms/pa OR lung cancer.mp	188189
3	Exp Radiotherapy/OR exp Dose Fractionation/OR exp Radiotherapy Dosage/	133052
4	Exp Palliative Care/OR exp Pain/RT OR Pain Management/mt OR (palliative OR palliation).mp OR painful adj3 metastasis\$ OR (palliative\$ ADJ3 radiotherapy).mp	94404
5	1 AND 2 AND 3 AND 4	88
		Limit 10 yr: 28
6	1 AND 3 AND 4	608
	Exclusion criteria: non-lung primary site cancers	Limit 10 yr: 256
7	(1 AND 4 AND radiotherapy.mp AND combined modality therapy/OR analgesics/OR treatment outcome/OR quality of life/OR exp Antineoplastic Combined Chemotherapy Protocols) NOT 6	129
		Limit 10 yr: 50
8	Total	156

pain response proportion increases by about 10% if per-protocol patients only are analyzed^[15]. Median time to onset of pain relief is 1-4 wk and median duration of response is 12-24 wk. After SF, 3.3% of patients fractured *vs* 3.0% after MF ($P = 0.72$). Two point eight percent of patients receiving SF and 1.9% of patients receiving MF experienced SCC ($n = 6$ trials; $P = 0.13$). There were significantly more retreatment episodes in the SF arms (20%) *vs* MF arms (8%) ($P < 0.00001$). Re-analysis of assessable patients did not alter conclusions. There is a trend for greater acute toxicity after MF which is not statistically significant, and which was not a primary endpoint for any of the source trials^[8].

In view of mounting evidence, 8Gy/1 has been repeatedly recommended as standard of care for uncomplicated BM in the practice guidelines of many international bodies, including specifically for lung cancer (see Table 2 for references; level 5 evidence). A secondary analysis of the Dutch trial reported that in patients with spine BM, SF and MF EBRT result in equivalent pain relief (level 1 evidence)^[32]. In general, neither anatomic location treated nor RT dose is predictive of degree of functional improvement after RT (level 4 evidence)^[33,34]. No subgroups of patients with uncomplicated painful BM have been identified that clearly benefit from a higher total dose (level 5 evidence)^[11]. Additionally, it is not standard practice to prophylactically irradiate an asymptomatic uncomplicated BM (to avoid toxicity).

Evidence summary - does histology influence outcome?

The most detailed data on outcomes in relation to histology were reported in a secondary analysis of the Dutch Bone Metastases trial related to reirradiation (level 1 evidence)^[35]. In the original trial, between 03/1996 and 09/1998, 1157 Dutch patients with painful BM were randomized between 8Gy/1 ($n = 579$) and 24Gy/6 ($n = 578$); 287 lung patients were accrued^[16]. Patients had a minimum pain score of 2/10. Eligibility criteria included BM treatable in one RT target volume, no previous RT, no fracture or impending PF needing surgery, no SCC, and no BM within the cervical spine. Participants completed weekly questionnaires \times 13 wk, then monthly to

a maximum of 2 years, reporting maximum pain at the treated site, analgesic use and acute side effects. No major differences were reported between SF and MF in overall response rates, duration of response or progression rates^[16].

267/287 lung patients were assessable for response to initial EBRT: 107 (39.8%) were non-responders and 162 (60.2%) were responders^[35]. Fifty-eight percent responded after initial SF and 62% after initial MF. Mean time to initial response was 3 wk and mean duration of remission was 11 wk. Overall there was no correlation between histology and initial response ($P = 0.69$). Of the 267, 78 experienced pain progression (29.2%).

Within the first year after randomization, 49 patients with lung cancer were retreated. Of initial nonresponders, 22% were retreated at a mean time of 10 wk and mean pain score of 7.7/10. Of initial responders, 7% were retreated at a mean time of 11 wk and mean pain score of 3.8/10. Of those who had progressed, 19% were retreated at a mean time of 5 wk and mean pain score of 7.2/10. Progressive patients with lung cancer were retreated most often and earliest after randomization of any histology^[35].

Response to retreatment could be ascertained for 40/49^[35]. Of those who did not respond to initial RT, 50% (8/16) responded to repeat treatment at a mean time to response of 7 wk. Of those responding to initial treatment, 67% (16/24) also responded to repeat treatment at a mean time of 5 wk. After progression, 12/14 (86%) responded to a second course of RT at a mean time of 4 wk. The mean duration of response in initial non-responders, responders and those with progressive pain was 8 wk, 12 wk and 6 wk, respectively. Including the effects of retreatment, overall response rates in lung cancer patients receiving SF increased from 58% to 62%, but did not change post-MF. This is likely because SF patients were retreated earlier than were MF patients. Almost three times more lung patients were retreated than breast patients (HR = 2.6; 95%CI: 1.7-3.8; $P < 0.001$), probably because they were less likely to both respond to initial RT and to receive systemic therapy. In multivariate analysis corrected for early death, primary tumour, PS and randomization arm remained predictive for retreat-

Table 2 Evidence supporting recommended dose-fractionation schedules

Indication	Recommended schedule	Selected references	Level of evidence
Uncomplicated bone metastases (spine, non-spine)	8Gy/1	[15]	1
Neuropathic pain	8Gy/1 or 20Gy/5	[10,32,39,41,60,61]	5
Impending SCC, RT alone	Multifraction	[38]	1
		[41]	5
		[36]	4
Impending pathologic fracture, RT alone	20-40Gy/5-20	[45]	1

RT: Radiotherapy; SCC: Spinal cord compression.

ment ($P = 0.01$, $P = 0.001$ and $P < 0.001$ respectively)^[35].

Results of other studies are contradictory: in one, lung cancer patients were less likely to experience an early pain response to EBRT [only 27% had responded by month two, *vs* 70% of breast and prostate cancer patients (level 4 evidence)^[34]], and in another, lung cancer patients had the highest response rate (level 4 evidence)^[36] (see below). Overall there is no convincing evidence that outcomes differ based on primary site as reported in the dose-finding randomized trials [reviewed by reference 6 (level 4 evidence)]. Similarly, none of the meta-analyses separated out treatment effects by histology (level 1 evidence)^[8,12,13,15].

Evidence summary - complicated bone metastases

Although neuropathic pain secondary to BM is not as predictively responsive to standard analgesics^[37], it does respond to RT^[38]. Roos *et al*^[38] compared a single 8 Gy *vs* 20 Gy/5 for 245 per-protocol patients with any primary site with BM causing neuropathic pain; 31% had lung cancer (level 1 evidence). Eligible patients had no other metastases along the distribution of the neuropathic pain, no cauda equina or SCC. Pain relief was seen in 53% of SF and 61% of MF patients (intent-to-treat) with 26%-27% experiencing CR at two months. Median time to treatment failure was longer in the fractionated arm (3.7 mo *vs* 2.4 mo), but, like the response rates, the difference did not reach statistical significance within the confidence limits set by this non-inferiority trial. Rates of SCC, PF and reirradiation were not significantly different. Therefore, for BM causing neuropathic pain, either 8Gy/1 or 20Gy/5 may be considered standard. The authors recommended the latter; however, patients with decreased PS, shorter expected survival or comorbidities, who would not be amenable to multiple hospital visits should receive SF^[38] (Table 2).

The treatment of an asymptomatic BM may be deferred unless the patient has a serious impending condition such as SCC or PF. Diagnosis of impending SCC requires radiologic evidence of indentation of the thecal sac at the level of local or radicular pain, without associated neurologic signs or symptoms^[39]. RT may prevent neurologic dysfunction in impending SCC^[40] although these patients were excluded from the vast majority of the clinical trials investigating RT doses for uncomplicated BM. There is insufficient evidence at present to

guide practice on the optimal dose schedule, although definitive EBRT for epidural tumour without neurological impairment, mechanical pain, or spinal instability should be fractionated (level 5 evidence)^[41]. Based on data from established SCC, MFRT may have advantages in terms of local control and/or in-field recurrence (level 1 evidence)^[8,42,43], and there is likely no advantage in offering more than 30Gy/10 (level 1 evidence)^[42] (Table 2).

An impending PF is defined as a BM that has a significant likelihood of fracture under normal physiological loads. In patients with lung cancer who have a painful BM affecting a weight-bearing bone, especially a solitary lytic lesion involving > 50% of the cortex circumferentially, an expected survival > 4 wk, and satisfactory health otherwise, surgical fixation is recommended (level 5 evidence)^[39]. Although at least one group has reported a marked increase in surgical involvement for metastatic bone lesions in recent years (level 4 evidence)^[44], a proportion of patients will not be candidates for operative intervention, or will decline. In that circumstance, multifraction RT should be delivered (level 1 evidence)^[45] (Table 2).

Harada *et al*^[36] reviewed results from a single institution to clarify the outcomes of RT for femoral BM (level 4 evidence). 72 consecutive patients (20.8% with a lung primary) with 84 femoral lesions (77/84 symptomatic) were treated (2002-2005). 39/84 lesions were lytic and 43/84 were considered impending PF. Median RT dose was 30Gy/10 (range 20-40Gy/5-20) and peri-RT systemic therapy was allowed. No reirradiation was performed. Median follow-up was five months (range 1-28 mo). Overall post-RT, 8 lesions achieved a radiological CR and 27 a radiological partial response (PR) on plain X-ray assessed independently by a radiologist and orthopedic oncologist. The best overall response rate (CR + PR) was 42% (35/84), with 30 lesions considered stable and 19 showing progressive disease. Of impending PF lesions, 15/43 showed a radiological response. Median interval from the start of RT to radiologic CR/PR was 3 mo and median duration was 10 mo. Administration of chemotherapy, hormone therapy or bisphosphonates significantly correlated with a favourable radiological response; RT dose and impending PF status did not. Response rates differed significantly based on primary site: lung cancer had the highest (65%) in comparison to breast (47%), prostate (42%) and other (28%) ($P =$

0.03). Eleven lesions eventually required surgery at a median of 3 mo of which 8 had actually fractured; seven of these had been classified initially as impending PF. Eventual fracture rate in the impending PF group (7/43; 16%) was significantly higher than in the no impending PF group (1/41; 2%) ($P = 0.03$). In the 77 symptomatic lesions at baseline, pain was classified as improved in 36, stable in 36 and as progressive in 5. There was no correlation between radiological response and pain relief ($P = 0.17$). Overall median survival was 7 mo (95%CI: 4-9 mo)^[36].

Results of survey studies

Since 1998, 12 hypothetical cases involving patients with lung cancer and BM have been reported in one abstract and five full publications^[6,9,46-49] (Table 3). Case histories included patients of both genders, ranging in age from 45-78 years, PS 1-2, previously treated with radical surgery +/- adjuvant chemotherapy or curative chemoRT, or metastatic at diagnosis. Histologic subtype of lung cancer was usually unspecified. Investigations diagnosing BM varied as did extent of non-osseous metastases. One publication described current pain score and response to analgesics^[47]. Overall, eight cases were of uncomplicated BM (one non-spine, seven spine), and four were complicated (three neuropathic pain, one impending SCC and impending PF). Fairchild Case 3 and Chow Case 2 were identical except for the patient's age, while Chow Case 2 and Hartsell Case 2 are presumed identical given that the Chow survey was designed based on Hartsell's questionnaire. Overall response rates ranged from 15.7% to 63.3% ($n = 5$ studies); response rate of the Nakamura survey was provided as a proportion of institutions rather than practitioners (Table 3).

Radiation Oncologists living in Japan, Italy, the United States, Canada, Australia, and New Zealand along with members of the American, Canadian, Australian and New Zealand Radiation Oncology professional groups were surveyed by mail^[48,49], internet^[9,46] or during attendance at national meetings^[6,47]. Responses to three surveys were anonymous^[6,9,47]. Trainees were included in the sampling frame^[6,47], excluded^[9], or not specified but likely excluded^[46,48,49]. A prespecified list of dose fractionation schedules was provided only by De Bari. Factors influencing dose prescribed were sought on the basis of case^[6,46,47], overall^[9], or not explored^[48,49].

Table 4 indicates the proportion of respondents who would deliver EBRT in each case and the specified dose fractionations, with shading indicating practice that would currently be considered evidence-based. For uncomplicated bone BM, 8Gy/1 would be used by 13.7% of Japanese respondents to treat a right shoulder, and between 5.9% and 39.6% of respondents internationally to treat a painful spinal lesion. Range of doses suggested for an uncomplicated spine metastasis was 3Gy/1 to 55Gy/22 with up to 78.4% using 30Gy/10; this was also the most common dose suggested by respondents in Hartsell and in the Nakamura survey. For a BM associated with neu-

ropathic pain, 0%-13.2% would use 8Gy/1 and 5.8% to 52.8% would use 20Gy/5 (range 3Gy/1-45Gy/18). Finally, for the patient with impending SCC and PF, 54% would use a multifraction schedule of either 20Gy/5 or 30Gy/10; the respondents using a dose in the 'other' category could also be considered to have evidence-based practice although the specific additional proportion cannot be differentiated from missing values.

In the four publications which explored factors influencing choice of dose fractionation based on direct questioning of respondents, the most commonly cited factors impacting treatment decisions were wish to minimize risk of neurologic progression/SCC, prognosis, literature results and wish to minimize the chance of recurrent pain. Among those factors impacting treatment decisions the least were waiting list, personal habits and financial aspects (Table 5). In the only study to explore it, 36.4% (uncomplicated BM) and 30.6% (complicated BM) of respondents were influenced in their decision to deliver RT by analgesic response^[47].

Statistical predictors of characteristics of respondents likely to use SF schedules were reported by Fairchild, Chow, and Roos; Hartsell reported predictors of use of <30 Gy (Table 6). For use of SF in uncomplicated spinal BM, time in practice was associated with use of < 30 Gy by Hartsell but not associated in the Chow or Roos publications. University/academic practice was associated with use of SF^[9,49], and private practice with less use of SF^[9], while no differences were found by Roos. Fairchild *et al*^[9] found that those trained in the United States tended to use SF less often while Chow found no correlation. Radiation Oncologists practicing in the Southwest United States, New Zealand and Australia tended to use lower doses more often^[49], with no differences between Australia and New Zealand or between Australian states^[6]. No trends were observed for treatment of neuropathic pain in the two surveys reporting statistical predictors (Table 6).

DISCUSSION

Treatment decisions regarding palliative EBRT for BM should be based on individualized considerations of symptom burden, extent of disease, life expectancy, PS, comorbidities, toxicity, prior treatment and patient wishes. A retrospective study of 33 patients dying within 30 d of hospital admission, 39.4% of whom had lung cancer and 94% with metastatic disease, reported planned *vs* actual EBRT treatment; sites were not specified^[50]. 90% were planned for ≥ 30 Gy but only 58.1% completed it; almost 25% died during treatment. Half of patients spent > 60% of their remaining life on therapy with the median treatment time equivalent to the cohort's median survival (15 d)^[50]. However, while palliative EBRT should be appropriately customized to patients, the choice of dose schedule should have a robust evidence base.

Despite multiple randomized trials and four meta-analyses showing efficacy of SF irradiation for uncom-

Table 3 Hypothetical cases utilized by previous surveys

Ref.	Case history	Methodology	Response rate
Nakamura <i>et al</i> ^[46] Case 1 (Japan, 2012)	A 65 yr old man was diagnosed with squamous cell lung cancer one year earlier and was treated by radical surgery. He now has pain in his right shoulder. Radiologic examinations detected osteolytic bone metastasis at the right scapula and multiple lung metastases. ECOG 1	Radiation Oncologist members of JROSG completed an internet-based survey Presumed trainees were excluded Not anonymous	NR
Nakamura <i>et al</i> ^[46] Case 2 (Japan, 2012)	A 65 yr old man was diagnosed with squamous cell lung cancer one year earlier and was treated by radical surgery. He now has back pain. Radiologic examinations detected osteolytic bone metastasis at L1 and multiple lung metastases. There is no evidence of vertebral collapse or spinal or thecal sac compression. ECOG 1		
Nakamura <i>et al</i> ^[46] Case 3 (Japan, 2012)	Same setting as in case 2 with the addition of paresthesias in a distribution consistent with the L1 dermatome, compatible with neuropathic pain		
De Bari <i>et al</i> ^[47] Case 2 ¹ (Italy, 2011)	68yo woman ECOG 1, right lung cancer in 2005, pT2N1M0 underwent lobectomy -> adjuvant chemotherapy. No previous RT. Negative F/U to today. Lumbar pain (L2-L3) underwent bone scan and spinal MRI, total body CT and CT brain. 3 new liver lesions. Bone scan: multiple sites of pathological uptake. MRI: multiple osteolytic spinal metastases including at symptomatic sites. No clinical or radiologic evidence of SCC and no risk of immediate fracture. VAS: 8 without analgesics, 3 after regular weak opioids	Questionnaires given to ROs attending the national congress at the time of registration and collected at the end of the congress Trainees included Anonymous Prespecified list of dose fractionation schedules provided as answer choices, or 'other'	122/300 (40.6%) ³
De Bari <i>et al</i> ^[47] Case 4 ¹ (Italy, 2011)	78yo man ECOG 2, left lung cancer in 2007, pT3NOM0 post left pneumonectomy -> adjuvant chemo x6. Negative f/u until today. Sudden thoracic (D5/D6, D10) and lumbar (L4) pain. No clinical signs of cord compression. No other symptomatic sites. MRI spine: multiple spinal secondary lytic lesions. Radiological signs of D10 spinal cord compression. Risk of pathologic fracture at C3. CT body: multiple liver and lung metastases. VAS: 9 without analgesics, 3 after regular opioids analgesics (transdermal fentanyl 50 ug) and prn NSAIDs	Factors influencing dose were sought for each case	
Fairchild <i>et al</i> ^[9] Case 3 (Intl, 2009)	A 55-year-old male was diagnosed with stage IIIA (T3N2) non-small cell lung cancer one year ago, and was treated radically with chemotherapy and thoracic radiotherapy. He now has pain in the lower back, and a bone scan shows a lesion at L3. His pain localizes to an area consistent with L3, and motor and sensory exams are unremarkable. There is a lytic lesion present and evidence of mild vertebral collapse, but no cauda equina or thecal sac compression on MRI scan	Web-based survey distributed to Radiation Oncologist members of ASTRO, CARO and RANZCR Anonymous Trainees, retirees excluded No prespecified list of dose fractionation schedules provided	962/6110 (15.7%)
Fairchild <i>et al</i> ^[9] Case 4 (Intl, 2009)	Same setting as in case above, with the addition of paresthesias in a distribution consistent with the L3 dermatome, compatible with neuropathic pain	General factors influencing dose were sought (not case-by-case) Bonferroni used	
Chow <i>et al</i> ^[48] Case 2 (Canada, 2000)	A 45 yr old male was diagnosed with stage IIIA (T3N2) large cell carcinoma of the lung one year ago, and was treated with chemotherapy and thoracic irradiation. He now has pain in his lower back, and a bone scan shows a lesion in the third lumbar vertebra. His pain localizes to an area consistent with L3, and does not radiate. Motor and sensory examinations are unremarkable. A CT scan of this area shows a lytic lesion, but no evidence of compression of the cauda equina or thecal sac	Survey mailed to all ROs in active practice in Canada Excluded retirees or those practicing outside of Canada No mention of including trainees Did not specify whether anonymous No prespecified list of dose fractionation schedules provided	172/300 (57.3%)
Chow <i>et al</i> ^[48] Case 3 (Canada, 2000)	Same setting as above, but instead of L3, the lesion is at L1 with no evidence of cord compression. Assume the external beam irradiation to the painful site in L1 would not overlap the previous radiation field	Factors influencing dose not explored	
Roos <i>et al</i> ^[6] Case 3 (Aust/NZ, 2000)	Male, age 63 with disseminated large cell lung cancer and bone scan positive L1-L3, several ribs and skull. There is pain in the upper lumbar spine only and no neurologic dysfunction	Survey distributed to delegates at 1998 Royal ANZ College of Radiologists Annual Scientific Meeting and returned before a presentation on bone pain Anonymous Trainees included Presumed no prespecified list of dose fractionation schedules were provided since cases were designed based on previous surveys Factors influencing dose sought for each case Used Bonferroni correction	53/114 (46.5%) ³
Roos <i>et al</i> ^[6] Case 4 (Aust/NZ, 2000)	Male, age 63 with disseminated large cell lung cancer and bone scan positive L1-L3, several ribs and skull. There is pain in the upper lumbar spine as well as pain and tingling in the right L2 distribution consistent with neuropathic pain		

Hartsell <i>et al</i> ^[49] Case 2 ² (United States, 1998)	A 45 yr old male was diagnosed with stage IIIA (T3N2) large cell carcinoma of the lung one year ago, and was treated with chemotherapy and thoracic irradiation. He now has pain in his lower back, and a bone scan shows a lesion in the third lumbar vertebra. His pain localizes to an area consistent with L3, and does not radiate. Motor and sensory examinations are unremarkable. A CT scan of this area shows a lytic lesion, but no evidence of compression of the cauda equina or thecal sac	Survey mailed to randomly selected radiation oncologists in United States (63.3%) Factors influencing dose not reported Did not specify whether prespecified list of doses was given Presumed trainees excluded Did not report whether anonymized
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¹Only surveys containing all the answers for at least 3 of the 4 clinical cases in the wider survey were analyzed; ²Few case details provided in abstract but Chow *et al*^[48] reports using the same case so clinical history is as described by Chow *et al*; ³Including trainees. NR: Not reported; CT: Computed tomography; MRI: Magnetic resonance imaging; SCC: Spinal cord compression; Intl: International; ECOG: Eastern Cooperative Oncology Group.

Table 4 Grey shading indicates dose-fractionation schedules which would be supported by current evidence

Case	N treating with EBRT	8Gy/1	20Gy/5	30Gy/10	Other	Median (Range)
Uncomplicated-non-spine						
Nakamura case 1 (Japan, 2012)	51	13.70%	9.80%	66.70%	9.80%	NR (NR)
Uncomplicated-spine						
Hartsell case 2 (United States, 1998)	229	4% recommended < 30Gy		76% recommended 30-35Gy 20% recommended > 35Gy		NR (15Gy/5 - 47.5Gy/25)
Nakamura case 2 (Japan 2012)	51	5.90%	3.90%	78.40%	11.80%	NR (NR)
Chow case 3 (Canada, 2000)	171	15.80%	66.10%	8.80%	9.4% ³	NR (8Gy/1 - 30Gy/10)
Chow case 2 (Canada, 2000)	170	15.90%	64.70%	8.20%	11.2% ³	NR (8Gy/1 - 30Gy/10)
Fairchild case 3 (Intl, 2009)	867	18.3% ²	19.8% ²	41.9% ²	20.00%	30Gy/10 (3Gy/1 - 55Gy/22)
De Bari case 2 (Italy, 2011)	107€	22.20%	50.10%	26.80%	0.90%	NR (NR)
Roos case 3 (Aust/NZ, 2000)	53€	39.6%€	35.8%€	15.1%€	9.4%€	NR (8Gy/1 - 40Gy/18)
Complicated-neuropathic pain						
Nakamura case 3 (Japan, 2012)	52	0%	5.80%	78.80%	15.40%	NR (NR)
Fairchild case 4 (Intl, 2009)	844	6.6% ²	29.0% ²	42.8% ²	21.60%	30Gy/10 (3Gy/1 - 45Gy/18)
Roos case 4 (Aust/NZ, 2000)	53€	13.2%€	52.8%€	24.5%€	9.4%€	NR (8Gy/1 - 40Gy/20)
Complicated-impending spinal cord compression and impending pathologic fracture						
De Bari case 4 (Italy, 2011)	113€	30.60%	25.80%	28.20%	15.4% ³	NR (NR)

¹41 specialists/12 trainees; ²Takes into account multiple dose fractionation schemes listed per respondent; ³Includes unknown/missing responses; €: Extrapolated from data reported or from figure. EBRT: External beam radiotherapy; Intl: International; NR: Not reported.

plicated BM and BM associated with neuropathic pain in patients with lung cancer, its use as reported by respondents to hypothetical case scenarios remains low. Most common practice internationally and over time continues to be a 30Gy/10 schedule, representing significant divergence from multiple clinical practice recommendations and consensus guidelines (Table 2). The consequences in terms of increased departmental workload, health care costs and patient burden are significant^[6].

For a complicated BM presentation where the literature is less robust but supports fractionation, a higher proportion of respondents' dose schedules are evidence-based. For a patient with good PS, a truly solitary BM and no visceral disease, multifractionated highly conformal treatment may be a reasonable option. In that circumstance, long term tumour control may be important^[7].

However most patients with lung cancer and complicated BM do not meet these criteria and palliation, not tumour control, is the goal^[7]. Additionally, patients who are not likely to benefit from irradiation, especially those who are not likely to complete the prescribed course, should not be offered treatment. Those with extensive metastatic disease, a short life expectancy or very poor PS should be considered for SF radiotherapy regardless

of type of BM lesion, if they are treated at all^[42]. Settings in which RT should be omitted entirely in favour of best supportive care are reviewed by Lutz *et al*^[51].

It is not completely clear why the results of literature confirming the equivalence of single to MF continue to be overlooked, although studies suggest that trial evidence is just one of the factors physicians use in determining dose fractionation schedules^[9]. Patient-, institution- and training-related factors, along with individual physician beliefs, also play a role (Table 7).

Patient-related

The number of respondents employing SF for neuropathic pain, which in fact decreased after the publication of the TROG trial in 2005, may be due to the risk of occult spinal cord/cauda equina compression, the belief that tumour shrinkage is required to alleviate pressure on nerves or the general exclusion of these patients from trials examining efficacy of SF EBRT^[6,38]. Half of respondents to Nakamura Case 2 were concerned about the possibility of SCC^[46]. Concerns over toxicity and other QOL issues have not emerged as major factors^[6,9], likely because acute toxicity is typically mild and long term toxicity uncommon^[7,8]. As many as 85% of respondents who

Table 5 Factors influencing choice of dose fractionation scheme based on direct questioning of respondents

Case	Most impact	Least impact
Uncomplicated-spine Nakamura Case 2 (Japan, 2012)	Factors influencing those who chose MF: Time until first increase in pain Incidence of spinal cord compression Incidence of pathologic fracture	NR
De Bari Case 2 ¹ (Italy, 2011)	Prognosis Performance status Radiologic appearance of lesions	Financial aspects Personal habits Waiting list
Roos Case 3 ² (Aust/NZ, 2000)	Factors influencing those who chose SF: Literature results Patient convenience Resource limitations Factors influencing those who chose MF: Minimize chance of recurrent pain Minimize risk of neurologic progression (tie) Optimize tumour regression Patient convenience	NR
Complicated-neuropathic pain Roos Case 4 ² (Aust/NZ, 2000)	Factors influencing those who chose SF: Literature results Patient convenience Resource limitations Factors influencing those who chose MF: Minimize risk of neurologic progression Minimize chance of recurrent pain Optimize tumour regression	NR
Complicated – impending spinal cord compression and impending pathologic fracture De Bari Case 4 ¹ (Italy, 2011)	Radiologic appearance of lesions Site of metastasis Prognosis	Financial aspects Personal habits (tie) Waiting list
Overall Fairchild (Intl, 2009)	Prognosis Risk of spinal cord compression Performance status Previous RT Published evidence	Departmental policy Waiting list Future retreatment Age Late toxicity

¹Literature results were not one of the prespecified options; ²Includes specialist and trainee responses. Intl: International; MF: Multiple fractions; NR: Not reported; SF: Single fractions.

recommended MFRT for Nakamura Case 2 regarded it as superior based on time until first increase in pain; participants in the Roos survey also cited this factor, which is not supported by level 1 evidence.

Physician-related

Some international guidelines still recommend multifraction schedules for uncomplicated BM. NCCN suggests, but does not provide supporting evidence for, the range of 8-30Gy/1-20 for an uncomplicated BM secondary to NSCLC^[52]. Japanese radiation oncologists prefer to learn from US resources resulting in similar patterns of practice^[46]. Despite evidence to the contrary, radiation oncologists may continue to believe that higher total doses, which can now be delivered with minimal toxicity *via* highly conformal techniques, are preferred^[2,53]. However, the mechanism of pain relief may be related to changes in the local microenvironment rather than direct tumour kill^[7]. Suboptimal quality of early studies (heterogeneity of patients, differences in endpoint selection, inconsis-

tent follow-up practices) may have contributed to lack of confidence in their results^[48].

Institution-related

SF is most frequently prescribed in university or government (*vs* private) centres and in large treatment facilities^[6,7,9,26,49].

Health Care System-related

Reimbursement system is one part of the wider cultural and bureaucratic context of location of practice/institutional structure^[24]. SF is more commonly used in countries using a budget or case-payment system (*e.g.*, Canada) compared to those with fee-for-service reimbursement, such as Japan^[7,46]. In a survey of 23/25 Belgian RT centres after changes in the Belgian reimbursement system in 2001 from fee-for-service to case payment, there was an increase in use of SF in 86% of centres^[11]. In Canada, where SF are used more often, the majority of departments are funded mostly by government while university

Table 6 Statistical predictors of use of single fraction schedules

Case	Factor	OR for use of SF (95%CI)	P
Uncomplicated-spine Hartsell Case 2 (1998, United States)	Respondents recommending doses < 30Gy: Longer time in practice Academic practice Practice in the Southwest	NR	NR
Chow Case 2 (Canada, 2000)	No differences based on country of specialty training or year training completed	NR	NR
Chow Case 3 (Canada, 2000)	University practice	2.08 (1.35-3.19)	0.001
Fairchild Case 3 (Intl, 2009)	Private practice	0.27 (0.12-0.61)	0.002
	Trained in United States	0.17 (0.10-0.28)	< 0.001
	Practice in Aust/NZ	2.44 (1.43-4.18)	0.001
Roos Case 3 (Aust/NZ, 2000)	No difference based on trainees vs specialists, public vs private practice, years of experience, % workload palliative, between Aust vs NZ or between Aust states	NR	NR
Complicated-neuropathic pain Fairchild Case 4 (Intl, 2009)	University practice	2.31 (1.33-4.00)	0.003
	Trained in US	0.22 (0.11-0.43)	< 0.001
Roos Case 4 (Aust/NZ, 2000)	No difference based on trainees vs specialists, public vs private practice, years of experience, % workload palliative, between Aust vs NZ or between Aust states	NR	NR

Aust: Australia; Intl: International; NR: Not reported; NZ: New Zealand.

and other funds make up < 10% support^[28].

A recently published patterns of care study characterized palliative RT dose and fractionation in a large US cohort of metastatic NSCLC patients (stage IV at baseline or metastatic at recurrence) and explored factors influencing RT delivery^[2]. The Cancer Care Outcomes Research and Surveillance Consortium prospectively enrolled 1574 patients (2003-2005) who participated in phone surveys and whose medical records were reviewed. 65% were male with a median age of 68. Eighty-seven point two percent had metastatic disease at diagnosis and the remainder was diagnosed with a distant first recurrence within 15 mo. Among 194 patients who received palliative EBRT to bone (218 courses), 50% received 6-10 fractions, 20% five fractions or fewer and 6% received SF. Among 206 patients with known dose, 49% received 21-30Gy. Patients younger than age 55, who had had surgery to a metastatic site and those receiving chemotherapy were more likely to receive RT to any site. Type of insurance was not predictive. Patients receiving RT to BM treated in integrated networks (HMOs, Veterans Administration) received on average 3.4 fewer fractions ($P = 0.001$) and 4Gy less dose ($P = 0.049$), although had similar rates of RT delivery^[2]. No information was provided about whether BM were complicated or uncomplicated. The authors concluded that a substantial proportion of patients received higher doses and more fractions than clinical trial data supports, despite the fact that this cohort had a short median survival. Patients treated in integrated networks received lower total doses and fewer fractions suggesting that provider characteristics, organizational structures and processes or financial incentives influenced clinical practice^[2].

However, arguments against SF have been almost entirely refuted by recent data, including multiple second-

ary analyses of the 1999 Dutch trial which randomized between 8Gy/1 and 24Gy/6, reporting no difference in outcomes^[16]. In terms of reirradiation, although SF and MF patients experienced equivalent response and progression rates, SF patients were retreated more frequently, at an earlier time during follow-up and at a lower pain score. This was interpreted by the authors as evidence that the differences resulted from practitioner bias rather than true differences in efficacy^[35].

Several economic analyses have compared different schedules of EBRT. A cost-utility analysis was conducted prospectively within the Dutch trial^[14]. SF RT provided an additional 1.7 quality-adjusted weeks and cost USD\$873 less than MF, including the effects of retreatment. When considering total societal including non-medical costs, the estimated savings was larger (USD\$1753) but not statistically significant^[14]. In a cost-effectiveness analysis of the TROG neuropathic bone pain study^[38] incorporating data to three months post-RT, although larger retreatment costs were associated with the SF arm, these were offset by savings in medication and hospital admission costs, as well as by the lower cost of initial RT^[54]. Through the use of a Markov model, Konski estimated that SF RT was more cost-effective for painful BM than either multifraction RT, chemotherapy (mitoxantrone and prednisone) or analgesics (oxycontin with senokot bowel routine). MF RT had only slightly more quality-adjusted life months than SF but cost USD\$1300 more^[55].

van der Linden *et al*^[56] compared patients with any histology accrued to the Dutch trial surviving > 52 wk from randomization. Responses were 87% after 8Gy and 85% after 24Gy, again including effects of retreatment ($P = \text{NS}$). Duration of response, time to response, and progression rates were also similar, indicating that patients do not outlive the benefits of SF.

Table 7 Reasons for reticence in use of single fractions

Factor	Ref.
Patient-related	
Neuropathic pain	[6]
Prevent or address neurologic symptoms	[46]
Maximize time to first increase in pain	[6]
Patient selection	[7]
Prognosis	[7]
Patient wishes	[7]
Fear of toxicity (acute/late)	[6]
Site of bone metastasis	[9]
Comorbidities	[9]
Physician-related	
Influence of global opinion leaders	[52]
Presumed dose-response	[7]
Professional membership affiliation	[9]
Country of training	[9]
Country of practice	[9]
Lack of experience with large fraction sizes	[9]
Lack of participation in related trials	[6]
Disbelief of early trial results due to quality	[48]
Institution-related	
Departmental policy	[9]
Longer wait times for RT delivery	[62]
Type of centre	[9]
Health care system-related	
Retreatment more often required	[35]
Increased costs due to retreatment	[14]
Reimbursement system	[24]

Two studies have examined the effect of palliative EBRT in patients during the last 12 wk of life^[57,58]. In a secondary analysis of 274 patients treated within the Dutch study, the proportion showing a pain response did not differ between the SF and MF arms^[57]. A retrospective review evaluated 232 patients dying within 3 mo of beginning treatment, 34% with lung cancer, 64% men, median KPS 60, median age 69, and 58% received SF. Overall response rates were 70% at one month and 63% at two months, controlling for analgesic usage. The authors concluded that despite limited lifespan, patients with painful BM with an estimated survival of three months should still be considered for RT^[58].

In terms of risk of pathologic fracture, 35% of the lesions classified as impending fracture in Harada *et al*^[36] study responded to multifraction RT, and 81% did not require surgical intervention. Both the degree to which recalcification is dependent on dose, and helps to prevent future fracture, remain unclear, however^[46].

Finally, no significant differences have been found in responses rates of elderly (≥ 65 years) compared to younger patients at one, two and three months after RT, when controlling for analgesic usage, supporting referral of patients regardless of age^[59].

Surveys are valuable in assessing practice when it diverges from published data, but have well-known limitations, reviewed in Fairchild *et al*^[9]. Exploration of differences in attitude may provide a more realistic basis for the construction of international consensus, leading to increased ownership. The results might not be entirely representative because it is not possible to conclude

whether answers accurately reflect practice. When the questionnaires were completed in comparison to when practitioners became aware of results of new published data cannot be determined. When facts are being solicited about existing systems, such as reimbursement method, accuracy of the answers cannot be checked. Some studies included trainee respondents, some did not, and some did not specify. Multiple calculations for associations are often performed, but rarely taken into account in the statistical analysis (*i.e.*, lack of Bonferroni correction). Possible explanations for differences in practice include a lack of histology-specific data^[19], demographics of respondents, contradictory definitions of uncomplicated BM, and varying availability of alternative treatment modalities such as vertebroplasty, radiofrequency ablation, radiopharmaceuticals and speciality surgery teams^[9]. Additionally, increasing use of systemic therapy in patients with advanced lung cancer may add local anti-tumour effects^[36], and case histories often did not describe planned or delivered systemic therapy. Overall, a survey is still a useful method of exploring attitudes, beliefs and practices, although it is not possible to extrapolate beyond the data reported.

CONCLUSION

Palliative EBRT has an essential role as a well-tolerated, minimally toxic, cost conscious and effective treatment for symptom control in this setting. However, survey studies concerning hypothetical cases of patients with lung cancer and both complicated and uncomplicated bone metastases continue to show a large discrepancy between published literature and patterns of practice. From a common sense perspective, the shortest RT regimen which maximizes outcomes in an evidence-based manner seems preferable. Schedules prescribed as multifraction courses, when SF would be appropriate, disadvantage all patients and overextend many centres' already strained resources.

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Erlotinib usage after prior treatment with gefitinib in advanced non-small cell lung cancer: A clinical perspective and review of published literature

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Abstract

Erlotinib and gefitinib are among the most widely researched, used and available molecularly targeted therapies for treatment of advanced non-small cell lung cancer (NSCLC). They are both tyrosine kinase inhibitors (TKIs) of the epidermal growth factor receptor (EGFR). In the past decade, there have been reports on clinical benefit from use of erlotinib after gefitinib failure in NSCLC patients. A review of published literature on this focussed topic is provided herein. Pooled analysis of published literature shows that majority of patients were female (60.6%), non-smokers (64.5%), had adenocarcinoma histology (88.3%) and were of East Asian ethnicity (92.3%). Presence of sensitizing EGFR mutation was detected in 48.4% of subjects. Disease control rates with prior gefitinib therapy and with subsequent erlotinib treatment were 79.4% and 45.4% respectively. Based upon our review, the most important predictive factor for clinical benefit from erlotinib identified was previous response to gefitinib. The exact explanations for the potential benefit from erlotinib use in this patient population is still not known and further studies are required to determine the role of molecular mechanisms

especially those related to resistance to initial EGFR TKI therapy.

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Key words: Gefitinib; Erlotinib; Non-small cell lung cancer; Epidermal growth factor receptor; Tyrosine kinase inhibitor

Core tip: This manuscript is focused on the controversial yet interesting topic of whether erlotinib provides clinical benefit amongst patients who have experienced disease progression after prior therapy with gefitinib—both drugs being tyrosine kinase inhibitors of the epidermal growth factor receptor. We have reviewed available literature on this topic, carried out a pooled analysis on available data and hope readers find it useful in clearing the confusion related to this topic.

Singh N, Jindal A, Behera D. Erlotinib usage after prior treatment with gefitinib in advanced non-small cell lung cancer: A clinical perspective and review of published literature. *World J Clin Oncol* 2014; 5(5): 858-864 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i5/858.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i5.858>

INTRODUCTION

Lung cancer remains among the most commonly occurring cancers in the world with majority of the cases being of non-small cell lung cancer (NSCLC)^[1,2]. As in other malignancies, molecularly targeted therapies are being increasingly researched and approved for clinical use in case of NSCLC especially adenocarcinoma. Among the molecularly targeted therapies available for advanced

NSCLC, the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) occupy a central place and form part of the standard treatment algorithms^[3]. Gefitinib, erlotinib and recently afatinib are the available, orally active agents in this group. Gefitinib was approved for use after two randomised trials-the Iressa Dose Evaluation in Advanced Lung Cancer 1 and 2 (IDEAL 1 and 2) studies-showed survival benefit as compared to placebo amongst patients with advanced NSCLC who had progressed after initial platinum based chemotherapy^[4,5]. However, based on a subsequent trial, *i.e.*, the Iressa Survival Evaluation in Lung Cancer study, which did not show an improvement in overall survival (OS), the use of gefitinib was disallowed in the United States by the Federal Drugs Authority (FDA)^[6]. Erlotinib was demonstrated to have efficacy in advanced NSCLC in the BR.21 trial. This was a randomised, double blind, placebo controlled trial in which the administration of erlotinib to patients with advanced NSCLC who had received prior chemotherapy led to an improvement in response rates (RR), progression free survival (PFS) and OS with an acceptable toxicity profile^[7]. Subsequently, erlotinib was approved by the FDA for use in advanced NSCLC. In all of the above studies, the clinical benefit and treatment response was more evident in certain subgroups namely female gender, non-smoker status, Asian ethnicity and adenocarcinoma. Subsequently, these subgroups were proven to have a higher incidence of sensitizing EGFR mutations which till date remains the most important predictive factor for clinical benefit with EGFR TKIs^[8].

Almost a decade ago, there was an initial report on the use of erlotinib after gefitinib failure in patients of non-small cell lung cancer^[9]. Following this, several case reports, case series, retrospective reviews and even a few prospective trials have been published related to use of erlotinib in patients who had received prior therapy with gefitinib. This is surprising, considering that the mechanism of action of both drugs is similar. No satisfactory explanation has been found till date, though many have been postulated. In the current article, we have reviewed published literature on this focused area and carried out a pooled analysis with the data available.

MECHANISM OF ACTION OF TKIS

EGFR is a transmembrane protein which functions as the receptor for the epidermal growth factor (EGF) pathway^[8]. The EGFR protein consists of three domains: an extra-cellular binding site for the EGF molecule, a transmembrane unit which spans the cell membrane and an intracellular unit which has tyrosine kinase activity. The binding of EGF causes conformational changes in the receptor which further leads to phosphorylation/dephosphorylation of the intracellular tyrosine kinase domain and subsequent downstream signalling. Mutations in the EGFR lead to a constitutive activation of the EGF pathway and subsequent activation of cell survival pathways^[10].

The mutations underlying the activation of this pathway were elucidated in the earlier part of the last decade^[11-13]. Based on the experience with imatinib in chronic myeloid leukaemia, inhibitors of the EGFR tyrosine kinase pathway were developed. The first of these was gefitinib, which acts by interfering with the tyrosine kinase activity of the intracellular domain of the EGFR. It was followed by erlotinib, which has a somewhat similar mechanism of action. These function as adenosine triphosphate (ATP) mimetic molecules and bind to the intracellular domain of the EGFR and block receptor phosphorylation and subsequent downstream signalling and activation of dependent pathways^[14].

LITERATURE REVIEW

We carried out a PubMed search for all published literature in the English language on this topic till 2013. Multiple case reports, retrospective reviews and prospective studies were identified that have been listed in Table 1. The studies included in this review used standard treatment protocols for gefitinib and erlotinib, *i.e.*, 250 mg/d and 150 mg/d respectively. Tumour response was assessed according to the Response Evaluation Criteria in Solid Tumours^[15]. The outcome measures reported in the studies included: RR, defined as combination of complete response (CR) and partial response (PR); Disease control rate (DCR), defined as the combination of CR, PR and stable disease (SD); PFS, defined as the period from the start of treatment to the date when disease progression or death was observed and OS, defined as the period from the start of treatment to the date of death (Tables 2 and 3).

Use of erlotinib after gefitinib failure was first reported in 2005^[9]. Subsequently, sporadic case reports were published which demonstrated similar findings^[16-18]. A prospective phase II study evaluated the use of erlotinib in patients of NSCLC who had been treated with chemotherapy followed by gefitinib and then had failure on gefitinib. A total of 21 patients were included in this study. Out of these 21 patients, who had progressed on gefitinib, 2 patients achieved PR and 4 patients SD while the remaining 15 patients had progressive disease (PD). The DCR was 28.6% (95%CI: 9.3% to 47.9%) while the median PFS and OS were 60 d (95%CI: 43 to 77 d) and 158 d (95%CI: 141 to 175 d), respectively. An interesting point noted was that the only predictor of a response to erlotinib was the presence of a prior response to gefitinib. Surprisingly, the presence of EGFR mutations was not associated with a response to erlotinib. In fact, 4 out of 6 responders to erlotinib did not have the EGFR mutation^[19].

A retrospective study analysed 14 patients who had received erlotinib after failure of gefitinib over a two year period. The initial DCR for gefitinib was 64.3% (9 of 14), while it was 35.7% (5 of 14) for erlotinib. Predictors for good clinical response to erlotinib included never smoker status, adenocarcinoma subtype, good response to initial

Table 1 Baseline characteristics of subjects in studies evaluating response to erlotinib after gefitinib

Ref.	Year	Country	Type of study	No. of patients	Gender (M/F)	Smoking status (smokers/NS)	Histology (A/S/other)
Garfield ^[9]	2005	United States	Case report	1	1/0	1/0	0/0/1
Viswanathan <i>et al</i> ^[16]	2005	United States	Case report	5	1/4	2/3	NA
Walther <i>et al</i> ^[17]	2006	United Kingdom	Case report	1	0/1	0/1	1/0/0
Chang <i>et al</i> ^[18]	2007	Taiwan	Case report	1	1/0	1/0	1/0/0
Cho <i>et al</i> ^[19]	2007	South Korea	Prospective	21	10/11	10/11	16/3/2
Gridelli <i>et al</i> ^[32]	2007	Italy	Case report	3	0/3	0/3	3/0/0
Kim <i>et al</i> ^[33]	2007	South Korea	Case report	1	0/1	0/1	1/0/0
Costa <i>et al</i> ^[23]	2008	United States	Retrospective	18	7/11	7/11	16/0/2
Lee <i>et al</i> ^[21]	2008	South Korea	Prospective	23	4/19	0/23	22/0/1
Vasile <i>et al</i> ^[22]	2008	Italy	Prospective	8	4/4	1/7	6/0/2
Wong <i>et al</i> ^[20]	2008	Singapore	Retrospective	14	4/10	1/13	10/1/3
Wong <i>et al</i> ^[34]	2008	Singapore	Case report	1	0/1	0/1	1/0/0
Wu <i>et al</i> ^[35]	2008	Taiwan	Case report	1	0/1	0/1	1/0/0
Katayama <i>et al</i> ^[36]	2009	Japan	Retrospective	7	2/5	NA	7/0/0
Sim <i>et al</i> ^[24]	2009	South Korea	Retrospective	16	0/16	0/16	16/0/0
Zhou <i>et al</i> ^[25]	2009	China	Prospective	21	14/7	10/11	8/9/4
Wong <i>et al</i> ^[26]	2010	China	Retrospective	21	2/19	1/20	19/0/2
Asami <i>et al</i> ^[27]	2011	Japan	Retrospective	42	13/29	14/28	42/0/0
Hata <i>et al</i> ^[29]	2011	Japan	Retrospective	125	49/76	55/70	117/NA/8
Masuda <i>et al</i> ^[37]	2011	Japan	Case report	3	3/0	NA	3/0/0
Shoji <i>et al</i> ^[38]	2011	Japan	Case report	1	1/0	NA	1/0/0
Song <i>et al</i> ^[39]	2011	China	Retrospective	20	9/11	5/15	18/0/2
Takenaka <i>et al</i> ^[40]	2011	Japan	Case report	1	0/1	0/1	1/0/0
Saito <i>et al</i> ^[41]	2012	Japan	Retrospective	21	9/12	9/12	19/0/2
Tetsumoto <i>et al</i> ^[42]	2012	Japan	Case report	2	0/2	0/2	2/0/0
Koyama <i>et al</i> ^[43]	2013	Japan	Retrospective	104 ¹	56/48	50/54	90/6/8

¹Of the 104 patients, only 54 had prior treatment with gefitinib. A: Adenocarcinoma; S: Squamous cell carcinoma; M: Male; F: Female; NA: Not available; smokers: Includes current and ex smokers; NS: Non-smokers.

gefitinib and presence of EGFR mutations^[20].

23 patients of metastatic or advanced NSCLC who had documented progression on gefitinib were studied in an open label, single institution, phase II study. All patients were never smokers and 22 out of the 23 patients were of the adenocarcinoma subtype. The initial DCR on gefitinib was 65.3% (15 out of 23). 2 patients responded to erlotinib, giving a DCR of 8.7% and a RR of 4.3%, while the rest 21 patients developed PD within 3 mo^[21].

Another study included 4 males and 4 females in their study, with a mean age of 70 years. All of these patients had received chemotherapy at least twice, followed by gefitinib. 4 patients had achieved PR with gefitinib and 4 patients SD, and the median PFS was 17 mo. Two (25%) patients achieved PR and 3 (37.5%) patients SD, while 3 developed PD. The median PFS and OS were 5.9 and 14.6 mo, respectively with erlotinib. The authors observed that patients who had a longer PFS on initial gefitinib therapy had better disease control with erlotinib^[22].

Another retrospective study published in 2008 included 18 patients. These patients had received primary chemotherapy and had subsequently been given gefitinib. The initial response to gefitinib included 14 patients with either CR or PR, 2 patients with SD and 2 with PD. After treatment with erlotinib, 14 out of the 18 patients developed PD, while 3 patients had SD and only 1 PR. The median PFS was 2 mo and no patient had a PFS over 6 mo^[23].

The utility of established predictive factors for re-

sponse to EGFR TKIs namely female sex, adenocarcinoma subtype, Asian ethnicity and never smoking status, were also assessed for their predictive value in the context of erlotinib use following progression on initial gefitinib therapy. They included 14 patients and noted a DCR of 68.8% (95%CI: 0.44-0.86) on initial gefitinib treatment and a rate of 25.0% (95%CI: 0.10-0.50) after treatment with erlotinib. The median PFS was 6.3 mo for gefitinib and 1.7 mo for erlotinib. The above mentioned factors were found to be unreliable for predicting response to erlotinib after treatment failure with gefitinib^[24].

A more heterogeneous population of 10 (47.6%) smokers, 9 (42.9%) patients with squamous cell carcinoma, 8 (38.1%) with adenocarcinoma and 4 (19%) with other NSCLC subtypes, totalling 21 were included in another trial. All of them had progressed on gefitinib therapy after chemotherapy. 6 of the 21 patients responded, with 2 (9.5%) showing PR and 4 (19.0%) showing SD, giving an overall RR of 9.5% and a DCR of 28.5%. The median PFS was 55 d and the median OS was 135 d. All of these 6 patients had also had disease control with prior gefitinib therapy, either PR or SD^[25].

All of the previously mentioned trials included patients who had received chemotherapy prior to initial gefitinib therapy. A retrospective review of 21 patients who had received gefitinib as first line therapy instead of chemotherapy was conducted. The patient population was selected based on clinical characteristics *i.e.*, never smokers, females, Asian ethnicity and adenocarcinoma

Table 2 Prevalence of epidermal growth factor receptor mutations and comparison of responses to gefitinib and erlotinib *n* (%)

Ref.	No. of patients	EGFR mutations no	E19 Del/E21 L858R	Response to prior gefitinib				Response to erlotinib			
				CR	PR	SD	PD	CR	PR	SD	PD
Garfield ^[9]	1	NA	NA				1(100)		1(100)		
Viswanathan <i>et al</i> ^[16]	5	NA	NA		4(80.0)		1(20.0)				5(100)
Walther <i>et al</i> ^[17]	1	NA	NA				1(100)		1(100)		
Chang <i>et al</i> ^[18]	1	1 (100)	1/0		1(100)				1(100)		
Cho <i>et al</i> ^[19]	21	5 (23.8)	5/0		6(28.6)	4(19.0)	11(52.4)		2(9.5)	4(19.0)	15(71.5)
Gridelli <i>et al</i> ^[32]	3	NA	NA			3(100)			1(33.3)	2(66.7)	
Kim <i>et al</i> ^[33]	1	NA	NA		1(100)				1(100)		
Costa <i>et al</i> ^[23]	18 ¹	17 (94.4)	4/13		11(84.6)	2(15.4)			1(7.7)	2(15.4)	10(76.9)
Lee <i>et al</i> ^[21]	23	3 (13.0)	3/0		15(65.2)	2(8.7)	6(26.1)		1(4.3)	1(4.3)	21(91.4)
Vasile <i>et al</i> ^[22]	8	NA	NA		4(50.0)	4(50.0)			2(25.0)	3(37.5)	3(37.5)
Wong <i>et al</i> ^[20]	14	7 (50.0)	4/3			9(64.3)	5(35.7)			5(35.7)	9(64.3)
Wong <i>et al</i> ^[34]	1	1 (100)	1/0		1(100)					1(100)	
Wu <i>et al</i> ^[35]	1	1 (100)	0/1		1(100)					1(100)	
Katayama <i>et al</i> ^[36]	7	6 (85.7)	4/2	2(28.6)	2(28.6)	3(42.8)			3(42.9)	3(42.9)	1(14.3)
Sim <i>et al</i> ^[24]	16	5 (31.3)	2/3		9(56.3)	2(12.5)	5(31.2)		1(6.3)	3(18.7)	12(75.0)
Zhou <i>et al</i> ^[25]	21	7 (33.3)	NA/NA	2(9.5)		8(38.1)	11(52.4)		2(9.5)	4(19.1)	15(71.4)
Wong <i>et al</i> ^[26]	21	3 (14.3)	0/3			18(85.7)	3(14.3)			12(57.1)	9(42.9)
Asami <i>et al</i> ^[27]	42	28 (66.7)	14/14		22(52.4)	17(40.5)	3(7.1)		1(2.4)	24(57.1)	17(40.5)
Hata <i>et al</i> ^[29]	125 ²	63 (50.4)	NA/NA	3(2.5)	68(56.2)	22(18.2)	28(23.1)		11(8.8)	44(35.2)	70(56.0)
Masuda <i>et al</i> ^[37]	3	3 (100)	2/1		3(100)				3(100)		
Shoji <i>et al</i> ^[38]	1	0 (0)	0 (0)				1(100)		1(100)		
Song <i>et al</i> ^[39]	20	5 (25.0)	3/2		5(25.0)	9(45.0)	6(30.0)			7(35.0)	13(65.0)
Takenaka <i>et al</i> ^[40]	1	1 (100)	1/0			1(100)			1(100)		
Saito <i>et al</i> ^[41]	21	12 (57.1)	0/12		16(76.2)	5(23.8)			2(9)	6(19)	13(62)
Tetsumoto <i>et al</i> ^[42]	2	2 (100)	1/1		2(100)				2(100)		
Koyama <i>et al</i> ^[43]	54	44 (81.5)	22/22	4(7.4)	32(59.3)	13(24.0)	5(9.3)	0 (0)	4(7.4)	30(55.6)	20(37.0)

¹Response to gefitinib and erlotinib was evaluable for 13 patients only; ²Response to gefitinib was evaluable for 121 patients only. EGFR: Epidermal growth factor receptor; PR: Partial response; CR: Complete response; PD: Progressive disease; SD: Stable disease; E19: Exon 19; E21: Exon 21; Del: Deletion; NA: Not available.

Table 3 Response rates and survival rates with erlotinib following gefitinib therapy

Ref.	RR	DCR	PFS	OS
Cho <i>et al</i> ^[19]	9.50%	28.60%	60 d	158 d
Costa <i>et al</i> ^[23]			2 mo	
Lee <i>et al</i> ^[21]	4.30%	8.70%		
Vasile <i>et al</i> ^[22]	25%	62.50%	5.9 mo	14.6 mo
Wong <i>et al</i> ^[20]		35.70%	97 d	
Sim <i>et al</i> ^[24]		25%	1.7 mo	
Zhou <i>et al</i> ^[25]	9.50%	28.50%	55 d	135 d
Wong <i>et al</i> ^[26]		57.10%	14.9 wk	40 mo
Asami <i>et al</i> ^[27]	2.40%	59.50%	3.4 mo	7.1 mo
Hata <i>et al</i> ^[29]	9%	44%	2 mo	11.8 mo
Song <i>et al</i> ^[39]	0	35%	31 d	4.2 mo
Saito <i>et al</i> ^[41]		38.10%		369 d
Koyama <i>et al</i> ^[43]	7.40%	63.00%	135 d	333 d

RR: Response rate; DCR: Disease control rate; PFS: Progression free survival; OS: Overall survival.

subtype. Only 3 of these 21 patients had EGFR mutation status known. Disease control was achieved in 18 patients (85.7%) with first line gefitinib therapy and in 12 out of these 18 patients (66.7%) who received erlotinib as salvage therapy. The overall DCR for erlotinib was 57.1% (12 out of 21). All 3 patients who progressed on gefitinib did not respond to erlotinib^[26].

In a retrospective review of 42 patients with lung adenocarcinoma receiving erlotinib with history of prior gefitinib treatment, PFS (4.7 mo *vs* 1.8 mo) and OS (9.2 mo *vs* 4.7 mo) were better in patients who had experienced prior response with gefitinib as compared to those who had not^[27]. On multivariate analyses for prognostic factors for OS, only prior response to gefitinib was found to be significant but not presence of EGFR mutations. Interestingly in this cohort, 69% of patients had a sensitizing EGFR mutation (exon 19 deletion or L858R mutation exon 21).

A systematic review included 3 prospective studies, 3 retrospective studies and 7 case reports leading to a total of 106 patients. Out of these 9.9% patients had PR, 18.9% SD and 70.8% PD. The DCR was 37.5% in patients who had EGFR mutations and 21.7% in patients without EGFR mutations, which was not statistically different. The mean PFS varied from 1.7 to 5.9 mo. Analysis showed that the only factors which predicted response to erlotinib were the presence of SD on initial treatment with gefitinib and the presence of PFS to initial gefitinib for more than 6 mo^[28].

In a large retrospective analysis of 125 patients all of whom had experienced disease progression following initial gefitinib therapy, the RR was 9%, DCR 44% and median PFS 2 mo with erlotinib treatment. Multivariate analysis showed that disease control was predicted by

Table 4 Pooled analysis of demographic profile, prevalence of epidermal growth factor receptor mutations and disease control rates with erlotinib following prior gefitinib therapy

Female gender : 60.6% (292 of 482)
Adenocarcinoma histology: 88.3% (421 of 477)
Non smokers: 64.5% (304 of 471)
East Asian ethnicity: 92.3% (445 of 482)
EGFR mutation positive status ¹ : 48.4% (224 of 463)
Disease control rate with prior gefitinib treatment: 79.4% (336 of 423)
Disease control rate with subsequent erlotinib treatment: 45.4% (194 of 427)

¹Indicates presence of sensitizing EGFR mutations (exon 19 deletion or exon 21 L858R mutation). EGFR: Epidermal growth factor receptor.

three factors: good performance status [Eastern Cooperative Oncology Group (ECOG) PS 0/1], EGFR mutation-positive status (or unknown) and benefit from prior gefitinib therapy. Longer PFS was predicted by insertion of cytotoxic chemotherapies between gefitinib and erlotinib therapies in addition to ECOG PS 0/1 and benefit from prior gefitinib therapy^[29].

In another retrospective review published recently, 44 of the 54 patients had a sensitizing *EGFR* gene mutation (exon 19 deletion or L858R mutation exon 21) and all 54 patients had received gefitinib initially. DCR of 63% was observed with subsequent erlotinib treatment. The authors observed no significant differences in erlotinib efficacy between EGFR-mutated NSCLC who had developed gefitinib resistance as compared to another 50 patients of NSCLC with wild-type EGFR in whom gefitinib had not been given earlier. This study also showed that presence of skin rash was associated with better outcomes—an observation that has been reported by others also^[30].

POOLED ANALYSIS

We also carried out pooled analysis of published literature involving patients who had received erlotinib treatment following prior gefitinib therapy. This has been summarized in Table 4. It was not possible to analyse objective RRs and SD separately since some of the publications included in this review had only provided DCRs which is the sum of objective responses (CR/PR) and SD. There are three important observations that are apparent from the pooled analysis. First, majority of the patients were of East Asian ethnicity, females, non-smokers and had adenocarcinoma histology all of which are clinical surrogates for presence of sensitizing EGFR mutations. Second, approximately one of every two subjects in the pooled population had a sensitizing EGFR mutation (exon 19 deletion or exon 21 L858R mutation). However, the true prevalence in this population database is likely to be higher than the figure of 48.4% that arose on pooled analysis because in several of the publications, results of EGFR mutation testing were either not mentioned or were performed in subgroup of patients included in the article. An indirect indicator that this was a highly enriched pooled population is the fact that approximately

Table 5 Potential factors predicting response to erlotinib following prior gefitinib therapy

Previous response to gefitinib (most important)
Longer duration of response to prior gefitinib
Female gender
Adenocarcinoma histology
Non smokers
East Asian ethnicity
EGFR mutation positive status ¹
Good performance status
Chemotherapy cycles in between gefitinib and erlotinib

¹Indicates presence of sensitizing EGFR mutations (exon 19 deletion or exon 21 L858R mutation). EGFR: Epidermal growth factor receptor.

four of five patients had initial disease control with gefitinib. Third, despite progressing on gefitinib treatment, approximately 45% of patients achieved disease control with erlotinib. However, it was not possible to carry out analysis regarding the molecular mechanisms that led to gefitinib failure or resistance since these have not been provided in majority of the publications.

RATIONALE

A satisfactory explanation as to cause of responsiveness to erlotinib after failure of gefitinib is yet to be given. However, several hypotheses have been put forward. One possibility is that gefitinib is usually given at a clinical dose of 250 mg, which is around one third of the maximum tolerated dose, about 750 mg. In contrast, the clinical dose of erlotinib is 150 mg, which is very close to the maximum tolerated dose. Therefore, the biological dose of these two drugs may not be equal. However, in the IDEAL 1 and 2 trials, higher doses of gefitinib were not associated with a better RR^[4,5,19].

Another possibility invokes the presence of both EGFR TKI sensitive and resistant clones at the start of treatment with gefitinib. The administration of gefitinib leads to the selective dying out of the sensitive clones and subsequent development of resistance. When the TKI is stopped, the sensitive clones again propagate and are the reason for the response to the subsequent TKI^[21]. This is borne out by case reports demonstrating that readministration of gefitinib to some patients after progression may sometimes lead to disease control similar to that achieved with erlotinib^[31].

Patients who develop resistance to EGFR TKIs, acquire common mutations such as T790M secondary mutation or amplification of the MET oncogene. Other secondary mutations have also been reported. Some mutations, such as the L748S or E884K mutation, may result in differing sensitivities to the oral EGFR TKIs, resulting in different tumour responses^[12,19,21].

An overview of the potential factors predicting clinical benefit with erlotinib is provided in Table 5. The most consistent predictive factor has been prior response to gefitinib. However the predictive role of presence of

EGFR mutations in this setting has not been as strong as it is for first line therapy. The clinical surrogates for presence of EGFR mutations namely, female gender, Asian ethnicity, adenocarcinoma histology and non-smoking status, are the other factors that remain associated with clinical benefit from erlotinib in this setting.

CONCLUSION

Oral EGFR TKIs are widely available drugs that are an important component of the therapeutic armamentarium against advanced NSCLC. The use of sequential EGFR TKIs, especially of erlotinib after prior treatment with gefitinib has remained a controversial area so far. Given the disease control rate of approximately 45% in the current pooled analysis, such an approach can be considered in carefully selected patients especially those in whom alternate treatment options are not being considered. The exact explanation of this response is still not known. Possible predictive factor for clinical benefit with erlotinib includes previous response to gefitinib. The predictive value of sensitizing EGFR mutations requires further evaluation and further studies are required to determine the underlying molecular mechanisms for observed clinical benefit with erlotinib in this setting.

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WJCO 5th Anniversary Special Issues (1): Lung cancer**Tumour suppressor HLJ1: A potential diagnostic, preventive and therapeutic target in non-small cell lung cancer**

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Abstract

Lung cancer is the leading cause of cancer-related mortality throughout the world. Non-small cell lung cancer (NSCLC) accounts for 85% of all diagnosed lung cancers. Despite considerable progress in the diagnosis and treatment of the disease, the overall 5-year survival rate of NSCLC patients remains lower than 15%. The most common causes of death in lung cancer patients are treatment failure and metastasis. Therefore, developing novel strategies that target both tumour growth and metastasis is an important and urgent mission for the next generation of anticancer therapy research. Heat shock proteins (HSPs), which are involved in the fundamental defence mechanism for maintaining cellular viability, are markedly activated during environmen-

tal or pathogenic stress. HSPs facilitate rapid cell division, metastasis, and the evasion of apoptosis in cancer development. These proteins are essential players in the development of cancer and are prime therapeutic targets. In this review, we focus on the current understanding of the molecular mechanisms responsible for HLJ1's role in lung cancer carcinogenesis and progression. HLJ1, a member of the human HSP 40 family, has been characterised as a tumour suppressor. Research studies have also reported that HLJ1 shows promising dual anticancer effects, inhibiting both tumour growth and metastasis in NSCLC. The accumulated evidence suggests that HLJ1 is a potential biomarker and treatment target for NSCLC.

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Key words: Non-small cell lung cancer; Metastasis; HLJ1; Anticancer; Biomarker

Core tip: HLJ1, a member of the human heat shock proteins 40 family, has been characterised as a tumour suppressor. Research studies have reported that HLJ1 shows promising dual anticancer effects, inhibiting both tumour growth and metastasis in non-small cell lung cancer (NSCLC). The accumulated evidence suggests that HLJ1 is a potential biomarker and treatment target for NSCLC. We propose a hypothetical model for the roles of HLJ1 stimulator in suppressing lung cancer tumorigenesis. Investigating the integrated and coordinated molecular mechanisms of HLJ1 may shed new light on the treatment of lung cancer. The development of drug targeting HLJ1 may be an effective approach for lung cancer therapy.

Tsai MF, Wang CC, Chen JJW. Tumour suppressor HLJ1: A potential diagnostic, preventive and therapeutic target in non-small cell lung cancer. *World J Clin Oncol* 2014; 5(5): 865-873 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i5/865.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i5.865>

INTRODUCTION

Lung cancer is the most common cause of cancer death in the world, accounting for 17% of all cancer deaths^[1,2]. Non-small cell lung cancer (NSCLC) is the predominant type of lung cancer^[3]. The three major types of NSCLC are squamous cell carcinoma, large cell carcinoma, and adenocarcinoma, but several other types occur less frequently^[4]. Traditional therapeutic strategies (chemotherapy and radiotherapy) are often associated with unsatisfactory outcomes in lung cancer patients, and the problem is exacerbated by early detection difficulties^[5]. A minority of patients (approximately 30%) with NSCLC present with an early stage of the disease and receive curative surgery. However, even in those patients, up to 40% will subsequently relapse within 5 years^[6,7]. Targeted molecular therapy has become an important part of therapeutic strategies for treating lung cancer. However, the major challenges confronting targeted cancer therapies are variable responsiveness and the development of drug resistance^[8]. There have been significant advances in cancer treatments owing to our knowledge of the mechanisms underlying cell signalling pathways; however, the prognosis for patients with locally advanced or metastatic disease is still ominous.

If lung cancer is diagnosed and treated before it metastasizes, the 5-year survival rate is approximately 50%-70%. Once metastasis has occurred, the 5-year survival rate drops to < 5%^[9]. Therefore, metastasis is the most critical parameter determining survival in lung cancer patients^[10]. Improving survival in lung cancer is a major challenge for modern oncology, particularly considering the 5-year survival rate remains under 15% across all stages of disease^[11]. Metastasis, the spread of tumour cells from their primary sites to secondary sites within the body, is a multiple-step process that requires the accumulation of altered expression of many different genes. This complex process involves cell adhesion, degradation of the surrounding extracellular matrix, migration, proliferation at a secondary site, and stimulation of angiogenesis^[12,13]. Many studies on cancer metastasis have been conducted, and several molecules that participate in tumour cell invasion and metastasis, such as SLUG, NM23, CD44, MTA1, MMPs, TIMPs, KAI1, E-cadherin, KISS1 and CRMP1, have been identified in various cancer types^[14-18]. There is still no effective strategy for preventing or combating these metastatic processes. Therefore, it is important to develop novel strategies that target both tumour growth and metastasis in NSCLC.

In this review, we focus on the current understanding of the molecular mechanisms responsible for the effects of HLJ1 in lung cancer. The role of HLJ1, a member of the human heat shock protein 40 (HSP40) family, in lung cancer development and progression has been extensively investigated^[19-21]. HLJ1 has been characterised as a tumour suppressor, and research studies have identified the protein's promising dual anticancer effects in NSCLC, inhibiting both tumour growth and metastasis^[19,22,23]. The accumulated evidence suggests that HLJ1 is a potential

biomarker for NSCLC and a potential target of drug development.

HEAT SHOCK PROTEINS

The heat shock response was first described in 1962^[24], and a number of investigations have revealed that the process is an essential defence mechanism for cellular viability. HSPs are named for their increased synthesis after heat shock. In addition to elevated temperature, HSPs are markedly induced by nutrient deprivation, oxidative stress, heavy metals, radiation, pathogen infection and other stress factors^[25]. Under normal conditions, HSPs perform essential biological functions such as modulating protein activity by changing protein conformation, serving as molecular chaperones in protein transport between cell organelles, promoting multiprotein complex assembly/disassembly, and ensuring proper folding of nascent and altered proteins^[25,26]. Many other more specific functions have been identified for particular HSP types and include roles in immunological processes, cell cycle regulation, transcriptional activation and signal transduction^[27-29].

Mammalian HSPs have been classified into the following six families according to molecular size: HSP100, HSP90, HSP70, HSP60, HSP40 and small HSPs^[30]. HSPs play fundamental roles in the processes of signal transduction, cell proliferation and survival, cell cycle progression and apoptosis, and in other features of malignant cells, including invasion, tumour angiogenesis and metastasis^[28,29]. In recent years, research studies have identified several different HSPs, in a variety of tumour types, that may be putative clinical biomarkers or molecular targets for cancer therapy. The dependence of cancer cells on HSP90 has been successfully exploited in therapeutics^[30,31]. While HSP40 therapies are in the nascent stages, HSP27 and HSP70 therapies have been successfully used with HSP90 inhibitors as part of dual inhibition treatments and with antineoplastic drugs in combinational therapy^[32].

HUMAN HSP40 AND CANCER

The HSP40 family, also known as DNAJ proteins, constitutes the largest and most diverse sub-group of HSPs^[33]. All DNAJ proteins contain the 70 amino acid J domain, which is essential for interaction with HSP70. The DNAJ proteins that have been identified in humans are divided into three subclasses. Based on the presence or absence of conserved domains, DNAJ proteins are classified as type I (DNAJA, 4 members), type II (DNAJB, 13 members) or type III (DNAJC, 32 members)^[34]. DNAJ proteins are widely recognised as regulators of HSP70 function, but they also have roles as co-chaperones in the HSP90 multi-chaperone complex^[35]. The function and role of DNAJ proteins is still poorly understood. Current research is focused on understanding the role of HSP40 isoforms in regulating HSP70 and their subsequent involvement in disease progression.

The DNAJ proteins are differentially expressed in hu-

man tissues and can act as both tumour suppressors and onco-proteins^[19,36,37]. There are approximately 49 genes that encode DNAJ proteins, and several of these genes encode for multiple splice variants that may have different biological functions and cellular locations^[34]. Tid1 (DNAJA3) has 2 isoforms, Tid1-L and Tid1-S, which may function differently. Tid1-L acts as a tumour suppressor by negatively regulating cell proliferation, cell invasion, and tumorigenicity in breast cancer, lung cancer, and head and neck squamous cell carcinoma^[36,38,39]. Tid1-L overexpression in lung cancer cell attenuates epidermal growth factor receptor (EGFR) signalling and inhibits cell proliferation, colony formation, and *in vivo* tumour growth. Low Tid1-L/high EGFR expression predicts poor overall survival in patients with lung adenocarcinoma. Tid1-L has been shown to act as a tumour suppressor (though Tid1-S has not) by inhibiting EGFR signalling through interaction with EGFR/HSP70/HSP90 and by enhancing EGFR ubiquitinylation and degradation^[36]. Research studies have also suggested that Tid1-L is a novel regulator of p53-mediated apoptosis and that the use of the enhanced Tid1-L function to promote mitochondrial localisation of p53 could be an effective therapy in many cancers^[40]. In contrast, overexpression of Tid1-S in renal clear cell carcinomas enhances MetR kinase activity, leading to an increase in hepatocyte growth factor-mediated cell migration. The binding of Tid1-S to MetR may stabilise the receptor in a ligand-competent state, and this stabilising function may influence conformational changes that take place during the catalytic cycle to promote kinase activation. Targeted inhibition of Tid1-S may be a useful therapeutic tool in the management of MetR-dependent malignancies^[37].

MRJ (DNAJB6) was found to reduce tumorigenicity and metastasis of melanoma and breast cancer cells^[41]. MRJ induces β -catenin degradation and may play a role in maintaining an epithelial phenotype^[42]. Furthermore, researchers have shown that loss of MCJ (DNAJC15) expression confers resistance to specific chemotherapeutics in ovarian cancer cells^[43,44]. In clinical ovarian cancer patients, low MCJ expression was correlated with poor prognosis and resistance to chemotherapy. ABCB1 (drug transporter) gene expression is mediated by increased levels of the c-Jun transcription factor in the absence of MCJ^[43,45]. In contrast to the data on HSP70 and HSP90, there is limited information available on the expression and function of most DNAJ proteins in cancer. However, it is evident from the specific DNAJ proteins that have been identified that these proteins may play an important role in affecting cancer properties.

HLJ1 EXPRESSION IN LUNG CANCER

HLJ1, consisting 337 amino acids, was first identified from the human liver cDNA library and was classified as a member of the HSP40 family^[46]. HLJ1, also known as DNAJB4, belongs to the type II homologues of the Hsp40 family and comprises the following four conserved functional domains: a highly conserved J do-

main, a glycine/phenylalanine-rich region, a cysteine-rich region, and a COOH terminal domain^[47]. Homology analysis showed that the amino acid sequence of HLJ1 has 84% similarity with HDJ1 (DNAJB1) isolated from human placenta^[48]. HLJ1 mRNA is highly expressed in skeletal muscle and in the heart, pancreas, brain, lung, and other organs but is weakly expressed in the liver and kidney. Although the *HLJ1* gene was cloned from the liver cDNA library, the expression of HLJ1 in liver tissue is relatively low^[46].

Gene expression profiles have been used to identify possible associations with lung cancer behaviour or clinical outcome to better predict patient prognosis. We have previously established a panel of lung adenocarcinoma cell lines with increased invasiveness (CL1-0, CL1-1, CL1-5 and CL1-5-F4) that were derived from a parent lung adenocarcinoma cell line by repeatedly selecting for more invasive cells^[49]. These selected sublines have shown greater invasive and metastatic potential than the parental cells. By using a complementary DNA microarray, we identified metastasis-associated genes on a genome-wide scale in model lung cancer cell lines^[50]. Cluster analysis of the complementary DNA microarray data revealed that 589 (6.1%) genes were positively or negatively associated with cancer cell invasiveness. Moreover, most of these genes were involved in angiogenesis, cell motility, adhesion and proliferation. *HLJ1*, one of the metastasis-associated genes identified by that study, has been characterised as a novel tumour suppressor, and high HLJ1 expression is associated with reduced cancer recurrence and prolonged survival in NSCLC patients^[19].

NSCLC is a heterogeneous disease, and patients with similar clinical-pathologic features may have a broad range of outcomes^[51,52]. It is important to identify a prognostic marker that can predict clinical outcomes in NSCLC patients. We have demonstrated that HLJ1 is a novel tumour suppressor in NSCLC. Primary cancer specimens from 71 patients with histologically confirmed NSCLC were analysed using reverse transcription-polymerase chain reaction, and the HLJ1 expression in tumour tissue was lower than in adjacent normal tissue in 55/71 (77%) of the patients. Moreover, in 49% of the samples, the tumour tissue showed at least a 2-fold decrease in HLJ1 compared to adjacent normal tissue. NSCLC patients with high HLJ1 expression had significantly longer overall survival and disease-free survival times compared to those with low HLJ1 expression. Clinically, HLJ1 is a significant, independent prognostic predictor of recurrence and overall survival in NSCLC patients^[19]. Tumour suppressor genes are frequently deactivated by genetic alterations, such as chromosomal deletions and loss-of-function mutations. The *HLJ1* gene is located on human chromosome 1P31.1. Loss of heterozygosity (LOH) on the short arm of chromosome 1 has been reported in many types of cancer^[53]. High frequencies of LOH and microsatellite instability in the HLJ1 region have been detected in NSCLC patients^[19].

Microarray analysis suggests that HLJ1 expression is negatively correlated with cancer invasiveness. HLJ1 is reportedly up-regulated in less-invasive cell lines than

in highly invasive cell lines^[20,50]. Restoration of HLJ1 expression in NSCLC cells inhibited cell proliferation, anchorage-independent growth, cell motility, invasion and tumorigenesis. HLJ1 slows cell cycle progression by increasing STAT1 and p21^{WAF1} expression and decreasing cyclin D1 expression. The same study also suggested that HLJ1 can affect the expression of many genes downstream in the STAT1 pathway, including p21^{WAF1}, ISGF3G, IFIT1, IFITM1, OAS3, and GIP2 and that HLJ1 can increase p21^{WAF1} expression by affecting a p53-independent pathway^[19,20].

E-cadherin is a well-known invasion suppressor in several types of carcinoma^[54]. In a previous microarray analysis, we found that expression of the Slug gene positively correlated with invasive ability. Overexpression of Slug suppressed E-cadherin expression and increased invasive ability. The study showed that Slug promoted metastasis in lung cancer by down-regulating E-cadherin, up-regulating matrix metalloproteinase 2 (MMP2) and enhancing angiogenesis^[17]. We also found that HLJ1 indirectly up-regulated E-cadherin expression by inhibiting the repressive effect of the Slug gene on the E-cadherin proximal promoter^[20]. Recently, research studies have also indicated that HLJ1 acts as a molecular chaperone of E-cadherin that is able to sense E-cadherin folding, providing stabilisation of native folded E-cadherin in the plasma membrane or degradation of an unfolded counterpart. Post-translational regulation of E-cadherin by the HLJ1 molecular chaperone is sufficient to influence gastric cell invasion. HLJ1 is a sensor of E-cadherin structure features that might contribute to gastric cancer progression. Additionally, the expression of HLJ1 and E-cadherin is concomitantly decreased in patients with human gastric carcinoma^[55].

Nucleophosmin (NPM1) is a nucleolar phosphoprotein that localises in granular regions of the nucleolus and is highly expressed in malignant and actively dividing cells^[56]. NPM1 shuttles continuously between the nucleus and cytoplasm and acts as a multifunctional protein that plays an important role in the increased nucleolar activity needed for cell proliferation^[56,57]. Studies have proposed that the NPM1 protein has both oncogenic and tumour-suppressing effects, depending on its level of expression and cellular localisation^[58]. HLJ1 modulates NPM1 oligomerisation and NPM1-AP-2 α multi-protein complex formation, which alter AP-2 α transcriptional activity. These changes then suppress the expression of downstream genes such as MMP2 and, as a result, decrease lung cancer cell invasiveness^[22].

Evasion of apoptosis is a hallmark of most cancers^[59]. Therefore, it is important to identify genes that promote apoptosis in cancer cells, either under normal or stressful conditions, such as radiotherapy and chemotherapy. Several recent studies indicate that HSPs play important cytoprotective roles and are involved in regulating the apoptosis pathway^[60]. HLJ1 can promote cancer cell sensitivity to ultraviolet (UV) stress-induced apoptosis by enhancing c-Jun N-terminal kinase (JNK) activation and caspase activity^[61]. Moreover, the HLJ1 protein is a novel

substrate of caspase-3, which is followed by protein degradation during the apoptotic process. HLJ1 appears to play an important role in apoptosis. However, further studies are necessary to determine the underlying mechanism of HLJ1 in UV-induced apoptosis and the effects of reduced HLJ1 in late apoptosis^[61].

TRANSCRIPTIONAL REGULATION OF HLJ1

Elucidation of the roles and the regulatory mechanism of tumour suppressors may facilitate the development of rational therapeutic targets that inhibit cancer cell proliferation, angiogenesis and metastasis. YY1 is a 65-kDa multifunctional zinc-finger transcription factor belonging to the human GLI-Kruppel family of nuclear proteins^[62]. It can bind to a specific DNA consensus sequence, 5'-CGCCATNTT-3', which is present in many promoters. YY1 can either activate or repress the target genes, depending on the cofactors that it recruits^[62,63]. YY1 is a complex protein that plays pivotal roles in cell development, differentiation, proliferation, and apoptosis^[64,65]. Because the expression and function of YY1 are intimately associated with cell cycle progression, its physiologic significance has recently been applied to models of cancer biology^[64]. YY1 overexpression has been demonstrated in several human cancers such as breast cancer, prostate cancer, cervical cancer, brain cancer and colon cancer^[65,66]. However, functional and clinical analysis of the YY1 in NSCLC remains unclear. As a transcription factor, YY1 regulates the expression of numerous genes that are mostly involved in tumorigenesis. The HLJ1 promoter contains four YY1-binding sites that positively regulate HLJ1 expression. When compared with the WT construct, the mutants of these four potential YY1-binding sites resulted in different levels of reduction in HLJ1 promoter activity, from 17% to 34%, respectively^[20]. However, deletion of all four YY1-binding sites reduced the HLJ1 promoter activity by 93%, indicating that YY1 regulation plays an important role in HLJ1 expression. Overexpression of YY1 in NSCLC cells indicated that up-regulates the HLJ1 expression by directly binding to the promoter region, thus inhibiting cancer cell invasion^[20]. In addition, an enhancer segment was identified in the *HLJ1* gene at -2125 to -1039 bp upstream of the transcription start site, which includes the activator protein 1 (AP-1) site. The activation and synergistic up-regulation of the tumour suppressor HLJ1 is the result of the binding of the transcription factors AP-1 and YY1 to the gene's enhancer and promoter regions, respectively^[21].

Hepatitis B virus (HBV) is a major cause of human hepatocellular carcinoma (HCC). HBV proteins promote migration-related factors such as MT1-MMP, MMP9, and hypoxia-inducible factor 1- α and contribute to the HCC metastatic process^[67-69]. However, HBV could also promote HLJ1 expression in HCC cells by up-regulating the transcription factor YY1. The role of the HLJ1 in HCC cells still unclear^[70].

HLJ1 AS A MOLECULAR TARGET IN ANTICANCER THERAPIES

Curcumin (diferuloylmethane), a natural compound derived from the spice turmeric (*Curcuma longa*), has been used in the treatment of various inflammatory diseases in traditional Indian and Chinese medicine^[71]. The anticancer or chemopreventive effects of curcumin are the result of a variety of molecular mechanisms, including its direct or indirect interaction with various transcription factors, regulatory proteins and enzymes that all play a central role in key cancer-related processes such as inflammation, proliferation, survival, migration, angiogenesis, invasion and metastasis^[72,73]. Curcumin may have potential as a multi-target drug in anticancer therapy^[74-76]. Previous evidence has shown that AP-1 complexes enriched with c-Jun and JunB may result in morphologic alterations and anchorage-independent cell growth, whereas complexes enriched with JunD showed anti-proliferative effects. It has been suggested that curcumin inhibits tumour growth by inhibiting AP-1 activation^[77,78]. Curcumin increases JunD expression, stimulates HLJ1 enhancer activity, and triggers HLJ1 expression, which may subsequently reduce filopodia formation and up-regulate E-cadherin expression. Curcumin may inhibit cancer cell migration and invasion not only by inhibiting MMP2, MMP9 and MMP14 but also through HLJ1/E-cadherin induction^[23,74]. Curcumin may be a template for new antitumor drug developments that target the tumour suppressor HLJ1^[23].

Dimethyl sulfoxide (DMSO) is an amphipathic molecule that has a highly polar domain and two apolar methyl groups, making it soluble in both aqueous and organic media^[79]. DMSO is commonly used as a very efficient solvent for water-insoluble compounds in biological studies and as a cryoprotectant for cultured cells^[80]. In particular, DMSO has been approved by the United States Food and Drug Administration for the treatment of interstitial cystitis^[81]. DMSO was also used to treat leukaemia for several years, based on its ability to induce cellular differentiation, which caused leukaemia cells to lose their proliferative properties^[82,83]. DMSO has been found to arrest the cell cycle at the G1 phase in lymphoid cell lines^[84]. Additionally, DMSO treatment can modulate AP-1 activity, and DMSO is involved in the suppression of intercellular adhesion molecule 1 expression in a rat model of peritonitis sepsis^[85]. Our results suggest that DMSO is an important stimulator of the tumour suppressor protein HLJ1, and DMSO works by activating JunB and JunD in highly invasive lung adenocarcinoma^[86]. These efforts will help us to develop not only novel anticancer drugs that affect lung cancer progression but also new therapeutic strategies for the disease. For instance, a therapeutic strategy combining both induced expression of HLJ1 by DMSO-derived analogues and irradiation would synergistically increase the efficacy of radiotherapy and prolong lung cancer patient survival.

Recently, several types of herbal compounds were proven to be potential anti-cancer drugs. These com-

pounds, including curcumin from a spice turmeric^[74], epigallocatechin-3-gallate from green tea^[87] and lycopene from tomato^[88], could target important mechanisms in tumour growth and metastasis^[89]. Screening drugs from traditional Chinese medicine has been suggested as a shortcut in searching for new leading compounds. Using the HLJ1 promoter and luciferase reporter assays, the HLJ1-targeting drug-screening platform was established to screen and identify traditional Chinese herbs that can target the novel tumour suppressor gene HLJ1. Among the herbal drugs identified, the andrographolide is a promising new anticancer agent that could significantly induced HLJ1 expression and suppress tumour growth and invasion in NSCLC^[90]. Andrographolide, a diterpenoid lactone isolated from the Chinese herbal medicine *Andrographis paniculata*, is known for its wide pharmacological activities, such as its anti-inflammatory, anti-angiogenesis, pro-apoptosis and anticancer activities^[91]. Moreover, *Andrographis paniculata* has long been perceived as safe in traditional Chinese medicine as well as in the traditional medicine in Thailand and India. *Andrographis paniculata* is genotoxically safe and has been applied to clinical investigations on the treatment and prevention of upper respiratory tract infections^[92,93].

Previously, andrographolide was reported to inhibit colorectal cancer cell invasion and migration by suppressing the activity of c-Fos and c-Jun and thus reducing MMP7 expression^[94]. Andrographolide was also reported to suppress invasion and migration in lung cancer cells through attenuation of the PI3K/Akt signalling pathway^[95]. The HLJ1-targeting drug-screening platform is useful for screening novel anticancer compounds. Using this platform, we identified andrographolide as a promising new anticancer agent that could suppress tumour growth and invasion in NSCLC. The onco-suppressive effects of andrographolide may be partially mediated by JunB-regulated HLJ1 expression, which modulates the transcription factor AP-2 α binding at the MMP2 promoter and represses the expression of MMP2^[90]. In addition, silencing HLJ1 partially reverses the inhibition of cancer cell invasion by andrographolide. The results also establish the potential for using andrographolide as a multi-target lead compound in developing anti-cancer therapies.

CONCLUSION

In the past decade, improved understanding of the molecular mechanism of HLJ1 in NSCLC progression has been greatly appreciated. Accumulated data support HLJ1 as a novel tumour suppressor and a potential druggable target for NSCLC. Restoration of HLJ1 expression in NSCLC cells inhibits cell proliferation, anchorage-independent growth, cell motility, invasion, and tumourigenesis. HLJ1 can inhibit cell cycle progression by increasing the STAT1 and p21^{WAF1} pathways and by decreasing cyclin D1 expression. The activation of the STAT1 pathway by HLJ1 was independent of p53. We

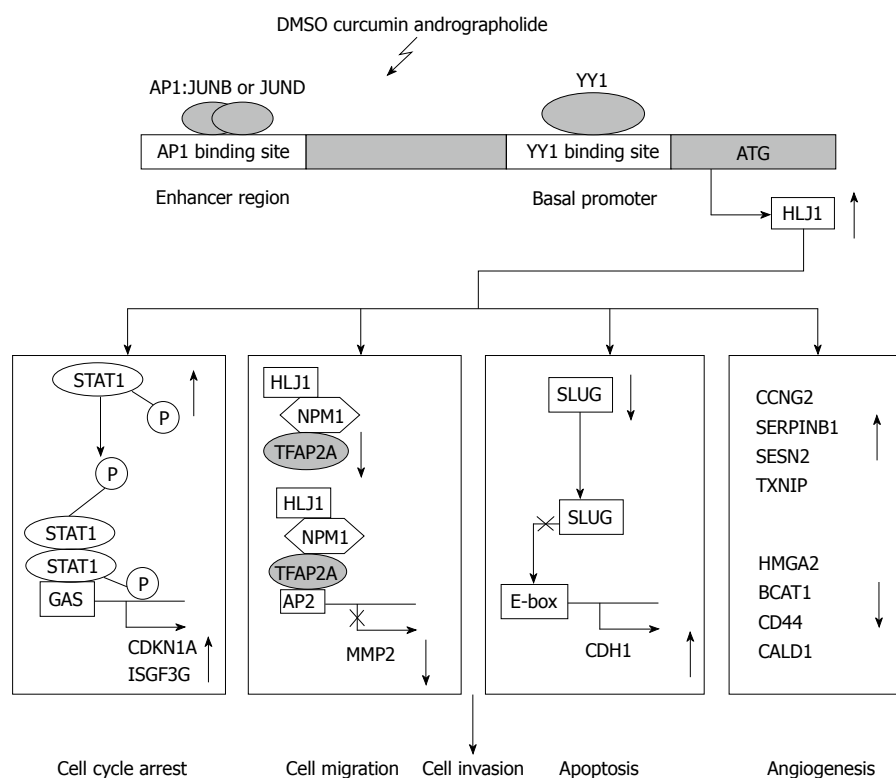


Figure 1 Schematic summary of the molecular mechanisms of HLJ1 in suppressing lung cancer tumorigenesis. The endogenous transcriptional expression of HLJ1 is the result of the binding of the transcription factors AP1 and YY1 to the gene's enhancer and promoter regions, respectively. HLJ1 inhibited lung cancer cell proliferation, anchorage-independent growth, cell motility, invasion and tumorigenesis through STAT1/p21WAF1 pathway, HLJ1/NPM1/AP2- α complex, SLUG/E-cadherin pathway and modulated cancer-related genes. DMSO: Dimethyl sulfoxide; AP1: Activating protein 1; GAS: Interferon-gamma activated sequence; CDKN1A: Cyclin-dependent kinase inhibitor 1A; YY1: Yin yang 1; STAT1: Signal transducer and activator of transcription 1; ISGF3G: Interferon-stimulated gene factor 3 gamma; NPM1: Nucleophosmin 1; TFAP2A: Transcription factor AP2 alpha; AP2: Activator protein 2; MMP2: Matrix metalloproteinase 2; SLUG: Snail homologue 2; CDH1: Cadherin 1; CCNG2: Cyclin G2; SERPINB1: Serine proteinase inhibitor B1; SESN2: Sestrin 2; TXNIP: Thioredoxin interacting protein; HMG2: High-mobility group AT-hook 2; BCAT1: Branched-chain aminotransferase 1; CALD1: Caldesmon 1; E-box: E-box transcription factor binding site.

also found that HLJ1 indirectly up-regulates E-cadherin expression through inhibiting the repression effect of the Slug gene on the E-cadherin proximal promoter. Increased HLJ1 expression is associated with prolonged disease-free and overall survival of patients with NSCLC. The endogenous transcriptional expression of HLJ1 is up-regulated through the binding of the enhancer AP-1 to its promoter YY1 with the co-activator p300 and the formation of bending DNA structure. Importantly, HLJ1 was reported to promote UV-induced apoptosis through JNK and caspase-3 activation in NSCLC. HLJ1 is a novel substrate of caspase-3 and is degraded at a late stage of apoptosis^[51]. In this review, we summarize the molecular mechanisms of the HLJ1 involved in lung cancer progression and propose a hypothetical model for the roles of HLJ1 stimulator in suppressing lung cancer tumorigenesis (Figure 1). Due to its tumour suppressor properties, HLJ1 is a potential target for anticancer therapy. Targeted induction of HLJ1 is a promising approach for cancer therapy, which also means that curcumin, DMSO, and andrographolide may serve as potential lead compounds or coordinated ligands for the development of novel anti-cancer drugs. Investigating the integrated and coordinated molecular mechanisms of HLJ1 may shed new light on the treatment of lung cancer. The develop-

ment of drug targeting HLJ1 may be an effective approach for lung cancer therapy.

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WJCO 5th Anniversary Special Issues (2): Breast cancer**Polymorphisms in base excision repair genes: Breast cancer risk and individual radiosensitivity**

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Abstract

Breast cancer (BC) is the most common cancer among women worldwide. The aetiology and carcinogenesis of BC are not clearly defined, although genetic, hormonal, lifestyle and environmental risk factors have been established. The most common treatment for BC includes breast-conserving surgery followed by a standard radiotherapy (RT) regimen. However, radiation hypersensitivity and the occurrence of RT-induced toxicity in normal tissue may affect patients' treatment. The role of DNA repair in cancer has been extensively investigated, and an impaired DNA damage response may increase the risk of BC and individual radiosensitivity. Single nucleotide polymorphisms (SNPs) in DNA repair genes may alter protein function and modulate DNA repair efficiency, influencing the development of various cancers, including BC. SNPs in DNA repair genes have also been studied as potential predictive factors for the risk of RT-induced side effects. Here, we review the literature on the association between SNPs in base excision repair (BER) genes and BC risk. We focused

on X-ray repair cross complementing group 1 (*XRCC1*), which plays a key role in BER, and on 8-oxoguanine DNA glycosylase 1, apurinic/apyrimidinic endonuclease 1 and poly (ADP-ribose) polymerase-1, which encode three important BER enzymes that interact with *XRCC1*. Although no association between SNPs and radiation toxicity has been validated thus far, we also report published studies on *XRCC1* SNPs and variants in other *BER* genes and RT-induced side effects in BC patients, emphasising that large well-designed studies are needed to determine the genetic components of individual radiosensitivity.

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Key words: Breast cancer; Polymorphisms; Base excision repair; Susceptibility; Radiosensitivity

Core tip: Single nucleotide polymorphisms (SNPs) in DNA repair genes may modulate DNA repair efficiency, influencing the development of various cancers, including breast cancer (BC). SNPs in DNA repair genes have also been studied as predictors for the risk of radiotherapy-induced side effects. We reviewed the literature on the association between SNPs in base excision repair (BER) genes and BC risk. We focused on X-ray repair cross complementing group 1, 8-oxoguanine DNA glycosylase 1, apurinic/apyrimidinic endonuclease 1 and poly (ADP-ribose) polymerase-1, which encode four important BER proteins. We also report published studies on SNPs in BER genes and individual radiosensitivity in BC patients.

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INTRODUCTION

Female breast cancer (BC) is the most prevalent malignancy worldwide, with 5.2 million cases diagnosed between 2004 and 2008^[1], and it is the primary cause of cancer-related death among women (<http://globocan.iarc.fr>).

Breast cancer is considered a multifactorial disease, and its occurrence is related to genetic, reproductive, environmental and lifestyle factors.

Breast cancer susceptibility has a complex genetic basis, which has been widely investigated over the past 20 years. Linkage analysis and a candidate gene approach have been used to identify susceptibility loci for BC with high and moderate penetrance. Rare mutations, principally in genes involved in DNA repair such as *BRCA1*, *BRCA2*, *TP53*, *ATM*, *CHEK2*, *PALB2* and *BRIP1*, are associated with a greatly increased risk of BC^[2].

Case-control and, in recent years, genome-wide association studies (GWAS) have identified more than 70 low-penetrance common variants [single nucleotide polymorphisms (SNPs)] associated with BC risk. Although many of the associations identified in GWAS are localised in non-coding regions of the genome, which are most likely involved in gene expression regulation, some common patterns have been identified among the BC susceptibility loci. Low-penetrance BC loci are related to mammary gland development, the DNA repair pathway, growth factors, the cell cycle, differentiation and apoptosis^[3].

Common SNPs in DNA repair genes have been extensively investigated in candidate-gene and case-control association studies in the context of breast carcinoma and other types of cancer. SNPs in such genes can affect the efficiency of the DNA repair machinery and contribute to genomic instability and cancer development^[4].

Among the different DNA repair pathways, base excision repair (BER) is responsible for the repair of bases damaged by the effects of X-rays, reactive oxygen radicals and alkylating agents. Many epidemiological studies have investigated the association between common variants in BER genes and human cancer, including breast cancer^[5]. Furthermore, DNA repair genes, including BER genes, have been extensively examined in several epidemiological studies to determine their association with radiation-induced toxicity in cancer patients undergoing radiotherapy (RT)^[6]. In fact, inter-individual differences in response to therapeutic radiation exposure have been observed, and this variability may be influenced by genetic factors affecting DNA repair efficiency^[7]. The side effects induced by RT in normal tissue largely depend on the capacity of cells to repair DNA damage caused by irradiation.

Here, we survey association studies on the most common variants in BER genes, evaluating their role in BC susceptibility and in the risk of developing adverse reactions after RT.

BER PATHWAY

The BER pathway is the primary mechanism that pro-

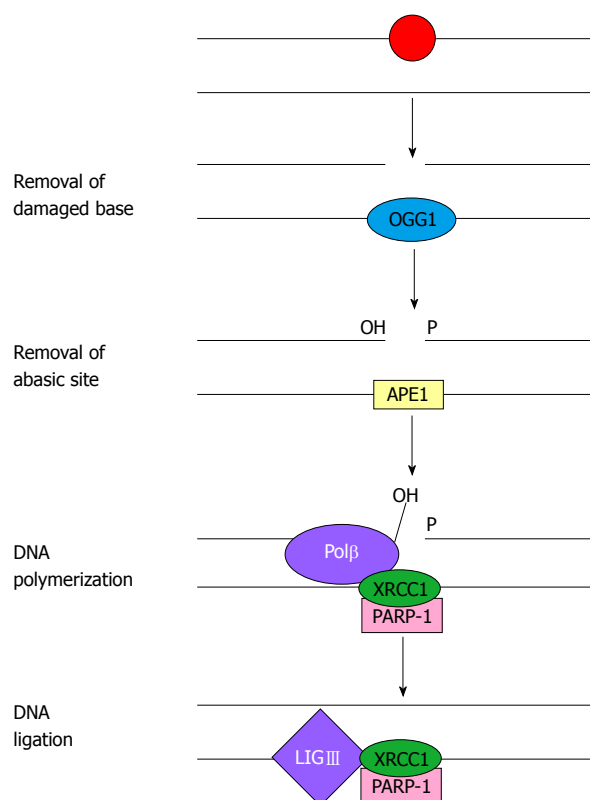


Figure 1 Base-excision repair. Simplified schematic representation of the short-patch base-excision repair pathway showing the key steps and main proteins involved in the repair of a damaged DNA base. Adapted from Costa *et al.*^[8]. OGG1: 8-oxoguanine DNA glycosylase 1; APE1: Apurinic/aprimidinic endonuclease 1; PARP-1: Poly (ADP-ribose) polymerase-1; XRCC1: X-ray repair cross complementing group 1; Polβ: DNA polymerase-β; LIG III: DNA ligase III.

ducts cells from oxidative DNA damage; it acts on small DNA lesions or modified bases, where it removes and replaces the damaged base^[4]. This process starts with the release of the damaged base by base-specific DNA glycosylases (*e.g.*, the oxidised base 8-oxoguanine is excised by 8-oxoguanine DNA glycosylase), followed by the cleavage of the sugar-phosphate chain, excision of the apurinic/aprimidinic (AP) site by endonuclease action, DNA synthesis and ligation (Figure 1). This pathway is referred to as short-patch BER and results in the replacement of the AP site *via* the incorporation of a single nucleotide. In contrast, the long-patch BER pathway produces a repair tract of at least two nucleotides^[8,9].

Enzymes involved in BER include 8-oxoguanine DNA glycosylase 1 (OGG1); AP endonuclease 1 (APE1 or APEX1), which excises the abasic residue; poly (ADP-ribose) polymerase-1 (PARP-1), which binds DNA-containing strand breaks; polynucleotide kinase; DNA polymerase-β (Polβ); and DNA ligase III (LIG III), which completes the restoration phase.

Another protein that plays a central role in BER is X-ray repair cross-complementing group 1 (XRCC1), which is a scaffold protein that coordinates both the initial and late steps of abasic site restoration *via* multiple protein-protein interactions^[10]. XRCC1 stabilises OGG1 on an AP site present in double-stranded DNA until APE1 is able to bind to the DNA, allowing the transfer

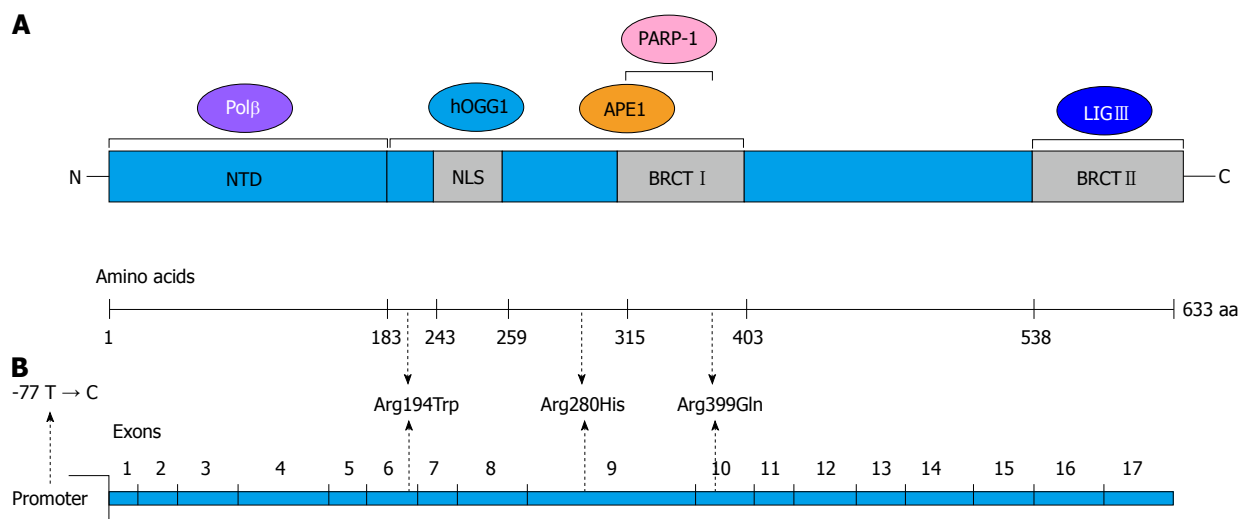


Figure 2 Domains of the X-ray repair cross-complementing group 1 protein and X-ray repair cross-complementing group 1 gene structure. A: The schematic diagram shows the regions of interaction with other base excision repair proteins; B: The diagram shows the structure of XRCC1 with the locations of the most common and well-studied single nucleotide polymorphisms: 77 T > C, Arg194Trp, Arg280His and Arg 399Gln. Modified from Sterpone *et al*^[14]. XRCC1: X-ray repair cross-complementing group 1; OGG1: 8-oxoguanine DNA glycosylase 1; APE1: Apurinic/apyrimidinic endonuclease 1; PARP-1: Poly (ADP-ribose) polymerase-1; Polβ: DNA polymerase-β; LIGIII: DNA ligase III; NTD: N-terminal domain; NLS: Nuclear localisation signal; BRCT: BRCA1 carbox-terminal domain.

of the DNA substrate from the DNA glycosylase product to the AP endonuclease. Polβ is then recruited by its interactions with APE1 and XRCC1. The binding of XRCC1 with PARP-1 also plays a role in BER. Moreover, XRCC1 is essential for the stabilisation of LIGIII.

Interactions between XRCC1 and its BER partners are mediated by different domains of the protein, which introduces the possibility that all four proteins, namely XRCC1, Polβ, PARP-1 and LIGIII, could form a single complex^[11]. Some of the interactions between BER proteins are illustrated in Figure 1.

Many polymorphisms have been identified in genes encoding proteins involved in BER and in other DNA repair pathways^[12]. In particular, many epidemiological studies have been performed to evaluate the association between XRCC1 SNPs and different types of cancer^[13].

SNPs IN BER GENES AND BREAST CANCER RISK

XRCC1 gene

The human XRCC1 gene maps to chromosome 19q13.2 and encodes a scaffold protein of 633 amino acids that plays a major role in the BER pathway and is also involved in other DNA repair mechanisms, such as single-strand break repair and non-homologous end joining. XRCC1 interacts with several BER proteins such as DNA polymerase β, APE1, OGG1, PARP-1 and LIGIII^[14]. A schematic representation of XRCC1 and its interactions is shown in Figure 2A.

Among the many SNPs found in the XRCC1 gene, three polymorphisms resulting in non-conservative amino acid substitutions have been identified: Arg194Trp (rs1799782), Arg280His (rs25489) and Arg399Gln (rs25487)^[15]. Another XRCC1 variant located in the 5'-un-

translated region (5'UTR), -77 T > C (rs3213245), was identified in 2004^[16]. The structure of the XRCC1 gene and the localisation of the most common SNPs are illustrated in Figure 2B. XRCC1 variants could affect the function of the protein and impair DNA repair efficiency. In particular, the XRCC1 Arg399Gln variant has been the subject of many case-control studies to investigate its possible association with breast cancer risk. The published data on this association, which are often contradictory, have been collected in several meta-analyses^[17-20] (Table 1). Huang *et al*^[17] showed that the Arg399Gln SNP is associated with a trend of increased breast cancer risk using both dominant and recessive models. In ethnic subgroups, and considering the recessive model, this polymorphism is associated with BC risk in Asians and Africans, although it is weakly related with breast cancer in Caucasians. The association between the Arg399Gln variant and BC risk was confirmed in Asian and African populations^[19], whereas other studies confirmed these data only among Asians^[18,20]. These findings suggest that the role of the XRCC1 Arg399Gln SNP as a risk factor for breast cancer may differ between Caucasian and Asian populations.

A very recent meta-analysis of 297 case-control studies evaluated the association between the XRCC1 Arg399Gln polymorphism and overall cancer risk^[13]; a significantly increased cancer risk was found in all genetic models. In stratified analyses by cancer type, significantly enhanced breast cancer risk was observed in Asians and particularly in Indians.

XRCC1 haplotypes

Some case-control studies assessing the association between XRCC1 haplotype and susceptibility to breast cancer were collected in a meta-analysis^[21]. Haplotypes for the most common non-synonymous XRCC1 SNPs

Table 1 List of meta-analyses on X-ray repair cross-complementing group 1 single nucleotide polymorphisms and haplotypes and risk of breast cancer

Ref.	XRCC1 SNPs	Number of studies analysed	Result
Huang <i>et al.</i> ^[17]	Arg399Gln	37	The 399Gln variant allele is associated with an increased risk of BC
	Arg194Trp	18	
	Arg280His	8	
Li <i>et al.</i> ^[18]	Arg399Gln	40	The recessive effect of the 399Gln variant allele increases the risk of BC (significant only in Asians)
	Arg194Trp	21	
	Arg280His	9	
Wu <i>et al.</i> ^[19]	Arg399Gln	44	This SNP is associated with increased BC risk in Asians and Africans
Saadat <i>et al.</i> ^[20]	Arg399Gln	36	This SNP is associated with increased BC risk in Asians
Yi <i>et al.</i> ^[13]	Arg399Gln	54	This SNP is associated with increased risk of BC in Asians and Indians
	XRCC1 haplotypes		
Saadat ^[21]	Arg399Gln	10	The Arg194-Gln399 haplotype is associated with increased BC risk in Asians
	Arg194Trp		

XRCC1: X-ray repair cross-complementing group 1; SNP: Single nucleotide polymorphism; BC: Breast cancer; Arg: Arginine; Gln: Glutamine; His: Histidine; Trp: Tryptophan.

(Arg194Trp and Arg399Gln) were considered, and the analysis showed a slight increased risk of BC associated with the Arg194-Gln399 haplotype in comparison with the Arg194-Arg399 haplotype. In the stratified analysis according to geographic location, the Arg194-Gln399 haplotype was significantly associated with breast cancer risk among individuals from Asian countries, while no association has been found between the *XRCC1* haplotype and BC susceptibility in Caucasian populations.

Thus far, a few *XRCC1* haplotype analyses have included the -77 T > C SNP in the 5'UTR^[22-24]. Two studies^[22,24], one performed on French and the other performed on Chinese BC patients and controls, considered *XRCC1* SNPs at position -77 and at codons 194, 280 and 399. Brem *et al.*^[22] found that the haplotype carrying the variant allele at codon 280 and the wild-type (wt) alleles at the other positions was associated with an increased risk of BC, although the association was not significant. In contrast, Liu *et al.*^[24] observed a significantly higher BC risk for the haplotype containing the variant allele at position -77 and the wt alleles at other positions.

In a case-control study on Caucasian BC patients, Sterpone *et al.*^[23] performed a haplotype analysis based on *XRCC1* genotypes at position -77 and at codons 194 and 399. The haplotype containing the wt allele at position 194 and the variant alleles at other positions was significantly associated with a higher BC risk in this study.

OGG1 gene

The human *OGG1* gene is located on chromosome 3p26.2 and encodes the key enzyme in the repair of 8-oxoguanine, which is one of the most common products generated by exposure to reactive oxygen species^[25]. Among the SNPs in *OGG1*, the most studied variant is the functional substitution Ser326Cys (rs1052133), which is located in exon 7 of the *OGG1* gene and causes an amino acid change (from serine to cysteine). Comparative functional analysis *via* a complementation assay of a defective *Escherichia coli* mutant revealed that the repair activity of the 326Ser protein was higher than that of the

326Cys protein^[26]. Therefore, this *OGG1* polymorphism may result in changes in DNA repair activity in human cells.

Many studies that have highlighted the association between the *OGG1* Ser326Cys SNP and breast cancer risk have produced conflicting results. To clarify the findings, several meta-analyses have been performed (Table 2)^[27-29]. Yuan *et al.*^[27] collected data published from 2003 to 2008 and evaluated the association between the *OGG1* Ser326Cys SNP and BC onset according to menopausal status and ethnicity by performing a stratified analysis. This meta-analysis suggested that the 326Cys allele had a significant protective effect against BC in European women, both in the additive (326Cys allele *vs* 326Ser allele) and dominant (Cys/Cys + Cys/Ser *vs* Ser/Ser) genetic models. No significant association was found between this SNP and menopausal status or other ethnicities. These results were discussed by Ding *et al.*^[28], who combined all of the studies on European populations and performed a new meta-analysis, which indicated a lack of association between the *OGG1* 326Cys allele and BC risk for this ethnicity. This result was confirmed by the meta-analysis performed by Gu *et al.*^[29], who identified 11 case-control studies published from 2003 to 2009; however, they did not observe any significant association between Ser326Cys SNP and BC risk, even in the analyses stratified by ethnicity, source of controls and menopausal status. An additional case-control study not included in the aforementioned meta-analysis also showed a lack of association between the *OGG1* Ser326Cys variant and BC susceptibility^[30].

Nevertheless, a recent study in a Korean population^[31] revealed that different SNPs in BER genes (including the *OGG1* Ser326Cys and rs2072668 SNPs) function in combination to increase the risk of breast cancer. Another recent study showed that *OGG1* 326Cys was significantly associated with an increased BC risk in specific subgroups of Chinese Han women (younger than 55 years, premenopausal, triple-negative or p53-positive)^[32].

The Ser326Cys SNP was also significantly associated

Table 2 List of meta-analyses on 8-oxoguanine DNA glycosylase 1, apurinic/apyrimidinic endonuclease 1 and poly (ADP-ribose) polymerase-1 single nucleotide polymorphism and risk of breast cancer

Ref.	BER gene	SNPs	Number of studies analysed	Result
Yuan <i>et al</i> ^[27]	<i>OGG1</i>	Ser326Cys	10	This SNP is significantly associated with a protective effect against BC in European subjects (additive and dominant model)
Ding <i>et al</i> ^[28]	<i>OGG1</i>	Ser326Cys	4	There was a lack of association between this SNP and BC risk in a European population
Gu <i>et al</i> ^[29]	<i>OGG1</i>	Ser326Cys	11	There was a lack of association between this SNP and BC risk
Wei <i>et al</i> ^[33]	<i>OGG1</i>	Ser326Cys	12	This SNP did not have a significant effect on BC
Wu <i>et al</i> ^[35]	<i>PARP-1 (ADPRT)</i>	Val762Ala	6	There was no association between this SNP and BC (all genetic models)
Wu <i>et al</i> ^[35]	<i>APE1</i>	Asp148Glu	5	There was no association between this SNP and BC (all genetic models)

OGG1: 8-oxoguanine DNA glycosylase 1; APE1: Apurinic/apyrimidinic endonuclease 1; PARP-1: Poly (ADP-ribose) polymerase-1; SNP: Single nucleotide polymorphism; BC: Breast cancer; BER: Base excision repair; Ser: Serine; Cys: Cysteine; Val: Valine; Ala: Alanine; Asp: Aspartic acid; Glu: Glutamic acid; ADPRT: ADP ribosyl transferase.

with overall cancer risk in a more recent meta-analysis^[33], and it showed a stronger association with lung cancer risk.

Therefore, there is evidence that the *OGG1* Ser326Cys polymorphism is associated with cancer risk, and in particular that it may be a low-penetrance susceptibility factor for lung cancer. Moreover, the Ser326Cys variation could interact with other factors such as age, triple-negative status and p53-positive status, thereby influencing breast cancer carcinogenesis.

PARP-1 and APE1 genes

PARP-1, also referred to as ADP ribosyl transferase, and APE1 are two of the most important enzymes in the BER pathway.

The human *PARP-1* gene is localised to chromosome 1q41-42 and encodes a nuclear protein that specifically recognises and binds DNA strand breaks. PARP-1 also recruits other BER proteins, including the XRCC1-LIG III complex, to facilitate the core BER reaction.

APE1 initiates the restoration step of the BER pathway by hydrolysing the 5'-phosphodiester bond of the AP site^[5]. The human *APE1* gene maps to chromosome 14q12^[34].

Several studies have assessed the association between common polymorphisms (*PARP-1* Val762Ala-rs1136410 and *APE1* Asp148Glu-rs3136820) in these two BER genes and BC risk, although inconclusive results were obtained. A meta-analysis of the literature, updated to 2011, was performed to obtain a more accurate estimate of this association^[35]. In total, 8 studies were included in the meta-analysis (Table 2). No association between *PARP-1* Val762Ala and breast cancer risk was found in any of the genetic models. Additionally, there was no association between BC risk and *APE1* Asp148Glu considering all genetic models; therefore, the analysis suggests that these two polymorphisms are not associated with BC susceptibility.

Two additional papers on *APE1* SNPs and breast cancer risk were recently published^[36,31]. A case-control study performed on a Chinese population reported no association between Asp148Glu and BC susceptibility, as

indicated by the above-cited meta-analysis. In contrast, a significant association between a SNP in the *APE1* promoter (-656 T > G) and decreased BC risk was found, suggesting that this polymorphism may influence breast cancer occurrence^[36]. In a study by Kim *et al*^[31], the association between *APE1* Asp148Glu and two *OGG1* SNPs, including *OGG1* Ser326Cys, and BC risk was evaluated. Whereas *APE1* Asp148Glu was weakly associated with BC risk, a combined analysis including the two BER genes revealed a significant effect on breast cancer occurrence, suggesting the importance of assessing a combination of SNPs in different genes and gene haplotypes for the prediction of BC risk.

SNPs IN BER GENES AND RADIOSENSITIVITY

Breast-conserving surgery followed by a standard RT regimen is the most common treatment for breast cancer. However, therapeutic exposure to IR can induce adverse reactions in normal tissue. These reactions show considerable variation among individuals, suggesting the involvement of genetic factors. Because IR hypersensitivity may lead to interruption of therapy, in the last several years, significant effort has been devoted to the identification of molecular factors that could increase radiotherapy-induced side effects.

SNPs in genes involved in processes such as DNA repair, cell-cycle control, apoptosis, cellular antioxidant defence and cytokine production may influence the individual radioresponse. Several experimental approaches, such as candidate SNP association studies and GWAS, are being used to investigate the genetic basis of normal tissue radiosensitivity^[37]. Radiogenomics studies are most numerous in breast cancer patients treated with RT and are aimed at identifying SNP profiles that can be used to select radiosensitive patients. In particular, several studies on BC patients evaluating the association between the risk of acute and late skin reactions to RT and SNPs in DNA repair genes (especially *XRCC1*) were performed; however, these studies yielded conflicting results. Eleven studies on *XRCC1* SNPs, mainly involving Caucasian patients, were collected in a recent meta-analysis^[38].

Table 3 Association between X-ray repair cross-complementing group 1 single nucleotide polymorphisms and radiotherapy-induced side effects in breast cancer patients

Ref.	XRCC1 SNPs	Number of studies analysed	Result
Xie <i>et al.</i> ^[38]	Arg399Gln	8	The 399Gln variant allele is associated with a higher risk of RT-induced toxicity (only in some subgroups of BC patients)
	Arg194Trp	6	No predictive value was found for this SNP
	-77 T > C	4	No predictive value was found for this SNP
	Arg280His	4	The 280His variant allele is protective against RT-induced toxicity (in BC patients treated with RT only)

XRCC1: X-ray repair cross-complementing group 1; SNP: Single nucleotide polymorphism; RT: Radiotherapy; BC: Breast cancer; Arg: Arginine; Gln: Glutamine; Trp: Tryptophan; His: Histidine; T: Thymine; C: Cytosine.

In these studies, the severity of acute and/or late side effects of RT was assessed according to various evaluation criteria, including the Common Terminology Criteria for Adverse Events (CTCAE) (http://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/ct-caev3.pdf), the criteria proposed by the Radiation Therapy Oncology Group (RTOG) and European Organization for Research and Treatment of Cancer (EORTC)^[39], the Late Effects of Normal Tissue-Subjective Objective Management Analytical (LENT/SOMA)^[40] and the Common Toxicity Criteria of the United States National Institutes of Health (NIH) ([http://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/ctcmanual_v4_10-4-99.pdf#search="ctc"](http://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/ctcmanual_v4_10-4-99.pdf#search=)).

To evaluate acute RT side effects in BC patients, the following clinical skin reactions within the radiation field of the breast were documented during treatment: erythema, epilation, desquamation and decreased sweating in addition to more severe morbidities such as edema, ulceration, haemorrhage and necrosis. Common late radiation effects (*i.e.*, effects that first occur at least 90 d after the initiation of RT) include fibrosis, telangiectasia and atrophy.

Both early and late normal tissue reactions are graded on a 5-point ordinal scale (0 indicating absence of a radiation effect and 5 indicating an effect leading to death). The grade of toxicity induced by radiotherapy was assessed according to different evaluation criteria, with high-grade toxicity considered as grade ≥ 2 (according to CTCAE, RTOG-EORTC and LENT/SOMA) or grade $\geq 2c$ (according to the NIH).

The results of the meta-analysis by Xie *et al.*^[38] are summarised in Table 3. No significant association between the XRCC1 Arg399Gln SNP and IR-induced toxicity was observed in the overall analysis. Nevertheless, stratified analyses showed that the Arg399Gln SNP was predictive of side effects in some subgroups of BC patients. For example, carriers of the XRCC1 399Gln allele were at higher risk of RT-induced side effects in studies using high-quality genotyping methods, in studies with mixed treatment regimens or when studies on only late toxicity were excluded. On the contrary, the XRCC1 Arg280His variant allele had a protective effect against RT-induced toxicity only in BC patients treated with radiotherapy alone. XRCC1 Arg194Trp and -77 T > C did not have any

predictive value.

This analysis indicated that large well-designed studies are needed to more clearly establish the predictive value of XRCC1 variants and SNPs in other DNA repair genes for radiation-induced side effects. The choice of genotyping method and the selection of well-characterised patient cohorts should be carefully considered.

Few studies investigating the association between SNPs in other BER genes and the risk of adverse reactions to RT in BC patients are available in the literature. Concerning the APE1 Asp148Glu SNP, the 148Glu allele was found to have a protective effect against the development of acute toxicity to radiation in Caucasian BC patients; however, this effect was only observed in the normal-weight subgroup of patients^[41]. Moreover, the authors observed that the APE1 148Glu and XRCC1 399Gln alleles exerted a combined protective effect. No association between APE1 SNPs and late complications in normal tissue after radiotherapy has been identified thus far for BC^[42,43].

The association of DNA repair SNPs with late RT effects in normal tissue has also been investigated in prostate cancer patients, although the radiogenomics studies reported thus far present interpretive difficulties because of numerous confounding factors^[37]. A GWAS was recently performed to identify SNPs associated with the development of erectile dysfunction following RT for prostate cancer^[44]. Twelve candidate SNPs identified in this study were associated with cellular functions such as adhesion, signalling and hormone metabolism rather than DNA damage repair. Therefore, the involvement of DNA repair SNPs in the radioresponse after RT for prostate cancer remains an open question.

Few studies have examined patient populations with tumours other than carcinomas of the breast or prostate, although significant acute and late toxicities are frequent in patients with squamous carcinomas of the head and neck who are treated with RT. The association of DNA repair SNPs with effects on normal tissue in the head and neck and in other types of cancers should be more extensively explored^[37].

The attempts made thus far to validate the published data on genotype and radiation toxicity did not confirm a clinically relevant predictive value for any published SNP^[45]. Currently, it remains controversial whether SNPs could significantly influence the risk of complications in normal tissue^[46].

CONCLUSION

In the last decade, there has been increasing interest in identifying associations between SNPs in DNA repair genes and susceptibility to various cancers, including BC^[4,5]. In this context, BER gene polymorphisms have been extensively investigated; however, their association with BC has not been clearly defined. The Arg399Gln SNP, which is the most common variant in the *XRCC1* gene, showed an overall weak association with BC risk that became stronger only for some ethnicities. In general, SNPs may contribute to the genetic risk for BC, although their effect is usually only slightly statistically significant. In some studies, SNP-SNP interactions have been examined to evaluate epistatic effects contributing to BC^[47]. SNPs in different DNA repair pathways, or in other pathways related to DNA metabolism, were selected, and specific SNP pairs showed a statistical association with BC risk. Significant trends in BC risk were also observed in association with an increasing number of risk alleles in different DNA repair genes^[48,23]. Concerning BER genes, *XRCC1* SNPs and haplotypes play an important role because they result in amino acid substitutions, which may affect the interaction of the protein with the other BER enzymes and alter DNA repair efficiency. Studies on the interaction between SNPs in BER genes should be encouraged because although a single SNP may have a negligible effect, interactions between different SNPs in genes of the BER pathway could significantly affect cancer risk.

Studies on the interactions between SNPs in genes of different DNA damage signalling and repair pathways should also be performed. Newly available techniques, principally GWAS, will help to explain the role of moderate-risk alleles and common lower-penetrance alleles in sporadic and familial BC risk.

Furthermore, gene-environment interactions should be investigated to elucidate the complex mechanism underlying BC carcinogenesis.

Similarly, it has been demonstrated that SNPs in BER genes (particularly *APE1* and *XRCC1*) may contribute to IR hypersensitivity. Until now, no association between SNPs and late toxicity has been confirmed, either for BC or for prostate cancer^[38]. Therefore, large, well-designed studies are needed to obtain more robust results.

Although the analysis of gene polymorphisms for the individualisation of cancer therapy is not yet widespread in routine clinical practice, understanding the genetic components of individual radiosensitivity remains an important goal. To properly assess the value of pre-treatment genotyping approaches, prospective collection of genomic DNA from patients enrolled in clinical trials should be planned to develop personalised radiotherapy protocols for both sensitive and resistant patients.

The establishment of gene polymorphism databases will significantly contribute to these tasks, and meta-analyses that collect a large amount of data will permit faster access to scientific results.

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WJCO 5th Anniversary Special Issues (2): Breast cancer

Association of survivin splice variants with prognosis and treatment of breast cancer

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Abstract

The purpose of this study was the overview of current knowledge regarding the use of survivin and its isoforms in prognosis and treatment of breast cancer. An advanced search of Medline was performed using the following search strategy: "(survivin isoforms) OR (survivin transcript variants) AND (breast cancer) AND (neoplasm OR tumor OR cancer OR carcinoma)". Relevant studies were retrieved and processed thoroughly in order to analyze the related data. Besides wild-type survivin full-length transcript, another six splice variants have been identified. Overexpression of survivin and its isoforms leads to shorter overall and disease-free survival; the transcript variants are correlated with apoptosis and could assist prognosis prediction. It has been proved through numerous studies that inhibiting survivin isoforms might become a promising target of drug therapy of carcinomas. Use of small molecule YM155 could offer new therapy for triple negative breast cancer patients, while, chemotherapy with 5-fluorouracil + epirubicin + cyclophosphamide and Tax-Epi could be guided by survivin splice variants measurements. Survivin transcript variants could become prognostic biomarkers and could provide information about clinical management

of patients suffering from breast cancer.

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Key words: Survivin gene; Isoforms; Therapy; Prognosis; Breast cancer

Core tip: Besides wild type survivin full length transcript, another six splice variants have been identified. Overexpression of survivin and its isoforms leads to shorter overall and disease-free survival; the transcript variants are positively correlated with apoptosis and could assist prognosis prediction. It has been proved through numerous studies that inhibiting survivin isoforms might become a promising target of drug therapy of carcinomas. Use of small molecule YM155 could offer new therapy for triple negative breast cancer patients while, chemotherapy with 5-fluorouracil + epirubicin + cyclophosphamide and Tax-Epi could be guided by survivin splice variants measurements. Survivin transcript variants could become prognostic biomarkers and could provide information about clinical management of patients suffering from breast cancer.

Pavlidou A, Kroupis C, Dimas K. Association of survivin splice variants with prognosis and treatment of breast cancer. *World J Clin Oncol* 2014; 5(5): 883-894 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i5/883.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i5.883>

INTRODUCTION

Globally, breast cancer is the most common type of non-skin human malignancy and the second cause of cancer-related deaths amongst women in the Western developed World. Breast cancer is responsible for 22.9% of all new cancer cases among women worldwide and 13.7% of

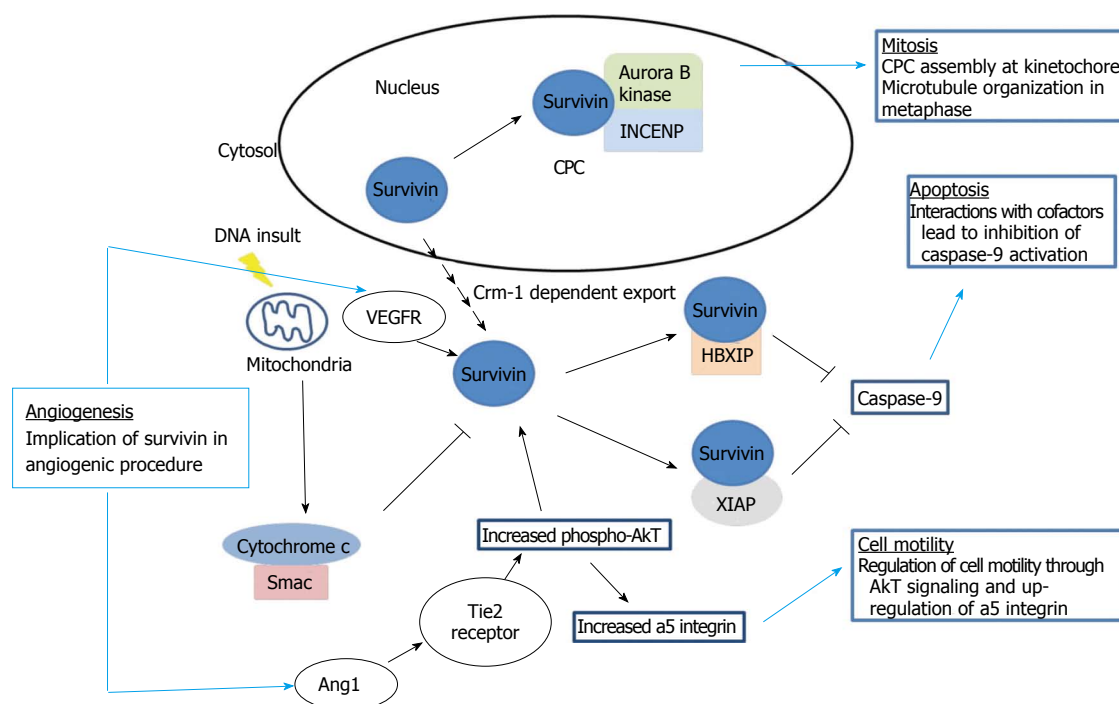


Figure 1 Survivin functions that contribute to tumor development and metastasis; chromosomal passenger complex, inner centromere protein, hepatitis B X-linked interacting protein and X-linked inhibitor of apoptosis protein, angiopoietin-1, vascular endothelial growth factor receptor, receptor tyrosine kinase 2 (modified from ref.[59]). CPC: Chromosomal passenger complex; INCENP: Inner centromere protein; HBXIP: Hepatitis B X-linked interacting protein; XIAP: X-linked inhibitor of apoptosis protein; Ang1: Angiopoietin-1; VEGFR: Vascular endothelial growth factor receptor; Tie2: Tyrosine kinase 2.

cancer deaths^[1]. There are numerous factors associated with the occurrence of breast cancer, such as: genetic susceptibility arising from mutations in high/moderate penetrance genes (such as *BRCA1/2*, *PALB2*, *CHEK2*, *BRIP1*, *RAD50*, *NSB1* etc.)^[2-3], hormone-associated reproductive factors such as increased menstrual cycles arising from either earlier age at menarche or later age at menopause or decreased parity, older age at first birth and use of hormone therapy; consumption of alcohol and type of diet; obesity; exposure to radiation; atypical hyperplasia of the mammary gland^[4].

Inhibition of apoptosis causes tumorigenesis through cell survival resulting in accumulation of genetic mutations that lead normal tissues to transformation. Apoptosis is a tightly controlled procedure of cellular death, which is crucial for tissue homeostasis. Proteins of the bcl-2 family and the inhibitors of apoptosis (IAP) family belong to key regulators of apoptosis^[5].

The main characteristics of the IAP family are the baculovirus IAP repeat (BIR) domains and the blocking of apoptosis by inhibiting directly caspases and procaspases. Till today eight proteins of IAP family were identified, survivin corresponds to the baculoviral IAP repeat-containing 5 domain (BIRC5). Among them, survivin and XIAP have attracted the research interest as therapeutic targets for various malignancies^[6].

Survivin is the smallest member of IAP family and is a multifunctional protein, which participates in the control of apoptosis, angiogenesis and proliferation (Figure 1). Additionally, since survivin is a member of the family

of the chromosomal passenger complex (CPC) proteins, it interacts with Borealin, the Aurora B kinase and the inner centromere protein (INCENP) in order to carry out substantial roles in cell division^[7]. Recently, a critical role for survivin in the control of autophagy was also reported^[8]. Survivin, normally expressed during embryonic and fetal development, is downregulated in adult tissues and overexpressed in a variety of human cancers^[9-12]. Inhibition of apoptosis by survivin is a predictor of poor prognosis and shorter survival in patients suffering from various carcinomas^[13].

In this review, we analysed the literature data regarding the correlation of survivin isoforms with clinicopathological characteristics of breast cancer and their prognostic significance in order to explore the inclusion of survivin isoforms -besides wild-type survivin- as prognostic biomarkers in emerging multiparameter technologies examining tissue RNA expression (analogous to Oncotype, Mammaprint, PAM50^[14,15] or their clinical use in target-oriented therapies.

SURVIVIN TRANSCRIPT VARIANTS AND FUNCTIONS

The survivin gene is located on chromosome 17q25 and up to seven alternatively spliced surviving transcripts have been detected so far^[7]. Alternative splicing of precursor messenger RNA (mRNA) is a process by which the exons are connected, so they could generate different mRNAs and proteins. Alternative splicing is a

Wild-type survivin

exon1	exon2	exon3	exon4
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Survivin-2b

exon1	exon2	exon 2b	exon3	exon4
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Survivin-ΔEx3

exon1	exon2	exon4	3'UTR
-------	-------	-------	-------

Survivin-2a

exon1	exon2	3'UTR
-------	-------	-------

Survivin-3b

exon1	exon2	exon3	exon 3b
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Survivin-2b+32

exon1	exon2	exon 2b+32	exon3	exon4
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Figure 2 Illustrative diagram of survivin isoforms exon structure. b: Sequence originated from intron; 3'UTR: Untranslated region, the red lines represent the baculovirus inhibitors of apoptosis repeat domain (modified from ref. [4] and [20]).

significant procedure which maintains the diversity of the genome. Over 95% of the human genes produce different splice variants which in many occasions present opposite tasks^[16]. Recently there has been evidence that differences in the splicing process can provoke myeloid cancer^[17], while comprehending this process is important in designing new therapies for cancer. One more research uncovered the fact that a common regulatory network is responsible for the simultaneous expression of a number of functionally associated splice isoforms^[18], which supports the idea that we should consider better as therapeutic targets the whole group of different transcript variants or their common regulatory network, rather than targeting on a single gene product^[19].

Except for wild type survivin full-length transcript, six other aberrant splice variants have been identified (Figure 2): (1) survivin-ΔEx3 that is created from the removal of exon 3 and also contains part of the 3' untranslated region; (2) survivin-2b that arises from the inclusion of part of intron 2 termed in this variant as “exon 2b”; (3) survivin-3b that originates from the inclusion of part of intron 3 termed in this variant as “exon 3b”; (4) survivin-2a stems from the insertions of exon 1 and 2 at the 5' end of intron 2, 197nt of intron 2 are added, of which 195nt are noncoding^[20]; (5) the recently described survivin-2b + 32 which combines intronic sequence 2b and an insertion of 32 additional nucleotides from intron 2 and therefore includes “exon 2b + 32”^[5] and finally the latest addition is (6) survivin-image (SI), which consists from a part

of the survivin gene (345 bp), a part of the image gene (155 bp) of eye cancer and another insertion of 7 bp. SI alternatively spliced isoform could be involved in other functional pathways related to tumorigenesis^[21].

The survivin variants present differential intracellular localization that possibly regulates their antiapoptotic potential. Co-expression experiments have showed that wild-type survivin can heterodimerize with its splice variants. Heterodimer formation can lead to specific subcellular localization patterns, implying that high expression in tumor cells can lead to formation of functionally distinct survivin complexes. Co-expression of wild-type survivin with survivin-ΔEx3 results in the recruitment of these complexes to the mitochondria, where they inhibit mitochondrial dependent apoptosis^[22]. The intracellular localization of survivin isoforms according to one study, depends on a Crm1-dependent nuclear export signal (NES) present in survivin, survivin-2b and survivin-3b, but absent in survivin-ΔEx3 and survivin-2a. These survivin isoforms enter the nucleus by passive diffusion since they lack an active nuclear import signal. The NES acting in consortium with an appropriate CPC formation are responsible for the cytoprotective activities of some of the survivin isoforms, as well as for their correct localization and function during cell division. Among all isoforms, only survivin-3b is cytoprotective and interacts efficiently with CPC proteins^[7].

The functions of the variants are not fully understood. Survivin-ΔEx3 has been described as an anti-apoptotic protein. Survivin-2b probably has pro-apoptotic action since it possesses a truncated BIR domain and still it dimerizes readily with wild type survivin causing the reduction of the anti-apoptotic effects of wild type survivin. Survivin-3b contains a complete BIR domain and therefore has a potential anti-apoptotic activity. Survivin-2a does not have the domain involved in inhibition of apoptosis and seems to have opposed activity to wild-type survivin.

Survivin-ΔEx3, is probably associated with cell immortality, or else remains inactive or assists angiogenesis. In the same way, survivin-2a and survivin-2b are cytoprotective, proapoptotic or sometimes they seem to be inoperative by conflicting manuscripts. Current research suggested that cancer development and survival of people suffering from tumors could be associated with the differential expression of survivin transcript variants. An explanation of this hypothesis could be that survivin variants display a trans-dominant negative (TD) phenotype enacted by the creation of silent heterodimers with survivin-wild type or by the titration of CPC proteins and/or Crm1. In conclusion, survivin-3b fulfils the molecular requirements, *i.e.*, interaction with Crm1 together with the capability to cooperate with all CPC components, demanded to perform “wt survivin-like” biological functions^[7].

It should be certainly noted that is difficult to unify all data concerning survivin variants, since results are controversial, but the accumulated information could be

Table 1 Summarized data from research articles that examined the expression of survivin transcript variants (used method is provided) accordingly to clinicopathological characteristics of patients and their survival

Ref.	Examined variants	Methods	Association with patient characteristics	Conclusion
Végran <i>et al</i> ^[22]	wt, sur2b, surΔEx3, sur3b, sur2a	Real time qPCR	Tumor grade, estrogen receptors, lymph nodes	High expression sur3b tumors had shorter OS and DFS
Span <i>et al</i> ^[23]	wt, sur2b, surΔEx3, sur3b, sur2a	Reverse transcription qPCR	Age, tumor grade, lymph nodes, steroid hormone receptors	High expression of sur2a, sur3b and wt indicate poorer prognosis
Ryan <i>et al</i> ^[10]	wt, sur2b, surΔEx3	Reverse transcription qPCR, Western blotting	Tumor size, nodal metastases, breast cancer type	Wt and surΔEx3 were correlated positively with apoptosis
Pavlidou <i>et al</i> ^[4]	wt, sur2b, surΔEx3	Real time qPCR	Tumor grade, estrogen receptors	Sur2b/wt had significant association with estrogen receptors
Athanassiadou <i>et al</i> ^[24]	wt	Immunocytochemistry, immunohistochemistry	Grade, lymph nodes, tumor size	increased wt may indicate a worse prognosis

wt: Wild-type surviving; sur2b: Survivin-2b; surΔEx3: Survivin-ΔEx3; sur3b: Survivin-3b; sur2a: Survivin-2a; qPCR: Quantitative polymerase chain reaction; OS: Overall survival; DFS: Disease free survival.

important for designing future strategies of therapy by using specific survivin transcripts as targets.

PROGNOSTIC VALUE OF SURVIVIN TRANSCRIPT VARIANTS IN BREAST CANCER

It is possible that survivin may be of great value in the prognosis of breast cancer patients. In this part of the review, we compiled the experimental data of four studies, examining the expression of survivin transcript variants accordingly to clinicopathological characteristics of patients with breast cancer and their survival (Table 1).

In the study of Végran *et al*^[22], survivin-3b variant expression provided the major findings in patient samples, presenting with inverse correlations with ten proapoptotic genes and five anti-apoptotic genes. These results may suggest a feedback loop at the gene levels with survivin-3b and could be a way that cancer cells use in order to counteract survivin-3b overexpression. The data are in agreement with the putative anti-apoptotic role of survivin-3b suggested initially by its amino acid sequence. The results also confirm previous observations concerning the higher expression of the antiapoptotic survivin-ΔEx3 and survivin-3b in p53-mutated breast tumors. However, some studies found out that survivin-ΔEx3 expression increases with histological grade and is more expressed in ER-negative tumors. Survivin-2b isoform is overexpressed in high-grade ER-negative and node-invasive tumors. This result is not in accordance with its putative proapoptotic role but it indicates that survivin-2b could be a marker of aggressiveness. Survivin-2a -probably due to its potential pro-apoptotic role- is more present in low-grade and non-invasive tumors. Végran *et al*^[22] results revealed that survivin, survivin-ΔEx3, and survivin-2b have no prognostic role in breast carcinoma. A pro-apoptotic role for survivin-2a has been ascribed; however, in this study it was found to be associated with the worst prognosis of breast cancer patients.

Span *et al*^[23] 2006 discovered that survivin-wild type, survivin-2a, and survivin-3b were associated in patient samples with poor relapse free survival. Survivin, survivin-ΔEx3 and survivin-2a variants were associated with younger age, advanced histological grade and steroid hormone-receptor-negative tumors, all factors that indicate poor prognosis for a breast cancer patient. Tumors from patients with many involved lymph nodes had lower amounts of the proapoptotic survivin-2b variant in the primary tumor than those with a limited number of involved lymph nodes. This negative correlation with lymph node status could suggest a role for survivin-2b in counteracting the antiapoptotic and/or cell cycle stimulating role of wild-type survivin. The data in this study confirm that some of the splice variants interact with each other and modify the prognosis of the patients. Specifically, high expression of the survivin-2a and survivin-3b, in addition to survivin indicate a poorer prognosis and the prognostic value of survivin is strongest in the presence of higher concentrations of these variants^[23].

In the report of Ryan *et al*^[10], both wild-type survivin and survivin-ΔEx3 mRNA correlated moderately with apoptosis in patient samples. Levels of the survivin-2b and survivin-ΔEx3 but not wild-type survivin were significantly higher in positive lymph nodes compared to the primary tumor. A weak but significant inverse relation was found between survivin-ΔEx3 and both tumor size and number of positive lymph nodes. This form of survivin was also detected more frequently in the ductal histological type compared to the lobular one. Previously, they have reported that caspase 3 levels were also significantly higher in ductal than lobular breast cancers. These findings suggest that the regulation of apoptosis is different in these two histological types^[10].

In a previous study of ours (Pavlidou *et al*^[4]) we have reported that survivin-2b, survivin-ΔEx3 and the ratio of survivin-2b/wild-type survivin showed *in vivo* a positive correlation with the grade of the tumor in breast cancer samples ($P < 0.05$). The two isoforms presented an increased expression in advanced histopathologic grade, a

finding that was expected for survivin-ΔEx3 but not for survivin-2b due to its pro-apoptotic activity. The fact that the two isoforms are elevated in high grade tumors makes them possible markers of tumor aggressiveness. Perhaps, these two isoforms have important but opposite roles in cancer development. The ratio of survivin-2b/wild-type survivin is increased in the early stages I and II, a result which may be due to different and antagonistic functions of the two proteins. Also it was found a significant association between the ratio of survivin-2b/wild-type survivin and estrogen receptors, which further reinforces the fact that this particular variation of survivin mRNA could predict survival of breast cancer patients.

Nectins are cell adhesion molecules involved in epithelial cell physiology. Nectin-4 is a new tumor-associated antigen and a reliable biomarker for breast carcinoma. The *in vivo* association of survivin and Nectin-4 with unfavourable prognostic indicators, and with one another, suggests that these proteins may also interact in breast carcinoma in order to exert their adverse effect. In conclusion, the combined survivin and Nectin-4 expression demonstrates a strong independent association with poor prognosis^[24].

GENOMIC EXPRESSION ASSAYS AND SURVIVIN

The search for novel prognostic and predictive biomarkers could deter cancer patients from unnecessary, inadequate and toxic therapy. This effort has culminated in the availability of few -but useful-commercial standardized assays for breast cancer patients. Besides the routine immunohistochemical measurements of hormone receptors and the HER2 oncogene protein product, only one other effort looked at the protein level: the Mammostrat test (by Clariant Diagnostic Services, GE Healthcare) that assesses 5 molecules: SLC7A5, HTF9C, P53, NDRG1, and CEACAM5 by IHC^[25]. It is not known whether the addition of survivin would strengthen the value of this approach (monoclonal antibodies to survivin now exist).

The majority of the other available assays have looked at the RNA level either with microarray expression arrays or with real-time PCR (RT-PCR) assays. The technology of microarray gene expression emerged as the best method to categorize patients based on their molecular signature and distinguish those who could use other therapy strategies. Nevertheless it is not yet inducted into the clinical practice due to some restrictions^[6,26]. An FDA approved kit, Mammaprint (by Agendia Inc.) assessing the mRNA expression of 70 genes from frozen breast cancer tissues is being extensively used in order to stratify patients into two distinct groups: low risk or high risk of distant recurrence (with no intermediate results). The tumor cell percentage and an RNA integrity score are also provided. Survivin (BIRC5) was included in the genes tested in the original validation study; however it was not selected for the final 70-gene panel^[27,28].

Other commercial kits employ RT-PCR assays for

selected genes from RNA extracted from formalin-fixed paraffin-embedded tissues: (1) an 8-gene panel from Avira (now Biotheranostics Inc) combining the Breast Cancer Index HOXB13:IL17BR ratio with the Molecular Grade Index consisting of the average expression of five cell cycle-associated genes (*BUB1B*, *CENPA*, *NEK2*, *RACGAP1* and *RRM2*)^[29]; (2) the PAM 50 assay (by ARUP Laboratories) that includes wild-type survivin among the 50 selected genes^[30]; and (3) the FDA-approved Oncotype DX Breast Cancer Assay (by Genomic Health). Oncotype DX testing may aid a patient by preventing redundant chemotherapy and its connected toxicity, while reduction of chemotherapy also leads to economical changes in health systems^[31,32].

It provides breast cancer patients with a Recurrence Score (RS) (a number between 0 and 100) that corresponds to a specific likelihood of breast cancer recurrence within 10 years of the initial diagnosis. This score is based on the relative expression of 16 different cancer genes measured in triplicate) normalized relative to a set of 5 reference genes within a range 0-15. A one unit increase reflects the doubling of expression. The Recurrence Score arises from the following equation^[31]:

$$RS = + 0.47 \times \text{HER2 Group Score} - 0.34 \times \text{ER Group Score} + 1.04 \times \text{Proliferation Group Score} + 0.10 \times \text{Invasion Group Score} + 0.05 \times \text{CD68-0.08} \times \text{GSTM1} - 0.07 \times \text{BAG1}$$

As seen in Table 2, survivin is one of the cancer genes belonging to the proliferation group and it corresponds to the highest positive factor in the RS equation (1.04). A low risk of recurrence is defined of less than 18, an intermediate risk as less than 31 and a high risk as 31 and higher.

In Stemmer's *et al*^[32] 2013 article, Oncotype DX testing seems to have significant impact on minimising chemotherapy in node positive/estrogen positive (N1+/ER+) patients with breast tumor. It has been proven that Recurrence Score is correlated with tumor grade; higher Recurrence Score results are associated with higher histologic grade. Only patients belonging to the high Recurrence Score group were prescribed for chemotherapy. The differences in the proportions of patients treated with chemotherapy between the low, intermediate, and high Recurrence Score groups were statistically significant^[32]. The clinical application of Oncotype DX testing was correlated with a decrease in chemotherapy and change of treatment recommendations^[32].

In another recent study, Solin *et al*^[33] 2013 used the so-called 12-gene Oncotype DX DCIS Score, which quantifies local recurrence risk and provides risk information independent of traditional clinical and pathologic characteristics. The treatment of ductal carcinoma *in situ* (DCIS; intraductal carcinoma) of the breast is variable, with concerns about both overtreatment and undertreatment. Among those 12 genes is survivin, in the group of cancer proliferation-related genes as shown in Table 2. The DCIS Score for proliferation group, where survivin belongs, arises from the following equation:

$$\text{DCIS Score} = + 0.31 \times \text{Proliferation Group Score} -$$

Table 2 Panel of 21 genes used in oncotype DX breast cancer assay

Proliferation group	Invasion group	Estrogen group	HER2 group	Reference genes
Ki-67 ¹	ST3	ER	GRB7 (growth factor receptor-bound protein 7)	GSTM1 ¹ <i>ACTB</i> ¹
STK15 ¹	CTSL2	PR ¹	HER2 [human EGF (epidermal growth factor) receptor 2]	CD68 <i>GAPDH</i> ¹
BIRC5 (Survivin) ¹ CCNB1 ¹		Bcl2 SCUBE2 [Signal peptide CUB (complement proteins C1r/C1s, Uegf, and Bmp 1)-EGF domain-containing protein 2]		BAG1 <i>RPLPO</i> ¹ <i>GUS</i> ¹
MYBL2 ¹				<i>TFRC</i> ¹

¹Genes used in 12-gene oncotype DX for ductal carcinoma *in situ*. ER: Estrogen receptor; PR: Progesterone receptor; Bcl2: B-cell CLL/lymphoma 2; ST3: Stromelysin 3; CTSL2: Cathepsin L2; Ki-67: Kinase inhibitor; STK15: Serine/threonine kinase 15; CCNB1: Cyclin B1; MYBL2: Myeloblastosis family transcription factor-like 2; ACTB: Beta-actin; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; RPLPO: Ribosomal phosphoprotein; GUS: β -glucuronidase; TFRC: Transferrin receptor; GSTM1: Glutathione S-transferase M1 gene; CD68: CD68 molecule; BAG1: Bcl2-associated athanogene 1.

$0.08 \times \text{PR} - 0.09 \times \text{GSTM1}$.

The DCIS Score predicts the risks of invasive local recurrence and provides information that complements traditional clinicopathologic prognostic factors for women with DCIS. The differences in the risks of developing local recurrence and invasive local recurrence between patients with a lower DCIS Score and a higher DCIS Score were statistically significant and clinically meaningful^[33].

In the future, it would be very intriguing to explore the inclusion of the other survivin isoforms as prognostic biomarkers in such emerging multiparameter technologies examining tissue expression (analogous to Oncotype, Mammaprint, PAM50 *etc.*^[14-15] since it has been proved that they possess potential importance in apoptosis and proliferation of breast cancer cells, especially when compared to wild-type full-length survivin.

APPROACHES FOR TARGETING SURVIVIN ISOFORMS

A lot of studies were designed in order to find out pathways and compounds, which can interact with survivin and its isoforms, since they seem to provide promising therapeutic targets in cancer. Initially these efforts had as an obstacle the structural properties of survivin, which assigned the molecule of survivin under the label “non-druggable”. Despite the difficulties, numerous ways concerning survivin inhibition have been utilized and depend mostly on indirect mechanisms, *e.g.*, by interference with the expression of the survivin gene, its mRNA processing, the intracellular localization and the binding partners, or by affecting stability of survivin protein or by provoking survivin-specific immune responses^[34]. Research is continuing in order to deploy drugs which cooperate with survivin and restrain its operation^[34,35].

TARGETING SURVIVIN mRNA

Numerous proteins and protein complexes were recognized and are necessary for the growth and survival of cancer cells. RNA interference experiments-for example-

can reduce the production of these proteins and confirm the fact that they are crucial for tumor cells in order to survive^[34,36].

RNA-based therapeutics comprise small interfering RNA (siRNA), antisense oligonucleotides, and microRNAs (miRNAs)^[19].

Chemically-modified antisense oligonucleotides consist approximately of 20 nucleotides and their annealing to mRNA permits its cleavage by ribonuclease H. A variety of alterations in their chemical structure was achieved, like using a phosphorothioate linkage instead of natural phosphates as a backbone of nucleotides and 2'-O-methyl residues. 2'-O-methoxyethyl residues or locked nucleic acids^[19,37] were also developed in order to improve pharmacological quality and structural stability. However, the chemistry-dependent toxicities due to their structures are a significant problem expected to be solved^[19].

siRNA is another structure of a double-stranded RNA which is composed by 21-22 nucleotides. The antisense strand of the siRNA after interacting with multi-protein RNA-induced silencing complex, anneals to the complementary mRNA and an endonuclease cleaves the annealed mRNA^[19]. There is data showing that survivin is a transcriptional target of HA-CD44 signalling, therefore the specific inhibition of CD44 expression by siRNA, resulted in a specific decrease in survivin expression. This correlated with a significant loss of breast cancer cell invasiveness. Simultaneous increased expression of both CD44 and survivin was detected in late-stage metastatic breast cancer cells, expression that was absent in normal breast tissue samples^[38].

Recently, a group of small non-coding RNAs, known as microRNAs, attracted cancer researchers^[39,40]. MiRNAs are small, non-coding RNAs of 19-25 nucleotides in length that are endogenously expressed in mammalian cells. miRNAs regulate gene expression post-transcriptionally, by pairing with complementary nucleotide sequences in the 3'-UTRs of specific target mRNAs. miRNAs act either as oncogenes or as tumor suppressors. More than 50% of miRNA genes are located in cancer-associated regions of the genome. Deletion or epigenetic

silencing of a miRNA that *e.g.*, normally suppresses one or more oncogenes could cause tumorigenesis, progression and invasion, as it was showed for miR-200, miR-122 and miR-203. Survivin was recognized as direct target of miR-203 in TNBC cells. Furthermore, the fact that overexpression of miR-203 can repress proliferation and migration of TNBC cells and is escorted by a reduction in BIRC5 expression, proves that miR-203 acts as a tumor suppressor in TNBC^[41].

SECONDARY ALTERATIONS OF SURVIVIN CONTROLLING ITS SUBCELLULAR LOCALIZATION AND FUNCTION

The multiple operations of survivin in individual subcellular compartments are probably fulfilled through the domain structure of the molecule and finely regulated by secondary alterations. The modifying enzymes could also become probable targets in order to investigate how the interference with survivin functions and its specific subcellular localization may be utilized in cancer therapy^[34,42].

INTERFERENCE WITH SURVIVIN FOLDING, STABILITY, AND INTERACTION PARTNERS

Heat shock proteins (HSPs) are molecular chaperones participating in the folding and stable protein conformation. One of their tasks is to block the creation of protein complexes and usually they are over-expressed in cancer cells. Survivin is a HSP90 client protein and the association among these proteins was used to generate preventive compounds. The formation of survivin-HSP90 complex is inhibited when shepherdin is bound to HSP90. Thus, incubation of glioblastoma cells with shepherdin triggered the irreversible destruction of mitochondria and HSP90 client proteins in the cytosol and finally tumor cell death. Targeting the action of HSP90 in different subcellular compartments might contribute a lot in cancer therapy, particularly when affecting its interaction with survivin^[34].

The transcription factor GATA 1 that was found to upregulate survivin expression in breast carcinoma could be a novel target of therapy. A research group revealed that the extraction of the grape seed regulates the function of a transcription factor, which is associated with the core promoter of survivin and can finally reduce its activity^[43].

IMMUNE RESPONSE AGAINST SURVIVIN

Isolating antibodies specific for survivin peptides seems significant, as the structure and sequence of the epitope binding groove of the antibody could be examined and possibly additional epitopes are discovered. Also this study could define with accuracy the peptide portion of

the survivin protein that is bound most efficiently and most commonly by humoral antibodies generated against survivin^[19]. This will lead to more specific survivin vaccines that would elicit better immune response, generate immune memory and offer protection from tumor progression^[44]. The development of antibodies is still costing a large amount of money and there are several considerations for their use^[19,45].

Comprehending the molecular principles of cancer and the function of immune defense mechanisms guided to development of novel therapeutic strategies. The most significant immune cells against the development of the tumor are the differentiated cytolytic CD8+ lymphocytes (CTLs)^[34]. Survivin is a tumor-associated antigen so it could be a potential target for immunotherapy^[34]. During the search for immunogenic tumor antigens, spontaneous CTL responses against survivin were identified^[45]. Survivin is an attractive target for vaccination since immune escape by down regulation or loss of expression of this protein would impair sustained tumor growth. The therapeutic effectiveness is improved by the availability of multiple survivin epitopes presented by different HLA restriction elements^[45,46]. Survivin-derived epitopes have been exploited in vaccination so that CD4+ responses were initiated in prostate cancer and melanoma patients. Tumor response and patient survival were correlated with the activities of survivin specific T cells^[34].

RADIOTHERAPY AND SURVIVIN

Radiotherapy enhances apoptosis and destroys cells once and for all. Survivin mRNA expression was inversely correlated with the sensitivity to ionizing radiation^[43,47,48]. Additionally, survivin could be a radio resistance factor since its production was increased by sublethal doses of radiation^[49]. Transfer of tumor-specific adenovirus-mediated PUMA gene using the survivin promoter enhances the radiosensitivity of breast cancer cells *in vitro* and *in vivo*^[43]. Also, signal transducer and activator of transcription 3 (Stat 3) could be used as target therapy in sensitization by radiation for patients with breast carcinomas, because these two molecules influence survivin. Radiotherapeutic sensitization approach has not been widely used in all clinical trials, despite the fact that survivin inhibitors might be a new group of antagonists, which might enhance the effects of radiotherapy when survivin is over-expressed^[43,50].

CHEMOTHERAPY AND SURVIVIN

In preclinical and clinical models, a variety of experiments were performed in order to block IAPs and therefore to lead breast malignant cells to apoptosis and to decreased size even in chemoresistant tumors, reinforcing their possible future utilization as cancer therapy targets^[6].

Overexpression of survivin has been shown to restrain apoptosis and to develop multi-drug resistance (MDR)^[51]. MDR in cancer therapy is represented by

resistance to a large variety of structurally unrelated cytotoxic compounds, resulting in insufficient death of tumor cells and chemotherapeutic failure in patients with solid tumors. Both breast cancer resistance protein (BCRP, ABCG2) and apoptosis-related molecules are associated with the evolution of MDR in tumor cells^[51].

It has been shown that survivin expression is significantly higher in cell lines after treatment with anticancer drugs. High levels of HER2 or survivin are related to chemo/endocrine therapy resistance and are predicting poor clinical outcome for breast carcinomas. Moreover, it was shown that low survivin expression levels increased the sensitivity of breast cancer cells to etoposide and 5FU^[43]. Prodigiosin (a bacterial metabolite), which down-regulates survivin transcriptionally, could be used in the treatment of paclitaxel-resistant breast tumors^[43].

It seems that p53 possesses an important role in the survivin-regulated BCRP expression: through downregulation of p53 expression, survivin reduces the inhibitory action of p53 on NF- κ B (p50) and then increases the expression of BCRP. In the majority of carcinomas, overexpression of survivin and loss of wild-type p53 expression/function take place simultaneously, hence BCRP overexpression and MDR to multiple compounds such as mitoxantrone, anthracyclines, methotrexate, topoisomerase I inhibitors, gefitinib, doxorubicin (dox), and 5-fluorouracil. Consequently, suppression of survivin expression can be a novel strategy to overpower BCRP-mediated MDR in tumors^[51].

1,25(OH)₂D3 (active vitamin D3) regulates genes involved in calcium homeostasis and bone formation through its interaction with the vitamin D receptor (VDR). Vitamin D3 inhibits survivin and TP73 isoforms in colon and breast carcinomas. *In vitro* and *in vivo* approaches speculate that the downregulation of TP73 isoforms by 1,25(OH)₂D3 could be survivin-dependent^[52].

Research is still in progress in order to design new agents aiming survivin either at the genomic level or at the protein level. A prominent member of these agents is YM155 (sepantronium bromide), a tiny molecule which inhibits survivin and decreases production of survivin by binding to the C-terminal region of interleukin enhancer-binding factor 3^[19]. LY2181308 is a second-generation antisense oligonucleotide with a phosphorothioate backbone and other structural alterations targeting the translation initiation site of survivin isoforms. YM155 and LY2181308 can inhibit expression of all survivin isoforms. Recently, it was found that amiloride can regulate the mechanism of different survivin splicing^[19].

It has been shown that survivin can block apoptosis when agents such as tamoxifen, paclitaxel and trastuzumab are used. It was also shown that a reduction of apoptosis caused by tamoxifen occurs after increasing the expression of survivin. Recent research in mammary tumors proved that survivin acts downstream the human epidermal growth factor receptor-2 (Her2) and (Her3)/PI3K/Akt pathway, so it seems to be essential in moderating paclitaxel impedance when Her2 is over-expressed^[6]. An additional study showed that cells overexpressing survivin

showed no decreased viability when treated with trastuzumab, providing evidence that survivin can overcome trastuzumab-induced cell growth inhibition^[6].

Despite the fact that doxorubicin, an anthracyclic agent of chemotherapy, is extensively used in the treatment of breast cancer, the research community has little information on the role of survivin in resistance to doxorubicin^[6]. Survivin-induced overexpression does not block dox-mediated lethal effects in invasive and non-invasive breast tumor cells. In the same way, silencing survivin by siRNA, with or without blocking XIAP too, cannot provoke cytotoxic stimuli and sensitize cells. In conclusion, these data imply that survivin and XIAP expression do not affect dox resistance in breast carcinomas^[6].

It has been well documented that the downregulation of survivin by chemotherapeutic agents sensitizes cancer cells to TRAIL-induced apoptosis. Consistently, it was also found that nemadipine-A potentiates TRAIL-induced apoptosis by reducing survivin expression in lung cancer cells. Although, the precise mechanisms are not clear, it was recently reported that survivin expression could be up-regulated by cellular calcium level^[53].

Moreover, derivatives of the natural alkaloid camptothecin have the ability to provoke proteasomal degradation of survivin in cells with defective p53 function and elevated XAF1 (another tumor suppressor) expression^[54].

Also, it was reported that overexpression of survivin-3b in breast tumor cell lines strongly inhibits 5-fluorouracil + epirubicin + cyclophosphamide (FEC) toxicity, a combination used widely in breast carcinoma treatment. Recently, the cytoprotective effect of survivin-3b after cisplatin treatment was reported. These results also showed for the first time the cytoprotective effect of survivin-3b after FEC treatment in less and in more aggressive cell lines by a p53-independent manner. In addition, increased expression of survivin-3b after one course of docetaxel/epirubicin treatment was associated with reduced disease free survival (DFS) of breast cancer patients. Indeed, high survivin-3b expression tumors had a shorter overall and DFS^[23]. All these findings suggest that the role of survivin is probably therapy specific in resistance. Recently, the complete pathologic response to GAT therapy (dox on first day, paclitaxel and gemcitabine on second day, every 14 d for 6 cycles) underlined its relation with tumor markers and unveiled a reduction in survivin expression in tumors after treatment^[6]. These results suggested that the decrease of survivin expression could be associated with the response to GAT^[6].

TRIPLE NEGATIVE BREAST CANCER AND CHEMOTHERAPY ENHANCED BY SURVIVIN ISOFORMS INHIBITION

Patients with local breast cancer could be cured by surgical resection combined with adjuvant therapy, including radiation, anti-estrogen therapy and Her2-targeting agents. Unfortunately, the management of metastatic breast cancer is far less successful than treatment of lo-

Table 3 Summarized data for research articles, which correlated survivin isoforms with breast cancer treatment

Ref.	Examined variants	Methods	Approaches for inhibition of survivin variants	Conclusion
Yamanaka <i>et al.</i> ^[55]	wt, sur2b, surΔEx3, sur3b	Real time qPCR	YM155	Survivin suppressing activity of YM155 may offer novel therapeutic option in TNBC
Boidot <i>et al.</i> ^[57]	wt, sur2b, surΔEx3, sur3b, sur2a	Real time qPCR	FEC/Tax-Epi	Alternative survivin transcript expression levels may be predictive markers in FEC and Tax-Epi treatment
Zheng <i>et al.</i> ^[58]	wt, sur2b, surΔEx3	MMT, flow cytometry	Recombinant plasmids pGEM-T	Feasibility of targeting wt and surΔEx3 in treating breast cancer

wt: Wild-type surviving; sur2b: Survivin-2b; surΔEx3: Survivin-ΔEx3; sur3b: Survivin-3b; sur2a: Survivin-2a; MMT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; YM155: Sepantronium bromide; FEC: 5-fluorouracil + epirubicin + cyclophosphamide; Tax-Epi: Docetaxel + epirubicin; TNBC: Triple negative breast cancer.

cal disease. Metastatic cancer -despite the progress in molecular-targeted therapies- is the first cause of breast cancer death and presents a true challenge for selecting optimal treatment. Triple negative breast cancer (TNBC) is a high-risk category with negative expression of both estrogen and progesterone hormone receptors and no Her2 protein overexpression (or c-erbB2 gene amplification) that has limited therapeutic options^[2]. TNBC patients represent approximately 15% of total breast cancers. This cancer type may be very aggressive, with rapid tumor growth, a high incidence of metastasis, an increased possibility of distant recurrence and a higher mortality rate than other breast cancers. The need to develop novel therapeutic options that are suitable for this subgroup of patients is obvious^[55].

Microtubule-targeting agents, like taxanes and vinca alkaloids are one of the most common classes of chemotherapeutic drugs for the treatment of TNBC. Survivin siRNA and a dominant-negative mutant of survivin enhanced the antitumor activity of taxanes in several types of cancer. Survivin suppression by YM155 increases susceptibility to apoptosis and enhances the disruption of mitosis, resulting in an enhanced response to microtubule-targeting agents. In previous studies, it was demonstrated that tumor regression induced by YM155 in combination with docetaxel was accompanied by a decrease in intratumoral survivin and an increase in apoptosis rate. These results support the conclusion that survivin inhibition might be an effective way to enhance the efficacies of microtubule-targeting agents against TNBC^[56].

EXPERIMENTAL DATA AND TREATMENT OF BREAST CANCER

In this section of the review, we present the experimental data of three studies correlating survivin isoforms with breast cancer treatment. The group of studies examined includes the articles of Yamanaka *et al.*^[55], Boidot *et al.*^[57] and Zheng *et al.*^[58] (Table 3).

Yamanaka *et al.*^[55], by using cell lines and mouse models, underscored the need for extensive investigation of the role of YM155 in breast cancer treatment and to develop this compound as a novel therapeutic option

for metastatic breast cancer patients. YM155 downregulated both levels of survivin mRNA and protein. RT-PCR revealed that YM155 suppressed the expression of survivin-2b, survivin-ΔEx3, and survivin-3b isoforms in human TNBC cells. The decrease of survivin, which was induced by YM155, was accompanied by spontaneous apoptosis. YM155 caused tumor regression with negligible systemic toxicity as evidenced by an absence of body weight loss. Furthermore, in this study there is the first evidence that YM155 may have therapeutic value in reducing the spontaneous metastasis of human TNBC cells *in vivo*. Considering the fact that survivin is overexpressed in high grade invasive and metastatic human tumors, it is suggested that dysregulation of survivin expression may confer an ability to evade immune responses and physical barriers to invasion of normal tissues. On the contrary, breast cancer cells may remain sensitive to apoptosis induction when survivin is downregulated by YM155, even after distant metastasis has been established. Taken together, therapeutic targeting of the survivin pathway may be beneficial for the treatment of TNBC patients^[55].

In their study, Boidot *et al.*^[57] examined with a real-time quantitative PCR technique, five survivin isoforms in breast cancer patients that were using as treatment either the combination of docetaxel + epirubicin (Tax-Epi) or the combination of FEC. Before therapy, survivin-2a was considerably higher in resistant than in sensitive tumors in the FEC treatment arm, suggesting for the first time that survivin-2a may be involved in resistance to FEC treatment. This result may be consistent with the finding that survivin can heterodimerize with its splice variants causing specific subcellular localisation patterns leading to formation of the functionally distinct survivin complexes. The ratio of survivin-ΔEx3 to wild type was also higher in sensitive than in resistant tumors in the Tax-Epi treatment arm. Increased measurements of survivin-3b after only one course of chemotherapy were significantly correlated with resistance in the FEC regimen cluster, and the ratios of survivin-ΔEx3 and survivin-2b to wild type were significantly higher in sensitive than in resistant tumors in the Tax-Epi treatment arm. Especially, elevated expression and ratio of survivin-3b, after one course of Tax-Epi, showed correlation with decreased

DFS and with reduced overall survival of the patients. These results indicate that an imbalance in the alternative transcript ratios may render the cells resistant or sensitive to apoptosis. They also show for the first time that measurements of alternative survivin transcript levels may become useful predictive biomarkers in FEC and Tax-Epi treatment in breast carcinomas^[57].

During their research Zheng *et al.*^[58] 2011 created four vectors by merging (1) the antisense gene of survivin; (2) the survivin gene (T34A); (3) the antisense gene of survivin-ΔEx3; and (4) the survivin-2b gene with enhanced green fluorescent protein gene (eGFP) in cell lines. Their data suggested that using survivin as a target had great effects on blocking cell development and promoting apoptosis. Utilizing survivin-ΔEx3 as a target resulted in reducing the anti-tumor action. This study uncovered the fact that the use of survivin as a target, by antisense RNA or survivin gene (T34A), was almost in the same manner efficient in prohibiting cell development and urging cell apoptosis in breast cancer cells (B-Cap-37). The advantage of survivin (T34A) could be that the dominant negative mutant competed with survivin, thus leading to phosphorylation-defective survivin. Antisense survivin-ΔEx3 notably prohibited the proliferation and promoted the apoptosis of breast cancer cells *in vitro*. These data suggest that restraining or preventing survivin may be a major step in designing drugs for breast cancer therapy and survivin-ΔEx3 may as well become a useful target for drugs against breast carcinomas^[59].

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WJCO 5th Anniversary Special Issues (2): Breast cancer

New concepts in axillary management of breast cancer

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Abstract

In the last decades, surgical treatment of breast cancer has evolved from more extensive procedures like radical mastectomy to less invasive breast conserving surgery. Similarly, surgical management of axilla has enormously changed from routine axillary dissection to sentinel lymph node biopsy. Traditional surgical approach to the axilla in case of sentinel lymph node negativity is to avoid completion axillary dissection. However, surgeons even avoid performing axillary dissection in selected patients with positive sentinel lymph node in clinical practice depending on the recent randomized controlled studies supporting this concept. All of the recent changes in the management of positive axilla necessitate surgeons to refresh their knowledge on this challenging topic.

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Key words: Breast; Cancer; Axilla; Sentinel lymph node

Core tip: In the last decades, surgical treatment of breast cancer has evolved from more extensive procedures like radical mastectomy to less invasive breast conserving surgery. Similarly, surgical management of axilla has enormously changed from routine axillary dissection to sentinel lymph node biopsy. Traditional surgical approach to the axilla in case of sentinel lymph

node negativity is to avoid completion axillary dissection. However, surgeons even avoid performing axillary dissection in selected patients with positive sentinel lymph node in clinical practice depending on the recent randomized controlled studies supporting this concept. All of the recent changes in the management of positive axilla necessitate surgeons to refresh their knowledge on this challenging topic.

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INTRODUCTION

In the last decades, surgical treatment of breast cancer has evolved from more extensive procedures like radical mastectomy to less invasive breast conserving surgery. Similarly, surgical management of axilla has enormously changed from routine axillary dissection to sentinel lymph node biopsy (SLNB). Introduction of modern imaging modalities and the increase in public awareness about breast cancer resulted in higher number of early breast cancer cases. This achievement helped more conservative surgical methods to overwhelm classic radical procedures in clinical practice. In addition, patients' preferences towards less invasive surgery and better cosmetic outcome forced surgeons to develop new surgical techniques to satisfy the patients' desires.

Currently, axillary dissection is not performed in patients with negative SLNB. On the other hand, traditional surgical approach to the axilla in case of SLNB positivity is to perform completion axillary dissection. However, surgeons avoid performing axillary dissection in selected patients in clinical practice. In addition, recent studies suggest that application of radiotherapy including the axillary field may be an alternative to axillary dissection

in the near future. Recent changes in the management of positive axilla necessitate surgeons to refresh their knowledge on this challenging topic.

SENTINEL LYMPH NODE BIOPSY

Sentinel lymph node biopsy concept has evolved to avoid unnecessary regional lymph node dissections in most probably tumor-free lymph node regions. This technique is based on the excision and pathological examination of sentinel lymph node(s) which is assumed to be the first lymph node(s) draining the primary tumor. Sentinel lymph node biopsy was first introduced into the treatment of penile cancer and malignant melanoma and feasibility of SLNB was later proven in breast cancer^[1]. In recent years, SLNB has largely replaced axillary dissection in the surgical treatment of breast cancer patients. Morbidity observed after axillary dissection such as seroma and hematoma formation, paresthesia, pain, lymph edema, restricted arm and shoulder function decreased in 6%-30% of patients treated with SLNB. Sentinel lymph node biopsy is primarily indicated in patients with clinically and radiologically normal axilla. Sentinel lymph node biopsy is a safe and accurate procedure to detect malignant cells in regional lymph nodes. However, 5%-10% false negativity rate in breast cancer has been disputed over the years, but this rate has definitely decreased to less than 5% in experienced centers. Sentinel lymph node biopsy can be performed using a blue dye and/or radiocolloid^[2,3]. Combined method increases the accuracy of sentinel lymph node detection and decreases the false negativity rate, especially during the learning stage of the procedure. Patent blue, isosulfan blue, and methylene blue are the agents used as blue dyes. On the other hand, technetium labelled sulfur colloid and albumin are utilized as radioactive agents.

Sentinel lymph node biopsy can be performed in almost all patients with few exceptions. Presence of a clinically and radiologically suspicious lymph node in the axilla is an absolute contraindication. Ultrasound guided fine needle aspiration biopsy should be performed in such patients in order to exclude axillary lymph node metastases. After a negative biopsy result, caution must be taken during SLNB. Any macroscopic lymph nodes should be excised even though they do not take up blue dye or radiocolloid.

Although they cannot be predicted before the procedure, allergic reactions to either blue dye or radiocolloid could be another contraindication to SLNB. Any cross-reactivity between the dyes and/or radiocolloid or drugs with similar chemical structure was not reported. Preoperative use of anti-allergic drugs does not prevent anaphylactic reactions, however, may decrease the severity of allergic reaction. In addition, administration of any blue dye during pregnancy and lactation is not accepted as a safe procedure. In contrast, radiocolloids can be safely utilized during pregnancy as the calculated fetal dose is low.

Previous surgery to the breast and axilla is a relative contraindication for SLNB. Surgical diagnostic method is extremely important for breast cancer patients. Core needle biopsy, especially under radiologic guidance, is the preferred method for diagnosis. Surgical open biopsies such as incisional or excisional biopsy affect further breast conserving and axillary surgery. False negativity rates of SLNB after excisional biopsy may increase and periareolar subdermal injections of blue dye and radiocolloid instead of peritumoral injections increase the success rate of this procedure. However, previous axillary surgery definitely increases the false negativity in SLNB^[4]. When SLNB is performed in patients with previous excisional biopsy or axillary surgery, the results should be carefully assessed.

NEW DEFINITIONS IN NODAL STAGING

After the introduction of SLNB in the axillary management of breast cancer, new concepts have been introduced into the nodal staging of breast cancer^[5]. Isolated tumor cells and micrometastases were the new definitions for nodal staging in addition to macrometastases. Isolated tumor cells were defined as cell clusters less than 0.2 mm in diameter or tumor cells fewer than 200 in number. On the other hand, micrometastases refer to malignant cell clusters between 0.2-2 mm in size or cells more than 200 in number. When the size of metastases is more than 2 mm, it is called as macrometastases. Presence of isolated tumor cells in an axillary lymph node is staged as N0 whereas micrometastases and macrometastases were accepted as N1. In addition, detection method of axillary metastases affects nodal staging and determines the significance of axillary metastases. Nodal metastases detected by either immunohistochemistry or molecular methods such as polymerase chain reaction are staged as N0 (+ or mol+). Clinical significance and impact on survival of metastases detected by immunohistochemistry or polymerase chain reaction is less important compared to metastases seen on hematoxylin-eosin sections.

AXILLARY MANAGEMENT IN CASE OF SENTINEL LYMPH NODE NEGATIVITY

The histopathologic examination result of SLNB determines the surgical approach to axillary lymph node basin. Main objective of SLNB is to prove that clinically and radiologically negative axilla is actually tumor-free after histopathologic examination. Previous prospective randomized studies reported false negativity rates of < 10% with SLNB^[6]. These results encouraged the surgeons not to perform axillary dissection in cases with negative SLNB. Without axillary dissection, detected loco-regional recurrences were much less than the predicted ones in patients with long term follow-up possibly due to beneficial effects of adjuvant radiotherapy and systemic treatment. Five-year axillary recurrence rate changes be-

tween 0.5%-1.5% in patients with negative SLNB^[7-9]. Axillary recurrence rates continued to be low even after ten years^[10]. In a meta-analysis of 48 studies including 14959 patients, axillary recurrence rate was reported as 0.3% after a median follow-up time of 34 mo^[11]. According to the results of previously mentioned studies, currently, axillary dissection is not performed in patients with a negative SLNB result to avoid possible morbidity due to dissection.

AXILLARY MANAGEMENT IN CASE OF SENTINEL LYMPH NODE POSITIVITY

On the other hand, axillary lymph node dissection has been the standard of care for patients with a positive sentinel lymph node. However, a meta-analysis including 8059 patients with a positive axilla from 69 trials reported that a sentinel lymph node is the only involved node in 40%-60% of cases^[12]. Frequency of non-sentinel lymph node positivity was dependent on tumor burden in sentinel lymph node and the detection method of metastases^[12,13]. Tumor size (> 2 cm), macrometastases (> 2 mm) and extracapsular extension in sentinel lymph node, number (> 1) and ratio (> 50%) of positive sentinel lymph nodes and lymphovascular invasion determine the probability of metastases in non-sentinel lymph nodes^[14]. Thus, axillary lymph node dissection in case of a positive sentinel lymph node has been questioned in recent studies. As a result, detection of isolated tumor cells or micrometastasis in sentinel lymph nodes is not accepted as a definite indication for axillary dissection. Even in case of macrometastasis in the sentinel lymph node, axillary lymph node dissection can be avoided in selected patients according to the results of recent studies.

Studies based on breast cancer patients' information from large data bases support this trend in daily practice^[15,16]. Axillary dissection is omitted in 16.4%-20.8% of the patients with sentinel lymph node positivity^[15,16]. In retrospective studies, older age, severe comorbidities, smaller tumor size, low grade, and hormone receptor positivity were reported as the most frequent reasons for avoiding axillary dissection^[15,17]. Besides, higher number of removed sentinel lymph nodes, lower percentage of positive sentinel lymph nodes and pathologic N stage support the decision on only SLNB^[18]. In addition, surgeons feel reluctant to perform axillary dissection when metastases in the sentinel lymph nodes were identified postoperatively. Low axillary recurrence rates reported in retrospective studies encouraged the surgeons not to perform completion axillary dissection^[18-21].

Presence of metastases in the remaining axilla in only 40%-60% of patients led to the development of nomograms to predict the patients with further metastases in the axilla^[12]. However, low sensitivity, specificity, and predictive values of these nomograms raised concerns about their use in clinical practice. Since each of these nomograms was developed according to the properties of the related patient population, applicability of the results to

every patient may be misleading. Besides, all of the tumor and sentinel lymph node characteristics utilized in nomograms may be unavailable during SLNB. This might challenge the surgeon's decision to proceed with or avoid axillary dissection during surgery.

In addition, results of the recent studies questioned the necessity of completion axillary dissection after positive SLNB. Several meta-analysis reported acceptably low axillary recurrence rates without any dissection in the axilla. Sentinel lymph node positivity should be categorized according to metastatic tumor load to definitely determine the risk of axillary recurrence. Recurrence rate is expected to be different for isolated tumor cells, micrometastases, and macrometastases.

ISOLATED TUMOR CELLS/ MICROMETASTASES AND AXILLARY DISSECTION

Surgeons first started to avoid axillary dissection in patients with isolated tumor cells or micrometastasis in sentinel lymph nodes. Primarily, patients with favorable tumor characteristics such as smaller tumor size and lower grade were selected. In a meta-analysis, 30 studies including patients with positive SLNB and without completion axillary dissection were reviewed^[22]. In these studies, 3468 patients with micrometastatic disease in SLNB were included. After a median follow-up time of 42 mo, only 0.3% of the patients developed an axillary recurrence. Another study including patients from Surveillance, Epidemiology, and End Results database reported even less regional recurrence rate of 0.1% among 1767 patients with micrometastatic disease and no further axillary dissection^[16]. Bilimoria *et al*^[15] evaluating the patients in the United States National Cancer Data Base reported an axillary recurrence rate of 0.6% in 530 patients with micrometastatic disease. On the other hand, axillary recurrence rate was reported as less than 1% after completion axillary dissection which is similar to the rates without completion axillary dissection^[7,15].

These results from the evaluation of various data bases led to the planning of randomized controlled studies to test the role of axillary dissection in patients with micrometastases in sentinel lymph nodes. IBCSG 23-01 study randomized 931 patients with micrometastases to either axillary dissection or no further surgical treatment. Disease-free and overall survival results were similar in both groups after a median five year follow-up^[23]. Patients treated with breast conserving surgery received radiotherapy whereas almost all patients were treated with systemic therapy, mostly hormonal treatment, in this study^[23]. In contrast, another study evaluating patients with isolated tumor cells and micrometastases in Netherlands Cancer Registry reported a significantly higher rate of regional recurrence for patients with micrometastases and without axillary dissection^[24,25]. More regional recurrences were detected especially in patients with shorter doubling time,

grade 3 tumors, and hormone receptor negativity^[24,25]. As a result of these studies, surgeons avoid completion axillary dissection in patients with isolated tumor cells and micrometastases in sentinel lymph nodes.

MACROMETASTASES AND AXILLARY DISSECTION

A meta-analysis reviewed 16 studies including 3268 patients with macrometastases in the axillary lymph nodes without completion axillary dissection^[22]. These patients were followed-up for a median duration of 43 mo. Overall, axillary recurrence was detected in only 0.7% of the patients. Type of surgery and application of adjuvant radiotherapy were not clearly reported in the relevant studies. These retrospective data encouraged surgeons to design randomized controlled studies on this issue.

There was a need for a randomized controlled trial to demonstrate the requirement for completion axillary dissection in case of sentinel lymph node positivity. ACOSOG Z0011 trial is the only randomized controlled trial comparing completion axillary dissection following SLNB and SLNB alone in axilla positive breast cancer patients. Although the study was closed earlier than expected with less patient accrual than initially planned, the results of this study certainly changed daily surgical practice. Patients with T1/T2 tumors, treated with breast conserving surgery, and with 1 or 2 positive sentinel lymph nodes were included in the study. Six hundred patients were randomized into two groups and disease-free and overall survivals of the patients were compared. Almost all of the patients (96%-97%) received adjuvant chemo- and radiotherapy. After a median follow-up of 6.3 years, no significant difference was reported between the two groups concerning disease-free and overall survival rates^[26].

Presence of axillary metastases determines the adjuvant treatment protocols in breast cancer patients. Detection of metastases in axillary lymph nodes generally is an indication for chemotherapy. Adjuvant systemic treatment affects the axillary metastases as observed in the neoadjuvant setting. Completion axillary dissection following sentinel lymph node positivity helps better staging of the patient and may be therapeutic. In addition, presence of further metastases in the remaining axilla will change decision on systemic chemotherapy. On the other hand, information obtained after completion axillary dissection such as the number of involved lymph nodes may affect the decision on adjuvant radiotherapy. Involvement of four or more lymph nodes requires adjuvant radiotherapy to supraclavicular and infraclavicular lymph nodes. Besides, patients treated with breast conserving surgery will receive adjuvant radiotherapy to the remaining breast tissue and axilla will be involved in the tangential field. Adjuvant chemotherapy and radiotherapy definitely have a role in the low recurrence rates detected in the axilla even after sentinel lymph node positivity without further dissection.

ALTERNATIVE APPROACHES TO AXILLARY DISSECTION IN POSITIVE AXILLA

Axillary dissection is the primary method used to obtain loco-regional control in breast cancer patients. Radiotherapy could be an alternative to dissection for controlling loco-regional disease in case of sentinel lymph node positivity. Previous randomized controlled studies reported non-significant differences between axillary dissection and radiotherapy to the axilla^[27-29]. Recently, axillary dissection and radiotherapy were compared in a randomized controlled study regarding efficacy in loco-regional control and decreasing morbidity in patients with positive sentinel lymph node^[30]. Patients with tumors 0.5 to 3 cm in size and clinically negative axilla were initially treated with SLNB. Patients with positive axilla after SLNB were randomized to either axillary dissection or axillary radiotherapy. Although the final results of the study in detail were not published, two treatment modalities seemed comparable.

CONCLUSION

The pathologic status of the axilla has a diminishing effect on the choice of adjuvant treatments. Sentinel lymph node biopsy supplies the necessary information to decide on the adjuvant treatments. Recently, presence of axillary lymph node metastases is not accepted as an absolute indication for adjuvant chemotherapy in breast cancer. Patients with smaller tumor size and favorable prognostic factors such as hormone receptor positivity, low grade and Ki-67 expression, absence of lymphovascular invasion may be spared adjuvant chemotherapy. On the other hand, prognostic factors determining the indications for adjuvant radiotherapy may require information about the remaining axilla in case of sentinel lymph node positivity. Although adjuvant radiotherapy was applied to patients with 1 to 3 metastatic lymph nodes in certain cancer centers, four or more positive nodes are accepted as a widely used indication in treatment. Completion axillary dissection can provide further information about the axilla in case of positive sentinel lymph nodes to assist on the decision of adjuvant radiotherapy. However, radiotherapy to axilla may replace axillary dissection if further randomized controlled studies report equal efficacy between the two treatment modalities.

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WJCO 5th Anniversary Special Issues (2): Breast cancer

Breast cancer as photodynamic therapy target: Enhanced therapeutic efficiency by overview of tumor complexity

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Abstract

Photodynamic therapy is a minimally invasive and clinically approved procedure for eliminating selected malignant cells with specific light activation of a photosensitizer agent. Whereas interstitial and intra-operative approaches have been investigated for the ablation of a broad range of superficial or bulky solid tumors such as breast cancer, the majority of approved photodynamic therapy protocols are for the treatment of superficial lesions of skin and luminal organs. This review article will discuss recent progress in research focused mainly on assessing the efficacies of various photosensitizers used in photodynamic therapy, as well as the combinatory strategies of various therapeutic modalities for improving treatments of parenchymal and/or stromal tissues of breast cancer solid tumors. Cytotoxic agents are used in cancer treatments for their effect on rapidly proliferating cancer cells. However, such therapeutics often lack specificity, which can lead to toxicity and undesirable side effects. Many approaches are designed to target

tumors. Selective therapies can be established by focusing on distinctive intracellular (receptors, apoptotic pathways, multidrug resistance system, nitric oxide-mediated stress) and environmental (glucose, pH) differences between tumor and healthy tissue. A rational design of effective combination regimens for breast cancer treatment involves a better understanding of the mechanisms and molecular interactions of cytotoxic agents that underlie drug resistance and sensitivity.

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Key words: Photodynamic therapy; Breast cancer; Tumor microenvironment; Treatment combination; Synergism

Core tip: Breast cancer is the most common cancer in women worldwide. However, effective therapies that reduce the high mortality rate and improve patient quality of life are still unavailable. In recent years, the use of photodynamic therapy has been examined for use in breast cancer treatment. Photodynamic therapy provides a new and promising antitumor strategy that could be implemented, alone or in combination with other approved or experimental therapeutic approaches, to a wide range of applications.

Lamberti MJ, Rumie Vittar NB, Rivarola VA. Breast cancer as photodynamic therapy target: Enhanced therapeutic efficiency by overview of tumor complexity. *World J Clin Oncol* 2014; 5(5): 901-907 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i5/901.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i5.901>

PHOTODYNAMIC THERAPY ON SOLID TUMORS: FOCUS ON BREAST CANCER

Photodynamic therapy (PDT) is one of the clinically approved and minimally invasive alternate methods for

treatment of various cancers, such as bladder, esophagus, respiratory tract and gynecologic cancers. PDT eliminates tumor cells by the combined use of nontoxic photosensitizers (PS) and light^[1]. Light activation of a PS results in energy transfer cascades that ultimately yield cytotoxic reactive oxygen species, which can then render cell death^[2]. Antitumor effects of PDT derive from three interrelated mechanisms: direct cytotoxic effects on tumor cells, indirect damage to the tumor vasculature, and induction of an inflammatory response that can activate systemic immunity^[3].

The photosensitizer is considered to be a critical element. In general, for solid tumor PDT, an ideal PS should meet at least some of the following criteria: a commercially available pure chemical, low dark toxicity but strong photocytotoxicity, good selectivity towards tumor cells, longer wavelength allowing deeper light penetration, rapid removal from the body, and multiple administration routes (oral, intravenous, intratumoral or inhalational). Although some PSs satisfy all or some of these criteria, there are currently only a few PDT photosensitizers that have received official approval around the world. Photofrin (630 nm; Axcan Pharma, Inc.), Levulan (prodrug of protoporphyrin IX, 630 nm; DUSA Pharmaceuticals, Inc.), Metvix (prodrug of protoporphyrin IX, 630 nm; PhotoCure ASA), Foscan (652 nm; Biolitec AG), Laserphyrin (664 nm; Meiji Seika Kaisha, Ltd.), and Visudyne (693 nm; Novartis Pharmaceuticals). Several second generation PSs (*e.g.*, HPPH, 665 nm; SnEt2, 665 nm; LuTex, 732 nm) have been investigated in many preclinical and clinical trials for various solid tumors, and in particular SnEt2^[4,5] and LuTex^[5] are clinically applied in the United States for breast cancer^[3]. These photosensitizers show the selectivity towards tumor cells and are ideal for cellular- and vascular-targeted PDT, and interference with cytoprotective molecular responses is of growing interest. Any interactions between PDT and PDT-sensitizing agents is confined to the illuminated area, thus, eliminating any potential systemic toxicity.

The majority of approved PDT protocols are for the treatment of superficial lesions of skin and luminal organs, such as actinic keratosis and Barrett's esophagus, whereas interstitial and intra-operative approaches have been investigated for the ablation of a broad range of superficial or bulky solid tumors located in the head and neck, brain, breast, lung, gastrointestinal, and genitourinary regions^[2]. Although breast cancer is the most common cancer in women's cancer in the worldwide. The effective therapies that would not only effective in both reduce reducing the high mortality rate and associated with the disease, but also improve improving patient the quality of life patients with breast cancer are still searching for have not yet been achieved.

In recent years possibilities of PDT has recently been examined for using in breast cancer treatment, though are analyzed, and the full-potential range of potential applications alone or in combination with other approved or experimental therapeutic approaches needed to be has

yet to be explored defined.

This article reviews article will discuss recent progress in researches focused mainly on concerning the efficacy's assessing of different various photosensitizers used in photodynamic therapy PDT, as well as the combinatorial strategies of various therapeutic modalities with non-overlapping toxicities, in order to improve the therapeutic index of treatments of parenchymal and/or stromal tissues of in breast cancer solid tumors. Any interactions between PDT and PDT-sensitizing agents will be confined to the illuminated area. Therefore, the potentiated toxicity of the combinations is not systemic

PDT COMBINED WITH CONVENTIONAL BREAST CANCER THERAPIES

Most women with breast cancer undergo some type of surgery as the main strategy for tumor removal, including breast-conserving surgery or mastectomy (removal of breast). The breast can be reconstructed at the same time as the surgery or later on. Radiotherapy or systemic therapy is commonly given as adjuvant treatment after surgery^[6]. Radiotherapy involves the external (sometimes internal) application of high-energy rays (ionizing rays) to destroy cancer cells, which is typically accompanied by short-term side effects such as swelling and heaviness in the breast, sunburn-like skin changes in the treated area, and fatigue^[6]. In addition, synergistic treatments offer favorable outcomes, such as increasing the efficacy, decreasing the dosage, avoiding toxicity, and minimizing the development of drug resistance^[7].

In recent years, researchers have become increasingly interested in combining antitumor therapies in order to improve the patient outcome and to avoid, at least in part, unwanted side effects. In this context, there are reports indicating that some PSs can act as radiosensitizers^[8,9]. With regard to breast cancer, several *in vitro* studies have shown a synergism between PDT and ionizing radiation in killing cells. The combined application of nontoxic doses of indocyanine green^[10], rhodamine 123 and its platinum complex^[11], zinc phthalocyanine and meso-tetrahydroxyphenylchlorine^[12] with light proved to be very effective and resulted in a nearly complete reduction of survival. These reports suggest that treatment of tumors with a combination of PS-mediated PDT and ionizing radiation could be superior to their individual use. The interaction of PDT and ionizing radiation could enhance the therapeutic effect, thus reducing the dose of radiation dose and potential side effects.

Systemic therapy, better known as chemotherapy, is a treatment with cancer-killing drugs that are given intravenously or by mouth. The drugs travel through the bloodstream to reach cancer cells in most parts of the body. There are several situations in which chemotherapy may be recommended in breast cancer patients: after surgery (adjuvant chemotherapy), before surgery (neoadjuvant chemotherapy) or for advanced breast cancer. In most

cases (especially adjuvant and neoadjuvant treatment), chemotherapy is most effective when combinations of more than one drug are used. Although many combinations are currently being used, there is no clear indication that any particular combination is more effective. The most common chemotherapeutics used for early breast cancer include the taxanes (such as docetaxel and paclitaxel) and the anthracyclines (epirubicin and doxorubicin). These may be used in combination with other drugs, such as cyclophosphamide and fluorouracil. Platinum agents (cisplatin, carboplatin) have also been useful for treating women with breast cancer. The type and amount of drug(s), as well as the length of chemotherapy treatment, determine the extent of side effects, which include nausea and vomiting, mouth sores, easy bruising, hair loss, change in appetite, increased chance of infections, low blood cell counts, bleeding and fatigue^[6].

Recently, the effect of PDT combined with traditional chemotherapy for the treatment of breast cancer has been studied. Low doses of cisplatin *in vitro*, which are unlikely to cause severe side effects, are more effective when appropriately combined with indocyanine green-based PDT^[13]. Additionally, another *in vitro* study showed that the combination of meso-tetrahydroxyphenylchlorine-mediated PDT and the chemotherapeutic 5-fluoro-2-deoxyuridine resulted in a lower cell survival than single-mode treatment^[14]. Benzoporphyrin derivative monoacid ring A-based PDT enhanced the antitumor effects of doxorubicin on breast cancer *in vivo*, which was associated with the cooperative regulation of extrinsic apoptotic pathways and the inhibition of tumor angiogenesis^[15]. However, the mechanisms involving the interactions between chemotherapeutic drugs and PSs, as well how they can be combined to increase cell killing while reducing side effects, needs to be examined in more detail.

We recently reported that the pre-clinical chemotherapeutic drug β -lapachone interacts with methyl aminolevulinic acid-PDT in breast cancer *in vitro*. However, we also demonstrated that the application scheme of both therapies have relevance on the outcome. Synergism was observed when chemotherapy was applied 24 h after PDT, due to the photodynamic induction of NQO1, the principal determinant of β -lapachone cytotoxicity. The combination of PDT followed by β -lapachone treatment is a potentially promising modality for the treatment of cancer^[16].

When treating cancer, cytotoxic agents are intended to exert their effect on rapidly proliferating cancer cells. However, cancer therapeutics often lack specificity, which can lead to toxicity and undesirable side effects. Many approaches have been designed to target tumors. Selective therapies can be established by focusing on distinctive intracellular and environmental differences between the tumor and healthy tissue. Additionally, a strategy to treat breast cancer involves the combination of drugs. The molecular interactions with cytotoxic agents, combined with increasing knowledge of the mechanisms underlying drug resistance and sensitivity, allow for the rational de-

sign of effective combination regimens for the treatment of patients^[17]. In this sense, combinations of PDT and tumor-targeted strategies will be reviewed.

PDT AND BREAST CANCER RECEPTOR-TARGETED AGENTS

The overexpression of many receptors in breast cancer cells, such as estradiol receptors, the human epidermal growth factor receptor 2, gonadotropin-releasing hormone receptors, and tistular factor VII receptors, is strongly associated with increased disease recurrence and a poor prognosis^[18]. Thus, therapies have been developed to target removal of ligands or inhibit their activation^[6]. As these receptors represent a potential site for directing receptor-mediated cellular uptake, photodynamic researchers utilized them as vehicles to selectively deliver photosensitizing agents. Thus, the overexpression of receptors in breast cancer was harnessed synergistically with the tumor-migrating effect of several PSs to selectively deliver target molecule-PS conjugates into breast tumor cells, and preferentially kill the tumor cells upon exposure to red light^[19-32] (Table 1). Developments in these specific types of receptor-targeting approaches highlight their potential advantages in the discovery of more effective cancer photochemotherapy agents.

PDT COMBINED WITH ANTI-APOPTOTIC STRATEGIES

PDT leads to the generation of cytotoxic oxygen species that appear to stimulate several different signaling pathways, some of which lead to cell death, and some that mediate cell survival. In this context, we observed that methyl-5-aminolevulinic acid-mediated PDT resulted in overexpression of survivin^[33], a member of the inhibitor of apoptosis family that inversely correlates with patient prognosis and whose role in resistance to anti-cancer therapies is a subject of intensive investigation. We demonstrated a specific role for survivin in modulating the PDT-mediated apoptotic response. Silencing survivin expression increased apoptotic indices and cytotoxicity exhibited by PDT on metastatic breast human cancer cells. In contrast, the overexpression of survivin increased cell viability and reduced cell death. Expression of another antiapoptotic protein, Bcl-2, was suppressed by genistein, and PDT with hypericin may represent a mutual therapeutic combination favoring apoptosis^[34]. The combination of genistein and PDT may therefore achieve a higher therapeutic outcome in human breast adenocarcinoma cell lines previously identified as PDT-resistant.

PDT AND MULTIDRUG-RESISTANCE INHIBITORS

One of the principal requirements of successful PDT is sufficient intracellular accumulation of the photosensi-

Table 1 Receptor-targeted photodynamic therapy on breast cancer cells

Targeted receptor	Photosensitizer	Result	Ref.
Estradiol receptor	Tetraphenylporphyrin	High-affinity conjugate protein binding	James <i>et al</i> ^[19]
Estradiol receptor	Tetraphenylporphyrin	High-affinity conjugate cell binding	Swamy <i>et al</i> ^[20]
Estradiol receptor	Pyropheophorbide a	Conjugate-selective cell death	Fernandez Gacio <i>et al</i> ^[21]
Estradiol receptor	Pyropheophorbide a	Conjugate-selective cell death	El-Akra <i>et al</i> ^[22]
Estradiol receptor	Pyropheophorbide a	High conjugate internalization	Sadler <i>et al</i> ^[23]
Estradiol receptor	Chlorin e6-dimethyl ester	Conjugate-selective cell death	Swamy <i>et al</i> ^[24]
Human epidermal growth factor receptor	Verteporfin and pyropheophorbide a	Conjugate-selective but less phototoxic	Savellano <i>et al</i> ^[25]
Human epidermal growth factor receptor	Verteporfin and pyropheophorbide a	Conjugate-selective cell death	Bhatti <i>et al</i> ^[26]
Human epidermal growth factor receptor	Zinc phthalocyanine (plus nanoparticles)	Conjugate-selective cell death	Stuchinskaya <i>et al</i> ^[27]
Human epidermal growth factor receptor	Sn-(IV) chlorin e6 monoethylenediamine	Conjugate-selective cell death	Gijssens <i>et al</i> ^[32]
Tisular factor (factor VII receptor)	Verteporfin	Conjugate-selective cell death	Hu <i>et al</i> ^[28]
Tisular factor (factor VII receptor)	Chlorin e6	Conjugate-selective cell death	Duanmu <i>et al</i> ^[29]
Gonadotropin-releasing hormone receptor	Zinc phthalocyanine	Conjugate-selective cell death	Xu <i>et al</i> ^[30]
Gonadotropin-releasing hormone receptor	Protoporphyrin IX	Conjugate-selective cell death	Rahimipour <i>et al</i> ^[31]

tizer drug. Mechanisms of anticancer drug elimination [or multidrug-resistance (MDR)] by tumor cells are mostly linked to the elevated expression and activity of drug efflux transporters that constitute a dominant impediment to curative cancer chemotherapy. Hence, novel strategies that overcome MDR modalities are considered a major goal of cancer research. The ATP-binding cassette protein ABCG2 (breast cancer resistance protein) effluxes some of the PSs used in PDT, and thus, has been associated with photodynamic resistance. It was reported from *in vitro* and *in vivo* experiments, that the tyrosine kinase inhibitor imatinib mesylate blocked ABCG2 function and enhanced the efficacy of PDT by increasing intracellular PS levels, and may therefore enhance the efficacy and selectivity of clinical PDT on breast cancer^[35].

ABCG2 is a putative cancer stem cell marker. Cancer stem cells, also known as tumor-initiating cells, are a small group of cancer cells involved in drug resistance, metastasis, and relapse of cancers and tumor-drug resistance^[36]. Hence, it is of importance to develop PSs that are not substrates of ABCG2, or design strategies to avoid ABCG2-mediated antitumor therapy resistance^[37]. Recently, ABCG2 was implicated in a mechanism that targets and kills cancer cells with an MDR phenotype. The MDR mediates extracellular vesicles (EVs) rich in ABCG2 in attached breast cancer cells that highly concentrate chemotherapeutics, thereby sequestering them away from their intracellular targets. The authors showed that the accumulation of photosensitive cytotoxic drugs, such as imidazoacridinones (IAs) and topotecan, damaged EV membranes and resulted in tumor cell lysis. Furthermore, the accumulation of IAs in lysosomes killed MDR cells by organelle rupture upon photosensitization. Therefore, a synergistic and cytotoxic effect resulting in MDR reversal is elicited by combining targeted lysis of IA-loaded EVs and lysosomes. In contrast, a selective photocytotoxic effect exerted by topotecan is achieved by accumulation in EVs of MDR cells. Thus, MDR modalities can be converted into a pharmacological, lethal Trojan horse to selectively eradicate MDR cancer cells by ABCG2-dependent drug sequestration within EVs^[38].

PDT AND NITRIC OXIDE (NO) SCAVENGERS

Photodynamic intervention generates reactive oxygen species that can destroy tumor cells. NO produced by photosensitized cells could be pro-carcinogenic by inhibiting apoptosis. It was shown that NO from chemical donor or activated macrophages made breast tumor cells sensitized by 5-aminolevulinic acid-generated protoporphyrin IX more resistant to photo killing by providing substantial protection against apoptosis^[39,40]. Additionally, it was demonstrated that PDT-treated breast cancer cells acquired the ability to upregulate inducible-nitric oxide synthase (iNOS) expression^[41]. In this sense, apoptotic cell killing was strongly enhanced by iNOS inhibition or knockdown and a NO scavenger^[42]. These findings strongly indicate that stress-elicited NO in PDT-treated breast tumors could compromise therapeutic efficacy and suggest that NOS-based pharmacologic interventions could prevent this.

PDT AND (BREAST) TUMOR MICROENVIRONMENT (TME) INTERVENTION

The TME is a well-defined ecosystem comprised of parenchymal (tumor) and stromal (non-tumor) populations that coexist and establish interspecific interactions, which contribute to malignancy^[43]. The TME of solid neoplasias is very different from those of normal tissues. The implementation of interstitial and estimation of PDT dosimetry relies on the complexity of the solid tumor. Moreover, the TME should be studied if stromal cells affected by photodynamic regimes extinguish the tumor ecosystem by destroying their network within tumor cells. In this sense, we have recently reviewed the term “Ecological Photodynamic Therapy” to emphasize the need to modulate PDT application regimens to exploit their effect on interspecific relationships and thus achieve complete tumor eradication^[43].

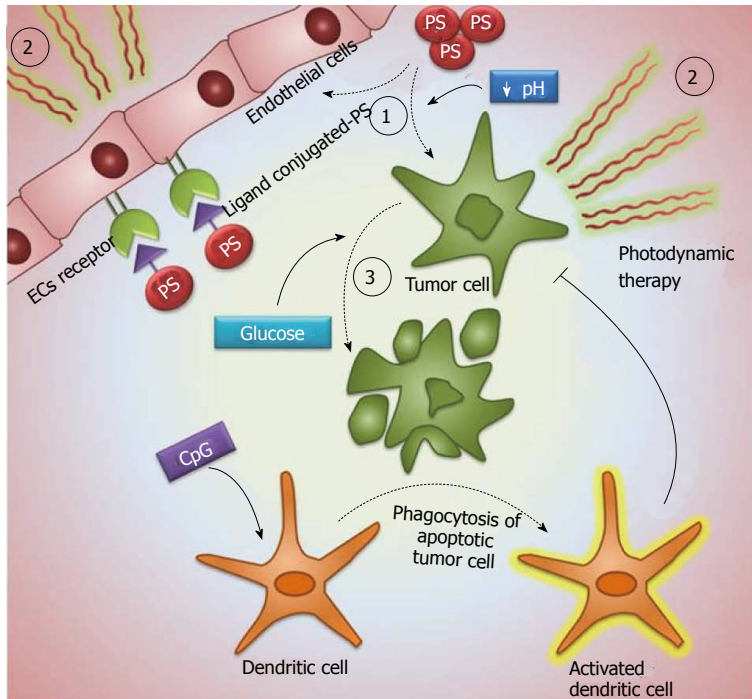


Figure 1 Photodynamic therapy (PDT) combined with tumor microenvironment intervention on breast cancer. PDT is one of the alternative methods for breast cancer treatment and involves: (1) administration of a photosensitizer (PS), which is internalized into either tumor cells or surrounding vasculature; (2) local irradiation at a wavelength corresponding to the absorbance peak of the PS; and (3) light activation of the PS, which promotes cell death mainly by apoptosis. Because of the benefits of improving PDT outcome, researchers have developed strategies to target the vasculature surrounding breast tumor cells by conjugating the PS with endothelial cell (EC)-specific ligands. Immunoactivation of dendritic cells using CpG increases phagocytosis of PDT-killed tumor cells and leads to their maturation and activation, thereby promoting an antitumor immune response. Regarding abiotic environmental factors, it was shown that photodynamic therapy sensitivity is reduced in glucose-deprived cells, and that a lower extracellular pH leads to increased PS uptake, reinforcing the photodynamic response.

Endothelial cells (ECs) are the stromal population whose principal function is to supply the TME with oxygen, hormones, nutrients, circulating cells and other fluids. A mutualistic interaction has been observed between ECs and tumor cells within TME: ECs provide nutrients and tumors cells develop a paracrine stimulation to finally sustain the angiogenic endothelial process^[43]; angiogenesis refers to the growth of blood vessels from pre-existing ones^[44]. Therefore, researchers have developed strategies to target the vasculature surrounding breast tumor cells. A novel and effective ligand-targeted PDT for breast cancer was synthesized by conjugating factor VII (fVII), a natural ligand with high affinity and specificity for tisular factor, with the PSs veterporfin^[28] or chlorin e6^[29] (Figure 1). The rationale for targeting tisular factor is based on its overexpression in breast cancer and its selective expression in pathologic neovascular ECs in cancer. fVII-targeted PDT improves the selectivity and efficacy of PDT for the treatment of breast cancer and induces apoptosis and necrosis as the underlining mechanisms of action. Moreover, fVII-targeted PDT was effective and safe for treatment of chemoresistant breast tumors *in vivo*, presumably by simultaneously targeting both the tumour neovasculature and chemoresistant cancer cells^[28,29]. Strategies to favor the vascular effect of PDT by targeting tumor vasculature are constantly evaluated. Recently, PSs conjugated to a peptidase-resistant peptide that targets neuropilins overexpressed in tumor angiogenic vessels were developed. Intravenously injected peptidase-conjugated PSs selectively accumulate in vascular cells with no degradation in plasma^[45] (Figure 1). This finding provides useful information for the future design of stable, targeted molecules to improve the outcome of PDT-treatment.

Treatment with PDT alone is often non-curative due

to tumor-induced immune cell dysfunction and immune suppression. This phenomenon has motivated a new approach of combining immunostimulants with PDT to enhance anti-tumor immunity. Thus, verteporfin-mediated PDT was combined with an immunomodulation approach using CpG oligodeoxynucleotide for the treatment of metastatic breast cancer *in vivo*. CpG primes immature dendritic cells *via* toll-like receptor 9 to phagocytose PDT-killed tumor cells, leading to dendritic cell maturation and activation. Peritumoral injection of CpG after PDT in mice gave improved local tumor control and a survival advantage compared to either treatment alone (Figure 1). In conclusion, CpG may be a valuable dendritic cell-targeted immunoadjuvant to combine with PDT^[46].

With regard to the TME, in addition to the cellular or biotic factors that modulate the photodynamic response, abiotic components also have a strong influence on PDT outcome. In this sense, the effect of chronic hypoglycemia on sensitivity to aminolaevulinic acid-induced PDT *in vitro* was studied in human breast cancer cells. It was shown that photodynamic therapy sensitivity was reduced in glucose-deprived cells^[47] (Figure 1). Additionally, tumors, due to their abnormal vasculature, are characterized by a more acidic environment compared to their surrounding normal tissues. The low pH can enhance the lipophilicity of several PSs, such as hematoporphyrin IX^[48]. It has been shown that increasing the lipophilicity of a drug leads to increased tumor uptake^[48] (Figure 1). As a result, it is possible to find a concentration gradient of the drug within the breast TME between the tumor tissue and the normal surrounding tissue. By injecting glucose, it is possible to further selectively reduce the extracellular pH value of tumors^[49], and to make tumor cells more sensitive to PDT treatment^[47]. This will, in

turn, increase the pH gradient between tumor and normal tissue and finally result in an increased concentration gradient for drugs that becomes more lipophilic at low pH values. If the low tumor pH explains the selective localization of such drugs, the clinical outcome of PDT can be improved by combining it with glucose injections. It is therefore necessary to characterize the interactions and biotic and abiotic components of the TME in order to achieve the disruption of ecological networks which finally can lead to the destruction of the ecosystem.

CONCLUSION

Despite major advances in the knowledge and treatment, breast cancer remains an enormous problem in terms of morbidity and mortality. It is expected the pharmaceutical industry and research institutes will continue to launch numerous clinical trials to evaluate applications of PDT in conjunction with, or as a replacement for, traditional methods for treating solid tumors.

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WJCO 5th Anniversary Special Issues (2): Breast cancer

Breast cancer phenotypes regulated by tissue factor-factor VII pathway: Possible therapeutic targets

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Abstract

Breast cancer is a leading cause of cancer death in women, worldwide. Fortunately, breast cancer is relatively chemosensitive, with recent advances leading to the development of effective therapeutic strategies, significantly increasing disease cure rate. However, disease recurrence and treatment of cases lacking therapeutic molecular targets, such as epidermal growth factor receptor 2 and hormone receptors, referred to as triple-negative breast cancers, still pose major hurdles in the treatment of breast cancer. Thus, novel therapeutic approaches to treat aggressive breast cancers are essential. Blood coagulation factor VII (fVII) is produced in the liver and secreted into the blood stream. Tissue factor (TF), the cellular receptor for fVII, is an integral membrane protein that plays key roles in the extrinsic coagulation cascade. TF is overexpressed in breast cancer tissues. The TF-fVII complex may be formed in the absence of injury, because fVII potentially exists in the tissue fluid within cancer tissues. The active form of this complex (TF-fVIIa) may stimulate the expression of numerous malignant phenotypes in breast cancer cells. Thus, the TF-fVII pathway is a potentially attractive target for breast cancer treatment. To date, a number of studies investigating the mecha-

nisms by which TF-fVII signaling contributes to breast cancer progression, have been conducted. In this review, we summarize the mechanisms controlling TF and fVII synthesis and regulation in breast cancer cells. Our current understanding of the TF-fVII pathway as a mediator of breast cancer progression will be also described. Finally, we will discuss how this knowledge can be applied to the design of future therapeutic strategies.

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Key words: Breast cancer; Blood coagulation; Tissue factor; Coagulation factor VII; Gene regulation; Therapeutic strategy

Core tip: Breast cancer is a worldwide problem. Difficulties with treating the disease and its recurrence persist due, in part, to a lack of therapeutic molecular targets. Blood coagulation factor VII (fVII) is generally produced in the liver. Tissue factor (TF), the cellular receptor for fVII, is an integral membrane protein that plays key roles in the extrinsic coagulation cascade. Formation of the TF-fVII complex causes contributes to the malignant phenotype of breast cancer cells. In this review, we summarize the breast cancer biology associated with the TF-fVII pathway. Further, we will discuss how these mechanisms can be targeted as therapeutics for this aggressive disease.

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INTRODUCTION

Breast cancer is a global health problem and remains a

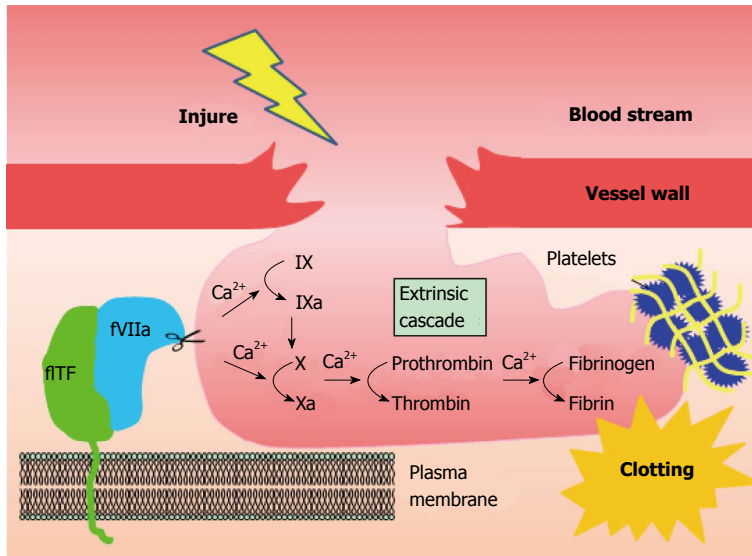


Figure 1 Extrinsic coagulation cascade initiated by the tissue factor-coagulation factor VII pathway. TF-fVIIa complex formation on the plasma membrane triggers an extrinsic coagulation cascade in response to injury. The TF-fVIIa complex located on the cell surface initiates the coagulation cascade by activating factor X via two different (coagulation factor IX-dependent or -independent) routes. This leads to fibrin deposition via the formation of thrombin. Blood coagulation completes by clot formation with other factors, such as platelets and red blood cells. FVII: Coagulation factor VII (fVII); TF: Tissue factor.

common cause of cancer death in women, worldwide^[1]. Currently, breast cancer may be treated using multiple chemotherapeutic programs depending on the histologic and molecular classification, such as the presence of hormone (estrogen and progesterone) receptors and/or epidermal growth factor receptor 2 (referred to as ERBB2 or HER2). However, difficulties associated with treating recurrent disease and triple-negative breast cancers that lack expression of apparent therapeutic molecular targets, remain^[2,3]. Therefore, a greater understanding of breast cancer biology is essential to translate these findings into novel, therapeutic strategies to combat aggressive breast cancers.

Coagulation factor VII (fVII) is a serine protease component of the extrinsic coagulation cascade, that is primarily synthesized and secreted by hepatocytes^[4]. Tissue factor (TF) is a 47-kD cell surface glycoprotein and a cellular receptor of fVII. fVII from blood plasma, associated with TF, gives rise to the activated complex, TF-fVIIa. This triggers a downstream coagulation cascade, eventually resulting in fibrin deposition (Figure 1). Previous studies have identified a correlation between cancer and blood coagulation, known as Trousseau's syndrome^[5]. It is likely that TF-fVII signaling is a major factor underlying this syndrome, although several other molecular mechanisms are possible^[5]. Indeed, hypercoagulation is a complication commonly associated with cancer patients and potentially contributes to patient mortality^[6]. Venous thromboembolism (VTE) is frequent in ovarian, pancreatic, and liver cancers^[7], and breast cancer during chemotherapy^[8].

Previous studies have revealed that plasma TF levels are elevated in cancer patients, including those with advanced breast cancer^[9]. Furthermore, breast cancer cells can release cell membrane-derived particles (generally referred to as microvesicles) with TF under various pathological conditions, leading to the hypercoagulation^[10]. A truncated form of TF, derived from alternative mRNA splicing, may be secreted into the blood stream^[11]. Therefore, TF-fVIIa complex formation may represent a major

cause of thromboembolic events. Numerous studies have also suggested that TF-fVIIa complex formation on the cell surface also contributes to the malignant phenotypes of cancer, including an increase in cell motility, invasiveness, cell survival, and angiogenesis^[12,13]. Recently, there is growing experimental evidence to suggest that TF also contributes to tumor initiation^[13]. Therefore, therapeutic strategies targeting TF may be advantageous to breast cancer, although the possible impairment of the physiological hemostatic process should be considered.

fVII is thought to penetrate hyperpermeabilized blood vessels around the tumor tissue^[14]. fVII may also exist in the lymph^[15]. This extravascular fVII may bind to TF, which is present on the surface of cancer cells. Notably, multiple breast cancer cells have been shown to ectopically synthesize fVII^[16]. This fVII is functional^[16], suggesting that aberrantly synthesized fVII may also contribute to the malignant phenotypes of breast cancer. In this review, we summarize the recent progress in breast cancer biology associated with aberrant coagulation mechanisms. In particular, we focus on the TF-fVII pathway, among the multiple coagulation mechanisms, because breast cancer phenotypes associated with platelets and fibrinolysis have been extensively reviewed^[17]. We also describe the mechanisms underlying TF and fVII overexpression and how their functions may be regulated in breast cancer cells. Finally, we discuss potential therapeutic strategies for breast cancer based on our current knowledge of the molecular mechanisms of TF-fVII signaling.

GENERAL BIOLOGY OF TF IN BREAST CANCER CELLS

TF-fVIIa signaling regulates breast cancer phenotypes

TF exists as either full-length or truncated forms, depending on the cell type. Full-length tissue factor (referred to hereafter as TF) is a 47-kDa membrane bound protein, essential for initiation of the extrinsic coagulation cas-

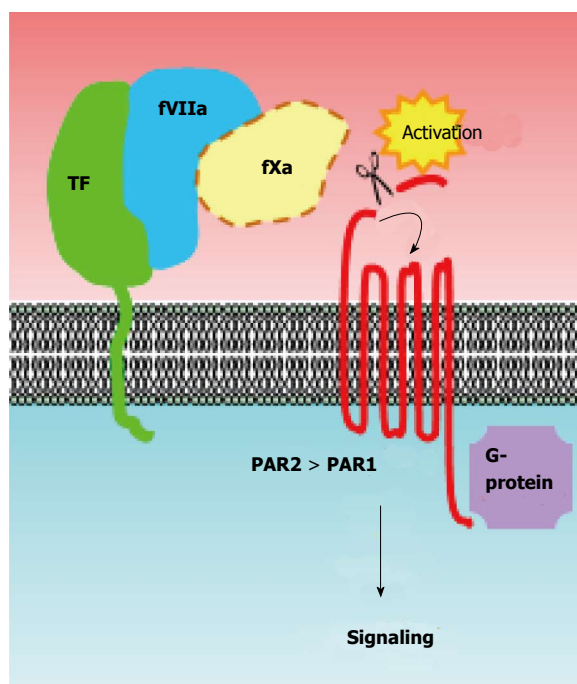


Figure 2 Activation of protease-activated receptors is a major mechanism of tissue factor-coagulation factor VIIa signaling in breast cancer cells. The proteolytic activities of the TF-fVIIa binary complex [potentially (designated as dotted line) ternary complex with fXa] cleave the N-terminal end of PARs. PARs are then activated via intra-molecular binding between the newly created N-terminus and an extracellular loop region of the receptors. Activation of these G-protein-coupled receptors subsequently activates downstream signaling cascades. A number of studies have indicated that PAR2 is crucial for activation of a TF-fVII-driven signaling cascade in breast cancer cells. The role of TF-fVII on PAR1 signaling in breast cancer cells is less evident. FVII: Coagulation factor VII; TF: Tissue factor; PARs: Protease-activated receptors.

cade (Figure 1). TF is widely, but selectively expressed in normal human tissues^[18] including normal breast tissue^[19]. In contrast, TF expression is minimally expressed in the liver^[18]. Thus, upon injury, the liver may use a coagulation pathway independent of TF-fVII formation^[20]. The role of liver TF remains elusive, however, because a recent study revealed that TF expression in mouse hepatocytes significantly contributes to thrombosis during liver injury caused by drug toxicity and hepatocyte transplantation^[21].

TF tends to be overexpressed in breast cancer tissues associated with malignant phenotypes^[22]. TF was initially classified as a member of the cytokine/growth factor receptor family owing to its amino acid sequence similarity^[23,24], suggesting that it may transmit intracellular signals. Indeed, TF-fVIIa is capable of transmitting intracellular signals *via* multiple pathways, predominantly those involving the activation of protease-activated receptors (PARs) (Figure 2). To date, a number of studies have made significant advances towards our understanding of how TF-fVIIa complex formation contributes to cancer progression^[12,13,25]. Until now, many studies concerning the biology of TF-fVIIa-dependent signaling were performed using breast cancer cell lines, possibly owing to their functional dependency on TF signaling^[26-29]. Among the breast cancer cell lines, MDA-MB-231 cells are well

characterized for their high TF expression, and are frequently used as a TF-dependent breast cancer model. Indeed, previous studies have shown that *in vitro* and *in vivo* phenotypes such as motility, invasiveness, and growth of MDA-MB-231 cells are highly TF-dependent^[26-30]. Moreover, in these cells, TF has been shown to act as an angiogenic switch, leading to breast tumor development in a spontaneous breast cancer model recapitulating the human disease^[31,32].

Regulation of TF expression in breast cancer cells

In human cells, the Sp1 transcription factor is a major regulator of the *F3* gene encoding TF, under normal conditions. Transcription may be affected by the presence of multiple single nucleotide polymorphisms (SNPs) within the *F3* regulatory region and these SNPs have been previously associated with disease characteristics^[33-36]. Immunohistochemical analyses demonstrate that TF is highly expressed in breast cancer tissues, in addition to ovarian and pancreatic cancer tissues^[9,22]. Although the detailed mechanisms are not clear, transcriptional activation appears to be a major mechanism of TF overexpression.

The mechanisms regulating *F3* gene expression are well characterized^[37]. Constitutively high *F3* gene expression is controlled by multiple transcription factors (Figure 3). It is likely that the aberrant activation of these factors causes higher TF levels in breast cancer cells, given that AP-1 and NFκB are proinflammatory transcription factors which are frequently activated in breast cancer cells^[38]. Indeed, previous studies have shown that these transcription factors strongly bind to the *F3* gene promoter in MDA-MB-231 cells. However, the promoter is poorly occupied in TF low-expressing MCF-7 cells^[39].

Breast cancer progression is dependent on sex steroid hormones. It was previously shown that TF expression increases in response to progesterone exposure^[40], resulting in breast cancer phenotypes *via* a TF-dependent pathway^[41]. Furthermore, a steady state level of TF mRNA in breast cancer cells may be determined by valance of its positive and negative regulatory mechanisms as it was found that PI3K/Akt and MAPK/ERK signaling pathways inversely regulate TF transcript levels in MDA-MB-231 cells^[42].

Inducible gene expression may also account for high TF expression in cancer tissues, although such mechanisms may not necessarily apply to breast cancer cells. For example, *F3* gene expression may be regulated by exposure to various environmental stimuli including cytokines^[13], growth factors^[13], and hypoxia^[13], resulting in the activation of AP-1 and NFκB.

It is likely that the tumor microenvironment and the associated blood supply affect the expression level of TF. In addition to serum factors, *F3* expression is also influenced by oncogenic events^[43-45] (Figure 3). Recent studies have shown that these factors play an important role in the regulation of *F3* expression in glioblastoma cells^[44,45], and similar mechanisms may also exist in breast cancer cells.

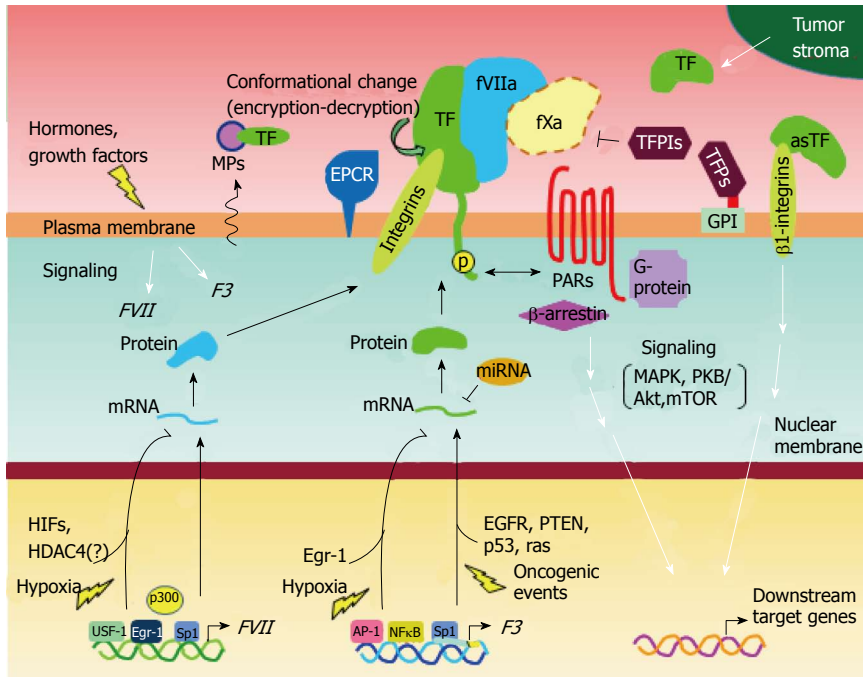


Figure 3 Possible mechanisms of expression and functional regulation of tissue factor and coagulation factor VII in breast cancer cells. Schematic overview summarizing the multiple mechanisms of gene expression, functional tuning, and intracellular signaling in breast cancer cells, as described in this review. It has been suggested that breast cancer cell phenotypes associated with the TF-fVII pathway may be specifically controlled via multiple autocrine and/or paracrine mechanisms, depending on tumor microenvironmental conditions. EGFR: Epidermal growth factor receptor; EPCR: Endothelial protein C receptor; FVII: Coagulation factor VII; TF: Tissue factor.

The Egr-1 transcription factor also plays a major role in controlling TF expression^[37]. As previously discussed, Sp1 largely controls the basal activation of *F3* gene. However, Egr-1 expression may be induced in cancer cells upon various stimuli within the cellular microenvironment. Because Egr-1 and Sp1 share a common DNA binding motif, Egr-1 may subsequently displace Sp1's occupancy of the *F3* gene promoter, thereby enhancing gene expression. A similar mechanism of *F3* activation may also apply under conditions of hypoxia (Figure 3). Hypoxia inducible factors (HIFs) are the major transcription factors responsible for adaptation to hypoxic environments within cancer tissues. Interestingly, however, it appears that Egr-1 rather than HIFs, is the major cause of *F3* activation under hypoxia, at least in glioblastoma multiforme cells^[45].

Recent studies also point to a role for microRNAs in the regulation of TF expression. In one study, miR-19 was shown to bind the 3'-UTR of the TF mRNA, repressing translation in breast cancer cells^[46] (Figure 3). This study also demonstrated that miR-19 is highly expressed in a TF low-expressing breast cancer cell line, MCF-7. Thus, regulation by this microRNA can determine TF levels.

Detailed mechanisms of TF-fVIIa signaling

Mechanisms of TF-fVIIa signaling potentially giving rise to malignant phenotypes can be classified as follows. In all cases, however, the TF-fVIIa-PAR axis plays a major role in the regulation of these cancer phenotypes (Figure 2). The first mechanism involves TF-fVIIa binary complex formation. Various studies indicate that the TF-fVIIa complex activates PAR2. This active PAR2 participates in determining the breast cancer phenotype, *via* activation of the mitogen-activated protein kinase (MAPK) cascade (Figure 3)^[27,28,30].

In the second mechanism, TF-fVIIa-dependent signaling may involve coagulation factor X (fX) for signal transmission (Figure 2). The TF-fVIIa complex can produce a TF-fVIIa-fXa complex^[26,47,48]. This tertiary complex can cause breast cancer phenotypes *via* not only activating PAR2-dependent signals, but also by activating PAR1 to trigger thrombin signals^[29]. In addition to MAPK, protein kinase B (PKB/Akt) is also involved in a signaling cascade mediated by the TF-fVIIa-fXa ternary complex^[48] (Figure 3). This ternary complex may also phosphorylate mTOR to activate components downstream of this signaling cascade in breast cancer cells^[29], thereby promoting cell migration. In addition, recent studies using non-breast cancer cells demonstrated that the endothelial protein C receptor (EPCR) supports this ternary complex to transmit signals^[49] (Figure 3).

The G protein-independent signaling pathway associated with PAR2 is one candidate mechanism for TF-fVIIa signaling in breast cancer cells^[50]. Indeed, enhanced breast cancer cell motility involves the recruitment of a scaffold protein, β -arrestin, to support cellular signaling^[51,52] (Figure 3). Which TF-fVIIa signaling mechanism is eventually used by breast cancer cells likely depends on the environmental conditions within the tumor, which may induce the expression of the downstream effectors of angiogenesis^[27,53]. Previous studies have shown that connective tissue growth factors such as Cyr61^[54], CTGF^[54] and the homeodomain DNA binding protein, CUX1^[55], are up-regulated in response to TF-fVIIa stimuli, however these experiments were performed using non-breast cancer cell lines.

cDNA microarray analyses in MDA-MB-231 cells also identified various factors downstream of the TF-fVIIa-PARs axis that may contribute to different breast cancer phenotypes. These studies identified novel target genes regulated by TF-fVIIa signaling, that result in a wound-

healing type response, including the chemokine ligand (angiogenic), CXCL1^[31,56], anti-apoptotic protein, Birc3^[56] and a component of basement membrane, CSF^[56].

MODULATORS OF TF FUNCTION IN BREAST CANCER CELLS

Conformational changes modulating TF function

Functional modification is an additional post-transcriptional mechanism regulating TF activity. Multiple mechanisms can control the procoagulant activity of TF by a conformational change referred to as encryption/decryption^[57] (Figure 3). Previous studies have shown that disulfide bond isomerization by protein disulfide isomerase (PDI), controls the function of TF as a mediator of coagulation or signaling, in endothelial and keratinocyte cell lines^[58]. However, this model appears to be controversial, because recent studies using the same endothelial cell line and MDA-MB-231 cells, indicate that decryption of TF is mediated *via* its interaction with anionic phospholipids^[59]. PDI has also been shown to activate the procoagulant activity of TF *via* its molecular chaperone activity^[60]. In conclusion, it is likely that the procoagulant activity of TF is specific for the decrypted form, while encrypted TF still transmits signals.

Tissue factor pathway inhibitors: negative regulators of TF-fVIIa activity

Tissue factor pathway inhibitors (TFPIs) are known to directly inhibit the enzymatic activity of TF-fVIIa complex^[61] (Figure 3).

Tissue factor pathway inhibitors (TFPI-1 and TFPI-2) are endogenous Kunitz-type inhibitors of TF-fVIIa-Xa complex formation, and thereby negatively regulate coagulation^[61]. TFPI-1 may exist as two alternatively spliced isoforms, TFPI α and TFPI β ^[62]. TFPI α is secreted or attached to the cell membrane *via* a glycosylphosphatidylinositol anchor, whereas TFPI β may be expressed as a membrane-bound form^[62]. Both forms are primarily synthesized in endothelial cells^[63]. Various cell types including macrophages, monocytes, platelets, and fibroblasts also produce TFPI-1^[64], thereby contributing to the physiological regulation of bleeding^[65]. TFPI-2 is highly expressed in the placenta, but is also synthesized in various normal tissues, including the endothelial cells of various blood vessels^[66].

Previous studies have shown that breast cancer tissues may also synthesize TFPI-1^[67] and TFPI-2^[66], and TFPI-1 expression levels were correlated with disease malignancy. In keeping with this observation, multiple breast cancer cell lines also express TFPI α and TFPI β ^[68]. Overexpression of both TFPI-1 isoforms induces apoptosis of breast cancer cells^[69]. Downregulation of TFPI-1 increases cell motility and breast cancer cell invasiveness^[70], suggesting that TFPI-1 acts as a suppressor of breast cancer phenotypes. In contrast, TFPI-1 may augment certain malignant phenotypes, such as adhesion of bladder cancer cells^[71]

and invasiveness of breast cancer cells^[66], *via* interaction with the TF-fVIIa complex. Thus, the therapeutic value of TFPIs is unclear, even though these anti-coagulants are aberrantly expressed in breast cancer tissues. Further *in vivo* studies are therefore required to completely understand the role of TFPIs in breast cancer progression.

POTENTIAL FUNCTIONS OF TF IN BREAST CANCER PROGRESSION

Tumor cell-derived TF

To date, numerous studies have been performed aiming to uncover the role of TF-fVIIa signaling in the malignant phenotypes of breast cancer using multiple cell lines. These cell lines represent useful models, because a number of tumor-associated processes such as motility^[28,29], invasiveness^[28,29], and survival^[48] are TF dependent. MDA-MB-231 is a good model cell line for TF-dependent breast cancer as this cell line synthesizes high levels of TF. Characteristics of this cell line, such as motility and invasiveness, are highly TF dependent *in vitro*^[26-28]. Furthermore, the growth of xenograft tumors derived from MDA-MB-231 cells is dependent on TF activity and subsequent PAR2 activation *in vivo*^[30]. This finding was supported by a PyMT mouse model characterized by spontaneous breast cancer development^[31]. These findings are also consistent with earlier studies using colorectal cancer cells, which revealed that TF does not contribute to cell proliferation *in vitro*^[43]. This suggests that host-tumor interactions are essential for the expression of a malignant phenotype in TF-driven breast cancer.

Another potential contributor of TF-driven breast tumor progression is EPCR (Figure 3). As previously discussed, EPCR can regulate TF-fVIIa signaling, and the importance of EPCR in breast cancer progression was recently demonstrated using both human xenograft^[72] and spontaneous murine breast tumor development^[73,74] models. Accumulating evidence indicates that TF largely contributes to the metastatic potential of breast cancer cells, and a recent study demonstrated that PAR1 signaling in both tumor and host cells is essential for TF-dependent lung metastasis of breast cancer cells^[74].

Breast cancer phenotypes regulated by TF-fVIIa complex formation are predominantly dependent on PAR2-dependent signals. However, PAR1 signaling may function to augment breast cancer cell invasiveness and tumorigenesis^[29,75-77], irrespective of TF dependency. PAR1 is a receptor of thrombin highly synthesized in metastatic breast cancer cells. Indeed, treatment of breast cancer cells with thrombin increases their PAR1-dependent invasiveness. PAR1 may also act as a receptor for matrix metalloproteinase-1, which is released from stromal cells within cancer tissues, and is capable of enhancing MDA-MB-231 cell invasiveness and tumorigenesis^[77]. Thus, the function of PAR1 and PAR2 signaling associated with breast cancer progression may vary, according to the cellular microenvironment and relative expression levels.

Exogenous TF

Expression of TF is not limited to tumor parenchyma cells, but is also expressed in the tumor stroma, where it promotes breast cancer metastasis^[78]. It was shown that transforming growth factor β released from cancer cells, may stimulate stromal cells to secrete TF, leading to the promotion of breast cancer progression in a paracrine manner^[78].

Exogenously synthesized TF in patients may affect a number of breast cancer cell characteristics. It was revealed that a paracrine effect of TF can influence the growth and metastasis of breast cancer cells^[79]. Treatment of cells with recombinant TF, mimicking stromal-derived TF (Figure 3), enhances the invasive and proliferative properties of these cells. This occurs *via* activation of β 1-integrins and/or PAR2-dependent signals, followed by inactivation of transcription of the estrogen receptor (ER) gene^[79] (Figure 3). The involvement of integrins in TF-driven phenotype expressions of breast cancer cells is similar to the findings that association of TF integrated into plasma membrane of keratinocytes and breast cancer cells bind integrins and may support growth of breast tumor^[30]. Furthermore, because ER positivity determines clinically distinct groups of breast cancer patients, ER gene regulation *via* TF signaling may affect the selection of therapeutic strategies used to treat breast cancer patients.

Previous studies reveal that TF expression within the stromal area of breast cancer tissues is due to production from immune cells^[80]. TF levels were closely associated with extravascular fibrin deposition and VEGF expression levels, suggesting that stromal-derived TF contributes to the angiogenic phenotype of breast cancer.

Truncated forms of TF

In addition to full-length TF, alternatively spliced, truncated forms of TF (asTF) also exist in humans^[81]. Recently, it was reported that asTF is highly expressed in breast cancer cells and contributes to the malignant phenotype^[82]. Similar to TF, asTF binds to integrins and therefore exists on the cell surface (Figure 3). However, the asTF-integrin complex augments cell proliferation, migration and anchorage-independent cell growth in a PAR2-independent manner^[82]. These results are in contrast to an earlier report that demonstrated that the growth of breast tumors derived from MDA-MB-231 cells is dependent on PAR2 signaling, downstream of membrane-integrated TF^[30]. It is possible that the distinct binding characteristics of these two TF isoforms underlie such differences.

TF EXPRESSION IN BREAST CANCER PATIENTS

TF as a serum component of breast cancer patients

In addition to asTF, breast cancer cells may also shed TF into the bloodstream as a component of microparticles (MPs), derived from plasma membrane in response to multiple stimuli^[83,84] (Figure 3). Thus, it is likely that these

TF-positive MPs contribute to plasma TF levels associated with clinical parameters^[9]. Indeed, TF-positive MPs may be secreted from human breast tumors in a mouse xenograft model, resulting in a coagulation-prone status^[10]. However, previous studies indicate that the risk of thrombosis during chemotherapy is independent of TF plasma levels in breast cancer patients, who display high levels of TF^[8,85]. Therefore, to date, it is not clear how, and to what extent, plasma TF derived from breast cancer cells, contributes to breast cancer malignancy. Conversely, a recent study demonstrated that circulating tumor cells (CTCs) of breast cancer patients may be detected by labeling cell surface TF^[86], suggesting that TF may be used as a diagnostic tool for breast cancer patients.

TF expression and its association with breast cancer patient clinical outcome

Activation of platelets^[8] and elevated plasma TF levels^[10] may be determinants of the thrombotic events observed in cancer patients. Indeed, VTE post-chemotherapy is a major event in breast cancer patients, prompting an investigation into the relationship between hemostatic markers and thrombosis^[8]. These studies revealed that plasma TF levels in patients were significantly elevated compared with those of non-cancerous individuals. However, TF levels did not increase during chemotherapy, indicating that chemotherapy-associated VTE does not correlate with TF, and more likely associated with neutrophil extracellular traps composed of cell free nucleic acids and neutrophils^[87].

TF is highly synthesized in breast cancer tissues as revealed by the analysis of clinical samples. In addition, TF is synthesized in the vascular endothelial cells of invasive breast cancer tissues. Thus, TF may be a marker for angiogenic phenotypes in patients^[22]. In addition to its expression in breast cancer tissues, TF levels are also increased in the plasma of breast cancer patients^[9]. Plasma TF levels were not significantly different between normal and benign tumors. However, TF levels were significantly higher in primary and recurrent cancer patients^[9]. Notably, this pattern of TF expression correlated with that observed in cancer tissues. The urine TF levels were also associated with poor prognosis of breast cancer^[88]. Taken together, these results suggest that the analysis of plasma and urine TF levels may be used to stratify patients into personalized treatment regimes.

Although the molecular mechanisms of the TF-driven breast cancer phenotypes may be explained by the various cellular events associated with the TF-fVIIa signaling cascade, recent studies indicate that post-translational modifications affecting TF expression also play an important role. Immunohistochemical analyses of breast cancer xenografts and clinical samples reveal that phosphorylation of TF (Figure 3) is correlated with the recurrence and aggressive phenotypes of breast cancer^[89]. It was found that the phosphorylation of TF in association with PAR2 expression correlates with breast cancer recurrence^[89]. In addition, *in vivo* studies using TF

cytoplasmic domain-deleted mice revealed interplay between the TF cytoplasmic domain and PAR2 signaling, to promote breast cancer by modulating the host angiogenic response^[32]. These studies provide a mechanism for the observed clinical association between TF phosphorylation and PAR2 signaling.

ECTOPIC EXPRESSION OF FVII IN BREAST CANCER CELLS

Constitutive expression

Similar to TF, the transcriptional regulation of human *FVII* has been extensively studied^[90-92]. In contrast to TF, biosynthesis of fVII in the mammalian body is limited. The primary site of fVII production is liver. Previous studies demonstrate that the human *FVII* gene promoter is typically bound by HNF-4 and Sp1 transcription factors, and is therefore highly activated in liver cells. The efficiency of *FVII* gene expression may be affected by genetic alterations, including SNPs and decanucleotide insertion^[93], as in the case of the *F3* gene. However, unlike *F3*, it appears that binding sites for inflammatory transcription factors such as NFκB and AP-1, do not exist within the *FVII* promoter region. Instead, the activity of *FVII* may be regulated in response to hormones such as estrogen^[94] and insulin^[95]. Plasma fVII levels are also known to associate with plasma lipid concentration^[96,97]. In addition to the liver, fVII may also be synthesized in monocytes and macrophages^[98,99], although the mechanisms of *FVII* regulation in these non-hepatocytic cells remains unknown.

fVII is primarily synthesized in the liver. However, various cancer cells may ectopically express fVII^[16]. Notably, multiple breast cancer cell lines constitutively synthesize high levels of the fVII transcript^[16]. Cancer cells with fVII expression exhibit pro-coagulant activity as TF-fVIIa complex is formed on the cell surface, suggesting that aberrantly synthesized fVII may be functionally active and contribute to breast cancer progression. Indeed, fVII expression is frequently observed in breast cancer specimens^[100].

Given the high expression of fVII in breast cancer cells and tissues, the molecular mechanisms of *FVII* activation were subsequently investigated using breast cancer cells. Binding of HNF-4 to the *FVII* promoter was shown to be essential for eutopic transcriptional activation. However, HNF-4 is not expressed in breast cancer cells^[100], suggesting that other factors are responsible for ectopic activation of *FVII*. Reporter gene assays revealed that reporter activity is fully activated by the authentic *FVII* promoter region in breast cancer cells^[100]. As expected, the HNF-4 binding site is dispensable for ectopic *FVII* gene expression in breast cancer cells. Further reporter assays revealed that an Sp1 binding site within the *FVII* promoter region is crucial for ectopic *FVII* gene expression. This study further demonstrated that the transcriptional regulators, USF-1 and Egr-1, also regulate ectopic expression of *FVII* in breast cancer cells^[100] (Fig-

ure 3).

Histone acetylation of gene promoters also plays a crucial role in the regulation of gene transcription, prompting analysis of such epigenetic modifications at the *FVII* gene promoter. These studies revealed that the histone acetyltransferases (HATs), p300 and CBP, predominantly occupy the *FVII* promoter region in breast cancer cells^[100]. In contrast, PCAF and SRC-1 HATs were also involved in the regulation of hepatocytic *FVII* expression^[100]. Thus, p300 and CBP may predominantly acetylate histones within the *FVII* promoter region, followed by accession of transcription factors responsible for transcriptional regulation in breast cancer cells. Conversely, various HATs may be responsible for eutopic *FVII* regulation.

Inducible expression under hypoxia

FVII transcription is inducible in ovarian cancer cells under hypoxic and hypoxia mimetic (CoCl₂ treatment) conditions^[16]. To date, the expression of fVII transcripts in response to hypoxia have been tested in several breast cancer cell lines^[100,101]. These studies revealed that fVII transcript levels are not enhanced in response to hypoxic stimuli in breast cancer cell lines with high fVII expression^[100]. Conversely, fVII mRNA levels were inducible in the breast cancer cell line, MDA-MB-468 under CoCl₂ stimuli^[101], suggesting a cell-type dependent induction of fVII.

The detailed mechanisms controlling *FVII* induction under hypoxic conditions were recently defined using ovarian cancer cell lines^[102], although it is not clear to what extent these mechanisms are applicable to breast cancer cells. These studies revealed that physical interaction between Sp1 and hypoxia inducible factor-2α (HIF2) may contribute to *FVII* activation in ovarian cancer cells, although HIF1 also indirectly affects *FVII* expression (Figure 3). This indicates that the promoter region occupied by HIF2 is devoid of a hypoxia response element (HRE)^[16,102], suggesting that HRE-independent mechanisms are responsible for *FVII* activation under hypoxic conditions. Indeed, chromatin immunoprecipitation analysis with MDA-MB-468 cells revealed that HIF2 predominantly associates with the *FVII* promoter region^[101], as in the case of ovarian cancer cells^[16]. Furthermore, this mechanism was synergistically induced following simultaneous exposure of ovarian cancer cells to hypoxic conditions and serum deprivation, *via* a HDAC4 (a class II histone deacetylase)-dependent pathway^[102]. These results suggest that the TF-fVII pathway is controlled by a stress-responsive, transcriptional mechanism, mediated by an HIF2/Sp1/HDAC4 network.

Exogenously supplied fVII vs autonomously produced fVII: Are there any functional differences?

Cell surface-bound TF binds fVII, irrespective of its source (eutopic or ectopic synthesis), raising the question of whether the TF-fVIIa complex functions differently depending on the source of fVII. Previous studies have

shown that the plasma concentration of fVII is quite low^[4]. Therefore, we can envision that self-production of fVII may facilitate TF-fVII complex formation compared with exogenously expressed fVII. This may be particularly important in hypoxic cancer microenvironments, where the supply of fVII from the bloodstream is likely limited because of poor and aberrant vasculature. In addition, it was recently described that ectopically synthesized fVII can augment the growth of breast cancer cells^[101]. This is an unexpected result as it had previously been observed that TF does not contribute to cell proliferation under *in vitro* cell culture conditions^[43]. Indeed, this study demonstrated that proliferation of breast cancer cells with high TF expression was not enhanced by exogenous supply of fVII^[101]. To date, however, the mechanisms regulating differential cell growth between cells exposed to exogenously supplied fVII and ectopically synthesized fVII remain unclear. Our knowledge concerning other functional differences in cancer cells associated with differential routes of fVII supply is currently poor; however, this represents an interesting field for future study.

TF-FVIIa SIGNALING AS A THERAPEUTIC TARGET

Potential therapeutic strategies targeting TF-fVIIa signaling

To date, several attempts have been made to inhibit TF-fVIIa activity associated with breast cancer *in vitro* and *in vivo*. One simple method used to block TF-fVIIa activity involves treatment with anti-TF antibodies. The use of monoclonal antibodies has been successfully used in breast cancer therapy to target cell surface HER2, and therefore represents a promising strategy^[103]. However, the major concern of this strategy is that blocking TF-fVIIa may also impair normal hemostasis, causing bleeding^[30]. Previous studies have shown that growth and lung metastasis of orthotopically transplanted MDA-MB-231 cells is profoundly suppressed by successive administration of the humanized anti-TF antibody, CNTO859^[104]. In this murine model, the CNTO859 antibody binds to human TF but not rodent TF, and therefore does not preclude normal hemostatic processes. However, the effect of this antibody in humans remains unclear. Similarly, a recent study demonstrated that the tick protein, Ixolaris, binds predominantly to the TF-fVIIa-fX ternary complex, thereby inhibiting downstream signaling involving PAR2 activation and suppressing tumor growth derived from MDA-MB-231mfp cells^[105]. However, this protein is unable to suppress the growth of murine breast cancer tumors, because Ixolaris does not bind the murine TF-fVIIa complex^[105].

One strategy to overcome the negative effects of TF-targeting antibodies on normal hemostasis is to use an antibody specifically inhibiting TF-fVIIa signaling. To date, we have identified a mouse monoclonal antibody, TF10H10, that fulfils this purpose^[30]. Similar to the CNTO859 antibody, growth of xenograft tumors

derived from MDA-MB-231 cells was shown to be effectively inhibited following treatment of cancer cells with a TF10H10 antibody prior to inoculation of mice^[30].

Inhibition of TF at the transcriptional level represents another strategy to target TF-fVIIa activity. As previously discussed, TF levels are transcriptionally controlled by various transcription factors regulating constitutive and inducible expression. Overexpression and/or functional activation of transcription factors such as Egr-1, NFκB, and AP-1 may be involved in this process. Previous studies have shown that curcumin, a major component of turmeric spice can inhibit binding of these transcription factors to gene promoter regions required for cell survival and invasion activities^[106]. Indeed, several studies showed that expression of the *F3* gene may be inhibited by this natural pigment in endothelial cells^[107,108] and monocytes^[109]. Thus, pharmaceutical inhibition by curcumin may suppress aberrant expression of TF in breast cancer cells.

Finally, there have been various attempts to combat breast cancer by targeting TF on the cell surface by increasing target selectivity. In these studies, fVII was conjugated with photosensitizers^[110,111], and the effect of this fusion fVII on tumor growth was monitored following injection into mice. Strikingly, tumor volume derived from breast cancer cells was markedly decreased in response to irradiation, compared with negative controls using non-fused fVII. This is likely because of the accumulation of photosensitizing drugs in tumor tissues with high TF expression. A similar approach was tested using fVIIa conjugated to a synthetic curcumin analog instead of photosensitizers^[112]. fVIIa successfully delivered curcumin to target breast tumor cells, thereby reducing toxicity and enhancing therapeutic efficacy.

Another interesting approach that may be harnessed to enhance tumor selectivity is the use of prodrugs that can be activated within the tumor microenvironment in a TF-dependent manner^[113]. In this study, doxorubicin-based prodrugs conjugated with albumin were used to treat tumors in a murine breast cancer model. These prodrugs penetrated tumor tissues and were predominantly activated by TF-fVIIa activity, because TF is highly expressed on the surface of cancer cells, leading to efficient suppression of breast cancer. Taken together, these studies demonstrate that increasing tumor selectivity of pharmaceutical compounds by targeting TF represents a promising strategy for cancer therapy, although careful control of dosage is necessary to prevent side effects, such as bleeding.

Possible strategies to inhibit ectopic fVII expression

Ectopic expression of fVII contributes to several breast cancer phenotypes *in vitro*. Thus, in addition to targeting TF expression, inhibition of fVII expression may also represent a potentially valuable therapeutic strategy. Importantly, the success of this strategy would rely on the selective inhibition of ectopic fVII, without inhibiting function of ectopically produced fVII in the liver.

Ectopic activation of the *FVII* promoter in breast cancer cells is associated with binding by p300 and CBP, while the *FVII* promoter in hepatocytes is occupied by various HATs, suggesting that targeting p300/CBP activities may selectively inhibit *FVII* expression in breast cancer cells^[100]. Curcumin is also capable of blocking the HAT activity of p300/CBP compared with other HATs. Indeed, curcumin markedly reduced fVII transcript levels in breast cancer cells in a dose-dependent manner, while normal expression of *FVII* in hepatic cells was only weakly impaired^[100]. Levels of constitutively expressed TF mRNA were not significantly diminished by curcumin treatment in these cells, consistent with the notion that curcumin specifically inhibits inducible TF expression. Furthermore, anacardic acid, another natural, small compound inhibitor of p300 and PCAF, did not selectively inhibit ectopic *FVII* expression, highlighting the specificity of curcumin for p300/CBP activity^[100]. The effect of curcumin on *FVII* expression was subsequently confirmed at the protein level. In contrast, HAT activity associated with the *FVII* promoter in hepatocytes, including p300 and CBP, is heterogeneous. It should be noted however, that selective inhibition of ectopic fVII synthesis was demonstrated using a limited number of cell lines. Therefore hepatocytic fVII synthesis may be considerably impaired by curcumin if p300/CBP is a component of the transcriptional machinery regulating the *FVII* gene in hepatocytes.

SUMMARY AND PERSPECTIVES

In this review, we describe various therapeutic strategies based on our current understanding of breast cancer biology associated with the TF-fVII pathway. The TF-fVIIa pathway in breast cancer cells may be regulated *via* multiple molecular mechanisms (summarized in Figure 3), enabling us to envisage a number of possible therapeutic strategies. Indeed, accumulating evidence suggests that TF is a promising target in breast cancer. Many breast cancer cell lines constitutively and perhaps inducibly express fVII. Given that TF-fVIIa signaling is a major mechanism underlying breast cancer-associated malignant phenotypes, strategies targeting ectopic fVII expression may also be considered in future therapeutic designs. How ectopic fVII expression affects breast cancer progression *in vivo*, however, remains an important question. Previous studies have shown that curcumin selectively inhibits ectopic fVII synthesis by removing p300/CBP from the *FVII* promoter region. Furthermore, animal studies have shown that curcumin can cure cardiovascular diseases^[114,115] and cancer^[116] by targeting p300 activity. Therefore, anti-p300 strategies using curcumin may be clinically applicable, without posing significant toxicity. Based on this, it may be of interest to investigate anti-p300/CBP strategies in fVII-expressing breast cancer models. It should be noted however, that anti-p300/CBP strategies may be compromised because HATs can also be targeted to the hepatocytic *FVII* promoter. The iden-

tification of novel molecular targets, specifically associated with ectopic fVII synthesis in breast cancer cells is therefore critical.

From a clinical point of view, the identification of relationships between fVII expression and various clinical parameters, such as chemoresistance, relapse, and overall survival, is essential to predict which patients may benefit from anti-TF-fVIIa treatment. Finally, many issues concerning the biology of ectopic fVII synthesis in breast cancer cells remain unresolved. Why do breast cancer cells tend to synthesize more fVII compared with other cancer cells? How does ectopically expressed fVII associate with TF to express the TF-fVIIa complex on the cell surface? It will be intriguing to investigate whether ectopically expressed fVII is equivalent to ectopically expressed fVII. A more detailed understanding of how ectopic fVII expression is regulated, and how it can contribute to breast cancer biology, is essential for translating our current knowledge to anti-breast cancer strategies.

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WJCO 5th Anniversary Special Issues (3): Cervical cancer**Image-based brachytherapy for cervical cancer**

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Abstract

Cervical cancer is the third most common cancer in women worldwide; definitive radiation therapy and concurrent chemotherapy is the accepted standard of care for patients with node positive or locally advanced tumors > 4 cm. Brachytherapy is an important part of definitive radiotherapy shown to improve overall survival. While results for two-dimensional X-ray based brachytherapy have been good in terms of local control especially for early stage disease, unexplained toxicities and treatment failures remain. Improvements in brachytherapy planning have more recently paved the way for three-dimensional image-based brachytherapy with volumetric optimization which increases tumor control, reduces toxicity, and helps predict outcomes. Advantages of image-based brachytherapy include: improved tumor coverage (especially for large volume disease), decreased dose to critical organs (especially for small cervix), confirmation of applicator placement, and accounting for sigmoid colon dose. A number of modalities for image-based brachytherapy have emerged including: magnetic resonance imaging (MRI), computed tomography (CT), CT-MRI hybrid, and ultrasound with respective benefits and outcomes data. For

practical application of image-based brachytherapy the Groupe Europeen de Curietherapie-European Society for Therapeutic Radiology and Oncology Working Group and American Brachytherapy Society working group guideline serve as invaluable tools, additionally here-in we outline our institutional clinical integration of these guidelines. While the body of literature supporting image-based brachytherapy continues to evolve a number of uncertainties and challenges remain including: applicator reconstruction, increasing resource/cost demands, mobile four-dimensional targets and organs-at-risk, and accurate contouring of "grey zones" to avoid marginal miss. Ongoing studies, including the prospective EM-BRACE (an international study of MRI-guided brachytherapy in locally advanced cervical cancer) trial, along with continued improvements in imaging, contouring, quality assurance, physics, and brachytherapy delivery promise to perpetuate the advancement of image-based brachytherapy to optimize outcomes for cervical cancer patients.

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Key words: Cervical cancer; Brachytherapy; Image-based brachytherapy; 3D-planning; Magnetic resonance imaging-based brachytherapy; Groupe Europeen de Curietherapie-European Society for Therapeutic Radiology and Oncology Working Group guidelines

Core tip: Brachytherapy is an integral part of radical pelvic radiation therapy for cervical cancer. While image-based planning has gained wide acceptance in external beam radiotherapy, the integration of image-based planning for brachytherapy has lagged significantly. More recently advances in planning software/hardware have lead to increased use of image-based brachytherapy. Herein, we highlight the clinical advantages of 3D brachytherapy planning for cervical cancer. We present multiple modalities for image-based brachytherapy including outcome data and dose constraints. Finally we outline practical guidelines for contouring target volumes and critical organs; and present future

directions in image-based brachytherapy aimed towards improving cervical cancer outcomes.

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INTRODUCTION

Cervical cancer is the third most common cancer in women worldwide with an estimated annual death rate > 275000^[1,2]. For early stage disease, prospective randomized data has established the equivalence of radical hysterectomy (\pm adjuvant radiation therapy based on risk factors) *vs* radical pelvic radiation therapy in terms of survival and disease control with increased toxicity with surgery \pm adjuvant therapy; however surgery is often the treatment of choice for early stage disease because of decreased treatment time, the potential opportunity for ovarian and/or fertility preservation, and a decreased risk of second malignancy^[3]. Definitive radiation therapy is the accepted standard of care for early stage patients not suitable for surgical resection and is integrated with concurrent cisplatin chemotherapy for patients with node positive or locally advanced tumors > 4 cm (all node positive and FIGO stage I B2, II A2, and higher). Definitive radiation therapy consists of a combination of external beam radiotherapy and brachytherapy; the addition of brachytherapy represents an integral part of definitive radiation therapy for cervical cancer shown to improve overall survival^[4,5]. Traditionally, as outlined in the International Commission on Radiation Units and Measurements (ICRU) 38 and the 2000 American Brachytherapy Society (ABS) guidelines for cervical cancer, brachytherapy dose was based on two-dimensional (2D) planning prescribed to a modification of the classical Manchester system point A for target coverage and conventional points for critical organs^[6,7]. The results for 2D X-ray based brachytherapy have been good in term of local control especially for early stage disease with acceptable toxicities, but there are unexplained toxicities and treatment failures^[8-10]. Additionally, the correlations between toxicities and critical organ point doses have not been consistent, limiting toxicity improvements through planning optimization in 2D brachytherapy^[10]. Computed tomography (CT) based three-dimensional (3D) planning and more recently more sophisticated image-based planning (Intensity Modulated Radiotherapy) has been widely accepted and implemented for external beam radiotherapy, however the acceptance and implementation of 3D image-based brachytherapy has lagged substantially. The relatively slow integration of 3D image-based brachytherapy can be attributed to a decreased availability of planning software/hardware, increased cost, and a lack of optimal training/expertise.

ADVANTAGES OF IMAGE-BASED BRACHYTHERAPY

Improvement in hardware and software for brachytherapy planning have more recently paved the way for 3D image-based brachytherapy which allows volumetric optimization improving tumor coverage and critical organ sparing which potentially increases local control, reduces toxicities, and helps predict outcomes. Early data for 3D planning has substantiated the potential improvements of 3D over 2D planning overcoming challenges in optimizing technique, reproducibility, uncertainties in target delineation, and the dosimetric planning processes. Several studies have compared the cervical tumor coverage and critical organ sparing by 3D image-based brachytherapy to doses delivered by 2D radiography-based brachytherapy^[11-18]. Investigators from University of Alabama Birmingham were some of the first to show that the 2D radiography-based approach using point A overestimates the tumor dose, especially in more advanced tumors where on average the gross tumor volume prescribed dose coverage was 98.5%, 89.5%, 79.5%, and 59.5% for stages I BI, I B2, II B, and IIIB, respectively^[11]. Others have shown that for smaller cervical tumors, the point A dose may achieve adequate tumor coverage, but over treats surrounding critical organ which can be improved with magnetic resonance imaging (MRI) image-based planning^[12]. These results were further validated by University of Pittsburgh data showing that the mean dose to 90% and its standard deviation was 83.2 ± 4.3 Gy that was significantly higher ($P < 0.0001$) than the mean dose 78.6 ± 4.4 Gy to Point A^[13]. Numerous early reports showed the orthogonal X-ray ICRU point doses underestimate doses to rectum and bladder as compared to CT based volumetric calculation by 1-5 folds (Table 1) especially for the bladder point doses^[19-22]. These results were later validated in a prospective study from MD Anderson, showing that the ICRU bladder point significantly underestimated the CT based highest dose to 2cc by a mean difference of 6.8 Gy, but did not differ significantly for the rectum with a mean difference of 0.21 Gy^[23]. Prospective studies from Korea and Vienna using all image-based brachytherapy correlated late changes in rectal mucosa on serial rectosigmoidoscopy with volumetric doses of 2cc, 1cc, and 0.1cc (D2cc, D1cc, and D0.1cc), showing significant dose cutoffs (Table 2) for both asymptomatic and symptomatic rectal changes^[24,25]. With longer follow-up the Vienna group has established well-defined dose-response curves for D2cc doses to the rectum and bladder (Table 3), which provide an invaluable risk-assessments for rectal and bladder constraints^[26]. While with 2D planning consistent validated constraints had been elusive impeding reductions in brachytherapy toxicity, these outcome data provide practical dose constraints for 3D brachytherapy optimization to limit the risks of late toxicity.

In addition to the well-established dosimetric advantages of 3D as compared to 2D brachytherapy planning

Table 1 Discrepancies between bladder and rectal doses as assessed by two-dimensional orthogonal films and computed tomography-image based planning

	Orthogonal film based <i>vs</i>	CT based
Ling <i>et al</i> ^[19]	Bladder	1.0 - 4.1x
	Rectum	1.4 - 2.5x
Schoepel <i>et al</i> ^[20]	Bladder	2.1 - 2.3x
	Rectum	1.3 - 1.6x
Stueckelschweiger <i>et al</i> ^[21]	Bladder	1.0 - 2.2x
	Rectum	1.1 - 1.6x
Kapp <i>et al</i> ^[22]	Bladder	1.0 - 5.4x
	Rectum	1.1 - 2.7x

Table 3 Correlations for volumetric doses for risk of grade 2+ late toxicity

	5% Risk	10% Risk	20% Risk	P value
Rectum				
D2cc	67	78	90	0.0178
D1cc	71	87	104	0.0352
D0.1cc	83	132	186	0.1364
Bladder				
D2cc	70	101	134	0.0274
D1cc	71	116	164	0.0268
D0.1cc	61	178	305	0.0369

D2cc: Dose to 2 cubic centimeters; D1cc: Dose to 1 cubic centimeter; D0.1cc: Dose to 0.1 cubic centimeter.

for cervical cancer, 3D planning additionally offers clinical advantages including: confirmation of applicator placement, decreased critical organs-at risk (OAR) dose for patients with a small cervix, accounting for sigmoid colon dose, and improved coverage for large volume disease while maintaining critical organ dosimetry. At the time of brachytherapy application, tandem placement can result in unsuspected uterine perforation despite the clinical impression of adequate tandem placement (Figure 1); 3D planning increases the diagnosis of perforation and avoids overtreatment of fundus/lower uterine segment^[27]. Patients with a small cervix represent a challenge to adequate brachytherapy delivery, where suboptimal weighting and positioning can lead to over-dosage of critical organs when using the conventional 2D planning one-size-fits-all optimization process (Figure 2). Investigators from Princess Margaret Hospital compared 2D *vs* 3D (MRI based) planning for patients with small cervix showing that tumor coverage (volume receiving 100% of the prescription dose > 95% of target) was adequately in 70% of the patients with the conventional 2D plans, respectively, and in 75% of the patients with the optimized plans and the minimal dose to the contiguous D2 cc of the rectal, sigmoid, and bladder wall volume was 16 ± 6.2 Gy, 25 ± 8.7 Gy, and 31 ± 9.2 Gy, respectively^[12]. While with MRI-guided brachytherapy optimization, it was possible to maintain tumor coverage and reduce the dose to the normal tissues, especially in patients with small cervix where the target volume treated to $\geq 100\%$ of the intended dose approached 100% in all cases, and the

Table 2 Dosimetric correlates for rectal toxicity in image-based brachytherapy

	D2cc (mean)	D1cc (mean)	D0.1cc (mean)
Koom <i>et al</i> ^[24]	75 Gy <i>vs</i> 69 Gy	80 Gy <i>vs</i> 73 Gy	90 Gy <i>vs</i> 85 Gy
VRS ≥ 2	(P = 0.02)	(P = 0.02)	(P = 0.04)
Georg <i>et al</i> ^[25]	72 Gy <i>vs</i> 62 Gy	76 Gy <i>vs</i> 65 Gy	88 Gy <i>vs</i> 75 Gy
VRS ≥ 3	(P < 0.001)	(P < 0.001)	(P = 0.002)
Georg <i>et al</i> ^[26]	72 Gy <i>vs</i> 64 Gy	76 Gy <i>vs</i> 67 Gy	88 Gy <i>vs</i> 77 Gy
Symptomatic	(P < 0.01)	(P < 0.01)	(P = 0.03)

VRS: Vienna rectoscopy score; cc: Cubic centimeters; Gy: Gray; D2cc: Dose to 2 cubic centimeters.

minimal D2cc of the rectum, sigmoid, and bladder was 12%-32% less than with conventional 2D brachytherapy planning^[12]. Dose to the sigmoid colon and small bowel was not accounted for in conventional 2D planning, which can receive > 70% of the point A dose^[28]. Image-based brachytherapy offers the added advantage of sparing dose to the sigmoid colon and small bowel, which potentially reduces the risk of stricture and ulceration (Figure 3). Large volume disease creates a challenge in achieving adequate coverage as portions of the disease extend larger distances from the central applicator with increasing tumor size; image-based planning in combination with a combined interstitial/intracavitary approach (Vienna applicator) help to create an asymmetric dose distribution improving tumor control and affording dose-escalation (Figure 4); which is especially important for larger tumors > 5 cm as highlighted in the Vienna group experience where comparing outcomes for cervical cancer patients prior to the introduction of image-based brachytherapy and the Vienna applicator, 3-year actuarial overall survival was 28% for tumors > 5 cm as compared to 58% ($P = 0.003$) with image-based brachytherapy^[29,30].

MODALITIES FOR IMAGE-BASED BRACHYTHERAPY-MRI

A number of imaging modalities have emerged for image-based brachytherapy planning. The Groupe Européen de Curietherapie-European Society for Therapeutic Radiology and Oncology Working Group (GEC-ESTRO) and ABS have developed guidelines to standardize contouring definitions and dosimetry for tumor targets and OARs^[31-33]. Both the ABS and GEC-ESTRO guidelines are based on MRI-based brachytherapy planning, with MRI offering superior soft tissue contrast^[34]. The largest experience with MRI-based planning comes from the Vienna group which incorporated MRI-based planning which each fraction, the mean dose to 90% of the target volume (D90) was 86 ± 16 Gy for the high-risk clinical target volume (HR-CTV) with a mean D2cc for the bladder, rectum, and sigmoid of 95 ± 22 Gy, 62 ± 12 Gy, and 62 ± 12 Gy^[35,36]. Similar dosimetric results have been published in the Aarhus and Leuven experience for definitive chemo-radiotherapy in advanced disease^[37,38]. The long-term outcome data from the Vienna experience

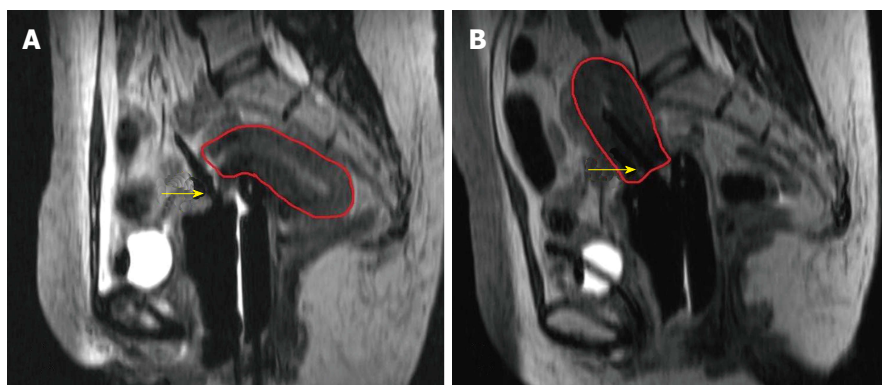


Figure 1 Uterine perforation despite perfect geometric applicator placement relative to bone anatomy. A: Shows the uterine perforation with retroverted uterus (red) diagnosed on magnetic resonance imaging (MRI) imaging for brachytherapy planning; B: Shows appropriate tandem placement (yellow arrow) ameliorated by placing the applicator under ultra-sound guidance and confirmed by MRI image-based planning.

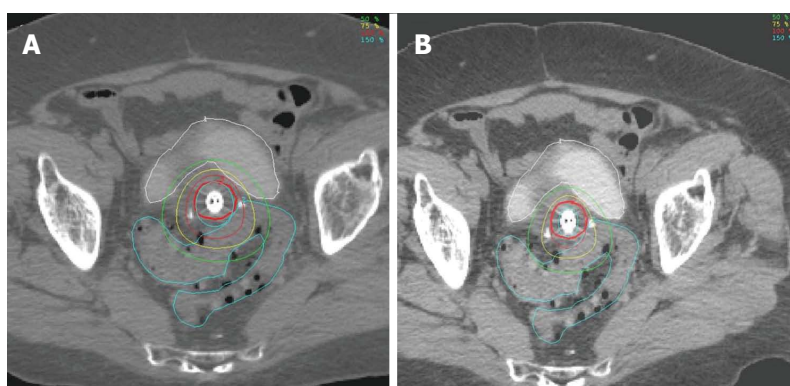


Figure 2 Conventional 2D point dose brachytherapy planning over doses critical organs for small cervix. Magnetic resonance imaging planning images showing over-dosage of critical organs with point-A image based prescription (A) for patient with small cervix which is improved with image-based brachytherapy (B).

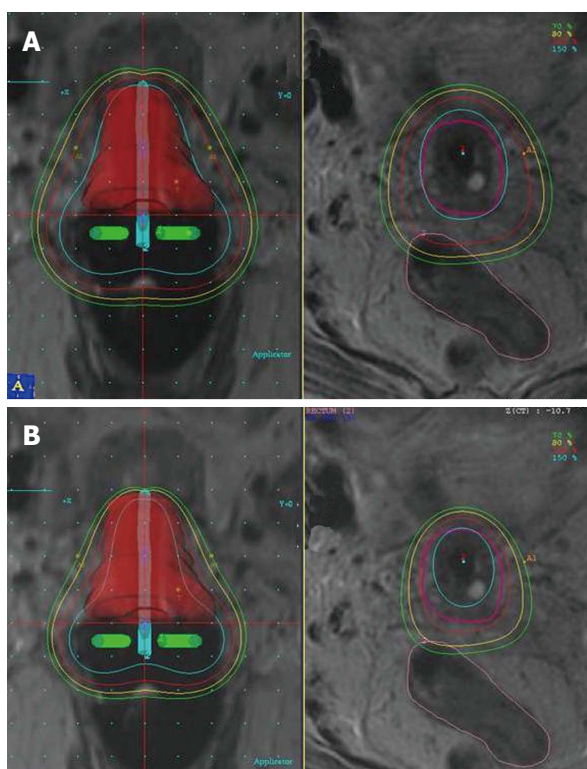


Figure 3 Sigmoid colon sparing with image-based planning. Magnetic resonance imaging planning images showing point A optimized plan with high doses to adjacent sigmoid colon (A), which is reduced with image-based 3D optimization (B).

showed an excellent 3-year 95% local control^[39].

MODALITIES FOR IMAGE-BASED BRACHYTHERAPY-CT

MRI is not universally available in radiation oncology departments; and the need for serial repeat imaging to account for changes in position of critical organs and tumor regression create logistical and financial impediments that have limited the universal applicability of MRI-based brachytherapy planning. Contrastingly, CT simulators are widely available in radiation oncology departments, thus interest grew in using CT based brachytherapy planning. To address these concerns, a prospective international cooperative group trial compared CT to MRI based planning showing that tumor height, thickness, and total volume measurements as determined by CT were not significantly different compared with the MRI volumes; similarly the MRI and CT dose-volume-histogram values of the D2cc, D1cc, and D0.1cc for the OARs were similar^[40]. However, the width measurements differed in HR-CTV for CT *vs* MRI based planning, resulting in statistically significant differences in the volume treated to the prescription dose or greater (MRI 96% *vs* CT 86%, $P = 0.01$) and dose to 90% of the treatment volume (MRI 8.7% *vs* CT 6.7%, $P < 0.01$)^[40]. Outcomes from Addenbrook, where a lack of access to MRI for brachytherapy

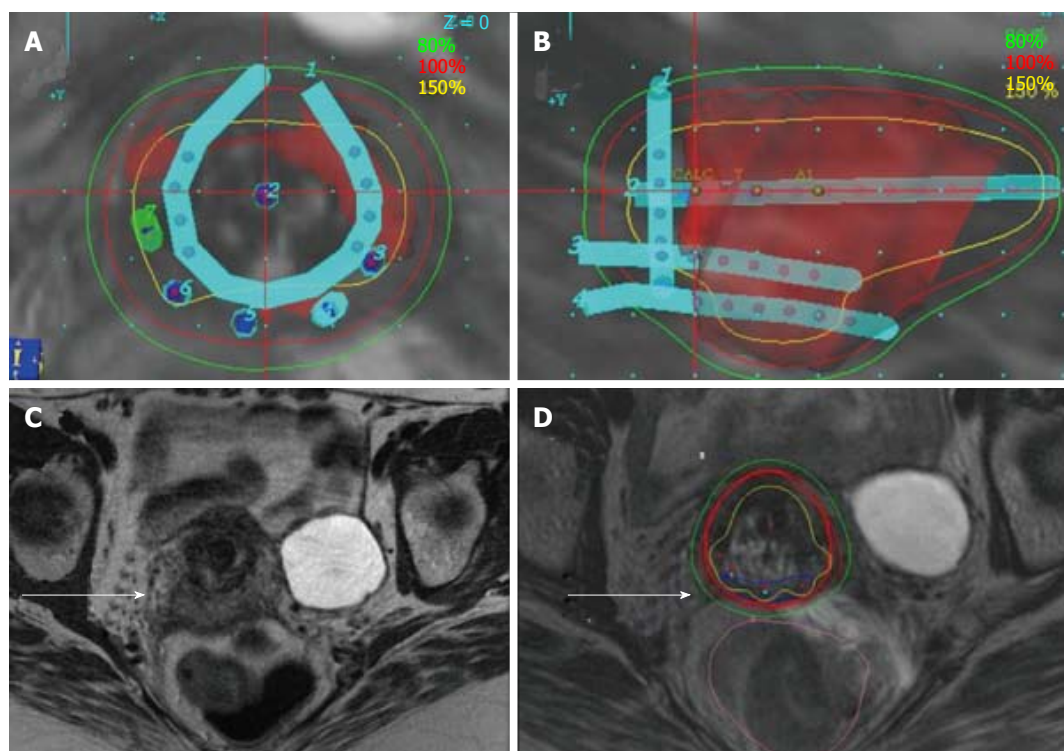


Figure 4 Use of the Vienna applicator to improve posterior disease coverage for locally-advanced cervical cancer. A and B: 3D reconstruction of Vienna applicator showing posterior needles; C and D axial magnetic resonance imaging (MRI) showing advanced disease with posterior extension, and improved coverage with combined interstitial and intracavitary MRI image-based planning.

planning forced CT image-based planning with each fraction, for 86% of the included patients the D90 was ≥ 74 Gy, with the only patient with local recurrence having a D90 of 63.8 Gy^[41]. When comparing their experience at Addenbrook with CT image-based planning to a previous institutional cohort of patients treated with chemoradiotherapy and 2D planning showed a significant 20% improvement ($P = 0.04$) in local control^[41].

MODALITIES FOR IMAGE-BASED BRACHYTHERAPY-CT AND MRI HYBRID

More recently, The University of Pittsburgh has shown a hybrid approach using MRI for first fraction and CT for subsequent fractions which allows for initial dose optimization based on the gold-standard MRI based imaging, with serial CT imaging to account for variations in applicator geometry and changes in OARs and target volumes throughout subsequent fractions^[42]. In the first report on a hybrid CT/MRI image-based approach in 42 patients we reported a mean D90 of 83.3 Gy with a mean D2cc for bladder, rectum, and sigmoid of 79.7, 57.5 and 66.8 Gy respectively. The complete response rate by PET/CT was 92.5%, with a 2-year local control rate of 88%^[43]. Dosimetric study from the Vienna group incorporating automated applicator based image registration compared the gold standard of MRI-based planning with each fraction to the hybrid MRI/CT approach, there was small systemic underestimation with the hybrid approach with

the mean difference in HR-CTV volume of $-1.7 \pm 6.6\text{cc}$ in HR-CTV, mean difference in D90 of -1.5 ± 4.3 Gy, and mean difference in D2cc for rectum, sigmoid, and bladder of 0 ± 4.9 Gy, 1.3 ± 1.2 Gy, and 1.1 ± 4.2 Gy, respectively^[44]. However all the outliers where the difference in D90 was greater than 1 Gy were large tumors requiring more complex applications (including Vienna applicator), thus the authors conclude that the hybrid approach is quite similar for small tumor and intracavitary applicators; however maybe suboptimal for larger tumors and more complex applicators^[44].

MODALITIES FOR IMAGE-BASED BRACHYTHERAPY-ULTRASOUND

Alternatively, investigators from Australia and Indian have incorporated trans-abdominal ultrasound for image-based planning, which also represents a more widely available and cost effective imaging modality that does not interfere with conventional stainless steel applicators^[45,46]. In a prospective planning study, investigators from Mumbai showed a reasonable correlation in trans-abdominal ultrasound and MRI-based planning^[45]. For outcome data, in the Australian experience planning was based on trans-abdominal ultrasound with each fraction, MRI was only available for one insertion and was used to assess response and later to validate the ultrasound volume. For ultrasound based planning, the mean D90 was 80.8 Gy with D2cc for bladder and rectum of 57.7 Gy

Table 4 Summary of clinical outcomes in published results for image-based brachytherapy for cervical cancer

	Local control	Disease free survival	Overall survival	Late toxicity (G3+)
STIC ^[47] (2-yr)	78.5%-100%	60.3%-89.7%	74%-96%	2.6%-8.9%
Vienna ^[39] (3-yr)	95%	74%	68%	7.7% crude
Pittsburgh ^[48] (2-yr)	90%	NR	82%	2%
Paris ^[49] (4-yr)	91%	86%	94%	0%
Addenbrooke ^[41] (3-yr)	96%	81%	82%	11% crude (14% actuarial)
Australia ^[50] (5-yr)	87%-88%	67%	60%	0.6%-4.6%
Korea ^[51] (3-yr)	97%	80%	NR	2%

NR: Not-reported; G3+: Grade ≥ 3 toxicity.

and 58.8 Gy, respectively^[46]. There was no significant difference in dosimetry between ultrasound and MRI planning, with a 90% local control rate^[46].

CLINICAL OUTCOMES FOR IMAGE-BASED BRACHYTHERAPY

While the integration of cisplatin chemotherapy to radical radiation therapy has improved outcomes, outcomes remain suboptimal especially for more advanced disease and novel chemotherapeutic agent development has been slow; as such improvements in radiation therapy, such as image-based brachytherapy which through improved target delineation and coverage, promises to be the next major step in improving cervical cancer outcomes. Recently international data for outcomes using image-based brachytherapy have been published (Table 4) substantiating the potential for improved outcomes suggested in prior planning studies^[39,41,47-51]. The international series outlined in Table 4 use a variety of treatment schedules, but highlight the potential advantages of image-based brachytherapy, high rates of local control 79%-100% with low rates of late complications 0%-14%. Building on these single institutional experiences, a prospective non-randomized multi-institutional series from the French Soutien aux Techniques Innovantes et Coûteuses with > 800 cervical cancer patients compared 2D PDR brachytherapy *vs* 3D (CT or MRI) PDR brachytherapy^[47]. Patients were divided into three groups based on the integration of radio-chemotherapy with surgery (pre-operative, post-operative, or no surgery). At a median 2-year follow-up, 3D planning significantly improved local (78.5%-100% *vs* 73.9%-91.9%, $P = 0.003$) and loco-regional (69.6%-96.1% *vs* 61.2%-87.9%, $P = 0.001$) relapse free survival which transcended treatment groups; there were trends towards improved disease-free (60.3%-89.7% *vs* 55.2%-86.5%, $P = 0.086$) and overall survival (74%-96% *vs* 65%-95%, $P = 0.27$) especially for the more advanced patients treated with radical radio-chemotherapy alone^[47]. Additionally 3D brachytherapy translated into statistically significant decreases in grade 3+ urinary (1.2%-5.5% *vs* 5.8%-9.2%, $P = 0.02$), gynecologic (1.4%-7.5% *vs* 5.7%-15.4%, $P = 0.01$), and global toxicity (2.6%-8.9% *vs* 12.5%-22.7%, $P = 0.002$)^[47]. The largest series using MRI based planning based on the GEC

ESTRO guidelines from Vienna group similarly showed excellent 3-year local control of 95% which represents a 65% relative improvement in local control from prior Vienna cervical cancer series using 2D planning, this local control advantage translated into 20%-30% improvement in disease-specific and overall survival primarily for more advanced tumors > 5 cm, respectively^[39]. One explanation for these improvements is dose-escalation with an increased mean dose to 90% of the volume (D90) from 90 Gy with conventional planning to 94 Gy with MRI-based planning, this is supported in a strong dose-response relationship which advocates that a D90 equivalent-dose-2 Gy (EQD₂) of at least 87 Gy is required for local control > 90% for advanced disease^[30,39,52].

RECOMMENDATION FOR PRACTICAL APPLICATION OF IMAGE-BASED BRACHYTHERAPY

For the practical application of image-based brachytherapy the guidelines published by the GEC-ESTRO and ABS working groups serve as invaluable tools for image acquisition, target/OAR delineation, and dosimetry/optimization^[31-33,53-55]. Briefly to summarize our institutional integration of these guidelines, for MRI-based planning it is recommended to use T2-weighted images, with either high signal intensity (if brachytherapy alone) or intermediate signal intensity (if brachytherapy following external-beam radiotherapy). Tumor target volumes consist of two clinical target volumes (CTV): the high-risk CTV (HR-CTV) which is optimized to receive a dose enough to sterilize macroscopic tumor representing the entire cervix plus presumed tumor extension (based on clinical assessment and/or residual grey zones on MRI) without safety margin and the intermediate-risk CTV which is optimized to received a dose enough to sterilize microscopic tumor (representing the HR-CTV plus a safety margin of 5-15 mm according to potential tumor spread-up to 5 mm anterior-posterior limited by bladder or rectum, 10 mm cranially in uterine corpus but only 5 mm if endocervical tumor, and 5-10 mm laterally into parametria). The rectum, bladder, sigmoid colon, and relevant parts of the small bowel loops adjacent to the target volumes are considered as the main OARs that are contoured with

each fraction. We typically initiate cervical brachytherapy during the 4th or 5th week of external beam radiotherapy with MRI-compatible Smit Sleeve placement. We primarily employ a ring and tandem intracavitary HDR technique (though also incorporate a Vienna applicator or template-based interstitial application where appropriate) with 5-6 Gy per fraction times 5 fractions (25-30 Gy) based on response to external beam radiotherapy using weekly fractionation during external beam radiotherapy and twice weekly after completion of external beam radiation therapy.

As outlined in the GEC-ESTRO and ABS guidelines we advocate MRI for each application; however if logistics preclude MRI-based planning with each fraction, alternative methods would be for MRI with the first fraction and serial CT-based planning for subsequent fractions^[43]. Alternatively if MRI-based planning is not available, outcome data for CT-based or US-based planning shows improved outcomes over 2D planning^[41,46,51]. An additional consideration would be to incorporate a diagnostic pre-brachytherapy MRI with CT-based or US-based brachytherapy planning to aid in soft-tissue delineation in brachytherapy planning^[56].

For optimization, we aim for a HRCTV D90 \geq 100% with a planned EQD₂ 80-85 Gy, except for patients with a poor response to external beam radiotherapy with large residual tumors where based on the Vienna dose-response data we push the dose to EQD₂ 85-90 Gy to attempt to improve local control^[52]. While based on the outcome data outlined in Table 2 and 3, we limit the rectum D2cc EQD₂ \leq 70 Gy, sigmoid D2cc EQD₂ \leq 70 Gy, and bladder D2cc EQD₂ \leq 90 Gy.

UNCERTAINTIES AND CHALLENGES IN IMAGE-BASED BRACHYTHERAPY

Despite the observed dosimetric and clinical benefits of image-based brachytherapy many uncertainties and challenges remain. Applicator reconstruction is a challenge to quality assurance in image-based brachytherapy, where a lack of a MRI compatible dummy catheter forces a reconstruction of source channels. A number of reconstruction methods have been purposed, but uncertainties in the reconstruction of source channels can generate both random and systematic errors in dose-volume-histogram (DVH) parameters^[57-59]. In an era of increasing emphasis on curtailing health-care costs, it is unclear how much the increase in demands on resources and total cost will limit applicability of image-based brachytherapy. Dosimetric uncertainties are challenged by reproducibility of mobile four-dimensional targets, OARs which can have dramatic inter-fraction differences based on distention/filling, and mobile applicators subject to intra- and inter-fraction motion^[57]. Finally the steep dose gradients of brachytherapy dose distributions place increased hopes on accurate contouring which is challenged by “grey zone” interpretation on MR imaging and evaluation of tumor response changes^[34].

FUTURE DIRECTIONS OF IMAGE-BASED BRACHYTHERAPY

To prospectively validate the adoption of image-based brachytherapy, a multi-institutional international trial, EMBRACE (an international study of MRI-guided brachytherapy in locally advanced cervical cancer) is currently accruing (<http://www.embracestudy.uk>). EMBRACE includes patients with FIGO stage IB-IVA cervical cancers, all patients receive concurrent cisplatin (40 mg/m² weekly) chemotherapy and conventionally fractionated external beam radiation therapy followed by MRI image-based brachytherapy according to the GEC-ESTRO guidelines with brachytherapy dose and DVH constraints at the discretion of the enrolling department standards. The study aims to enroll 600 patients over 3-year and promises to establish a benchmark for cervical cancer management in terms of tumor control, complications, dose specification, and a prospective assessment of quality-of-life. This trial along with continued improvements in imaging, contouring, dosimetry, quality assurance, physics, and brachytherapy delivery promise to perpetuate the advancement of image-based brachytherapy to optimize outcomes for locally-advanced cervical cancer patients.

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WJCO 5th Anniversary Special Issues (3): Cervical cancer**Clinical application of DNA ploidy to cervical cancer screening: A review**

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Abstract

Screening for cervical cancer with DNA ploidy assessment by automated quantitative image cytometry has spread throughout China over the past decade and now an estimated 1 million tests per year are done there. Compared to conventional liquid based cytology, DNA ploidy has competitive accuracy with much higher throughput per technician. DNA ploidy has the enormous advantage that it is an objective technology that can be taught in typically 2 or 3 wk, unlike qualitative cytology, and so it can enable screening in places that lack sufficient qualified cytotechnologists and cytopathologists for conventional cytology. Most papers on experience with application of the technology to cervical cancer screening over the past decade were published in the Chinese language. This review aims to provide a consistent framework for analysis of screening data and to summarize some of the work published from 2005 to the end of 2013. Of particular interest are a few studies comparing DNA ploidy with testing for high risk human papilloma virus (hrHPV) which suggest that DNA ploidy is at least equivalent, easier and less expensive than hrHPV testing. There may also be patient management benefits to combining hrHPV testing with DNA ploidy. Some knowledge gaps are identified and some suggestions are made for future research directions.

Key words: Cervical cancer screening; DNA ploidy; Automated quantitative image cytometry; High risk HPV testing

Core tip: Although application of automated quantitative image cytometry to screen for cervical cancer was first developed in Canada, the United States and Europe, it is most widely used clinically in China where it is applied to about one million tests annually. Over sixty papers reporting the clinical results have been published in Chinese since 2005. As the first review of this topic in any language, in addition to the usual goals of a review, it has the opportunity to increase the awareness of the Chinese clinical experience for those outside of China and to increase awareness of the technology background for English readers in China.

Garner D. Clinical application of DNA ploidy to cervical cancer screening: A review. *World J Clin Oncol* 2014; 5(5): 931-965 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i5/931.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i5.931>

INTRODUCTION

In a 2005 paper based on a study conducted in China^[1], this reviewer and coworkers stated the study objective as: "To establish if measurements of DNA ploidy could be used to assist cytopathologists and cytotechnologists in population based cervical cancer screening programs in countries where manually reading the slides is impossible due to the lack of sufficient skilled cytotechnologists." Based on the study results, we concluded that it could work. Since that time, DNA ploidy technology has been approved for cervical cancer screening, endorsed by several medical societies and fairly widely disseminated throughout China. The goal of this paper is to review the current status of automated quantitative image cytometry

(AQIC) to measure DNA ploidy as applied to cervical cancer screening.

The structure of this review is: (1) A very general introduction to the technology, with some historical perspective and with discussion of some practical issues; (2) A detailed development of a framework for evaluation of screening tests, mostly as a suggestion for how published data analysis might be made more clinically relevant. This section also attempts to alert readers to various well known and less well known pitfalls these evaluations are prone to and to estimate them to discriminate those that are important from those that are not; (3) A summary of existing published results, mostly from China, including reworking some of the published data in terms of the proposed framework; and (4) A discussion of some research still to be done especially in light of the huge advances in cervical cancer control made in the past decade due to the developments of human papilloma virus (HPV) vaccines and HPV testing. Does the objective from the 2005 paper still apply? Does DNA ploidy still have a potential cervical cancer screening role to substitute for nonexistent cytologists in this world of HPV vaccines and HPV testing?

TECHNOLOGY BACKGROUND

DNA ploidy

“Ploidy” is the genetics term for the number of basic sets of chromosomes in the nucleus of a cell. Cells that have an integer multiple of the basic set of chromosomes are “euploid”. Most human cells are euploid and have 46 chromosomes or two times the basic set of 23 chromosomes (one set from the mother and one from the father), referred to as “diploid”. Some human heart, liver and other cells are euploid with 92 chromosomes or 4 times the basic set and are known as “tetraploid”^[2]. Human gametes have one set of the 23 chromosomes, unpaired, and so are also “euploid”. Curiously, mature red blood cells in mammals have no chromosomes.

Cells which do not have an integer multiple of the basic set of chromosomes are “aneuploid”, simply meaning “not euploid”. Most human embryos that have an extra or missing single chromosome do not survive gestation, but some do^[3]; for example, Down’s syndrome occurs when there are three instances of chromosome 21 in all cells.

Some degree of aneuploidy is observed in virtually all solid tissue cancers^[4] in a “mosaic”; that is, the normal cells remain “euploid” but the cancer tumor cells are “aneuploid”. (This contrasts with “non-mosaic” aneuploidy like Down’s syndrome in which almost all cells are aneuploid.) Elucidation of the role of aneuploidy in cancer has a fascinating history, briefly sketched next, and remains an active area of study today. The key point is that aneuploidy is the hallmark of cancer cells in general and, in the case of cervical cancer, is present both in the early “pre-cancer” or “pre-invasive cancer” phases as well as in the later “invasive” phases. Generally, to detect aneuploid cells is to detect cancer cells.

Aneuploidy and cancer: A brief history

By the year 1890, chromosomes had been discovered and, although their function was not yet proven, it was known that the material responsible for heredity was contained in the cell nucleus where chromosomes are found. The German pathologist, Hanseemann^[5], published a paper in 1890 entitled “About asymmetric cell division in epithelial cancers and its biological significance” and another in 1891^[6] entitled “About pathological mitoses”; both papers are available online and contain wonderful hand drawn illustrations of asymmetric mitoses, as well as other phenomena, such as what is now known as apoptosis. Cells in the process of division are more commonly seen in tumors than in normal tissue and the division in normal tissue is almost always symmetric, producing identical daughter cells, whereas in cancer, one daughter cell often has more chromosomes than the other. These observations had been noted earlier by others, but Hanseemann suggested that the defining characteristic of cancer cells is that they lose their ability to divide symmetrically and, in the process, cease to have tissue specialization and increase their ability to live more autonomously, as cancer cells do in metastasis^[7].

Although both cameras and light bulbs were invented before 1890, they were not commonly available for microscopy and the illustrations in the journals were usually hand drawn by the author, sometimes assisted by “Abbe’s drawing apparatus”, rather than printed photomicrographs. Electric light commercialization slowly started in the 1880s, but incandescent light bulbs were not very practical until the invention of the tungsten filament in 1904. Also, it was not until 1893 that Köhler^[8] published the method that bears his name and is still used on most modern transmission microscopes to evenly illuminate a microscope slide without also seeing an image of the light source superimposed on the specimen image. Yet, with minimal technology and very limited knowledge of chromosomes, genetics and cancer, Hanseemann was able to make accurate observations and to formulate valuable hypotheses on the cellular mechanism of cancer.

A German zoologist named Boveri^[9] studied multicentric cell division in double fertilized sea urchin eggs and, when he learned of the work of Hanseemann on asymmetric cell division in cancer, he proposed a chromosomal theory of cancer in 1902 (an English translation is available online^[10]). In essence, the theory is that cancer cells come from normal cells that divide asymmetrically for various reasons and, although this is usually fatal for the daughter cells, sometimes a daughter cell will survive and become the progenitor for all the subsequent cancer cells. The Boveri theory of cancer is that aneuploidy, resulting from an error in the division of a normal cell, is the cause of cancer.

Boveri was jointly credited with the American, Walter Sutton (working independently), in 1903 with the discovery that chromosomes are the vectors for heredity.

Boveri^[11] published a more complete theory of cancer in 1914 (an English translation is available online^[12]) and

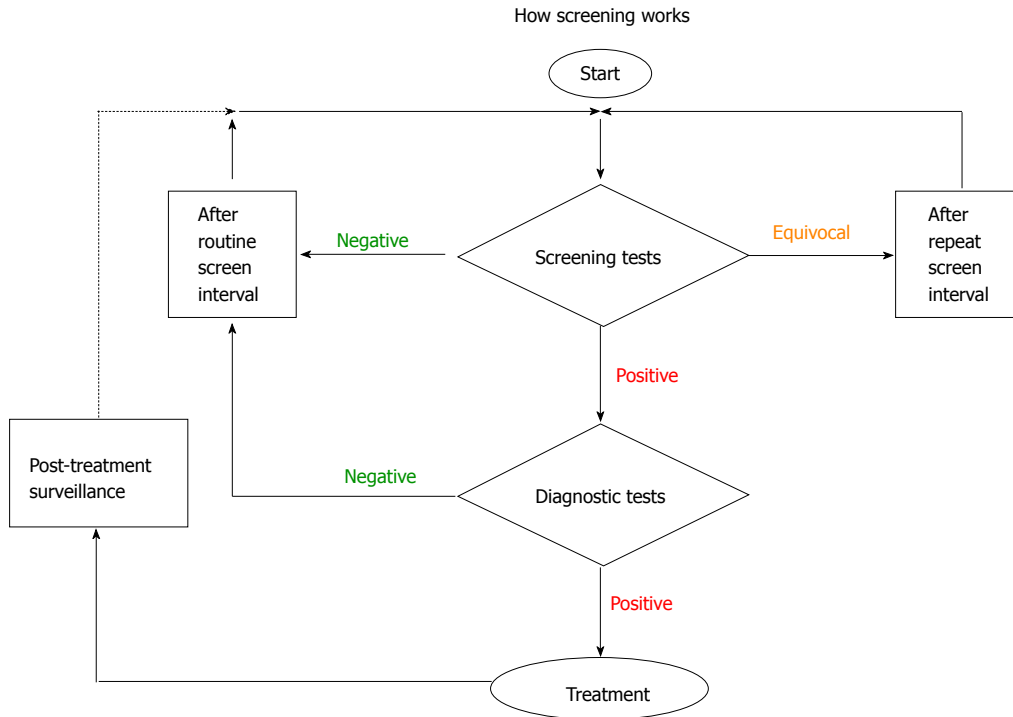


Figure 1 A simplified flow diagram of the relationship between screening and diagnosis showing that screening generally has not 2 but 3 outcomes: positive, negative and equivocal, which is managed by time and re-testing.

he died a few months later. Partly due to World War 1, this work was largely ignored. A translation into English was published by his wife in 1929^[13] but received only limited attention outside of Germany until the 1970s. This paper included 20 important insightful hypotheses about cancer, anticipating such concepts as the existence of oncogenes, tumor suppressor genes and cancer induced by infectious agents. Arguably, all 20 hypotheses were subsequently shown to be substantially correct, starting some 50 years after his death^[14]! The zoologist Boveri never studied or experimented with tumors or tumor cells and it has been speculated that it was because he was an “outsider” that he was able to form intuitive, imaginative and correct insights, uninhibited by the medical orthodoxy of the day^[14].

Boveri’s hypothesis that aneuploidy is the cause of cancer remains unresolved today, but is a topic of active study^[3,15-17]. Recent work shows that aneuploidy can be both a promoter and an inhibitor of cancer, depending upon the degree of chromosomal instability^[18].

Again, the key point for this review is that generally to detect aneuploid cells is to detect cancer cells.

It should be noted that “definitive diagnosis”, including tumor type, is usually determined by a diagnostic test, not by the screening test (Figure 1). In the case of cervical cancers, colposcopy directed biopsy is the usual diagnostic test. Screening by detecting DNA aneuploidy alone cannot determine what type of tumor is present, although advanced cytometry techniques, beyond the scope of those discussed here, could make such determination.

DNA Cytometry

“Cytometry” means “cell measurement” which comes in two basic technology flavors: “flow” and “image” (sometimes called “static”) cytometry. There are a broad range of techniques to measure chromosomes, both in flow and image cytometry, ranging from the simple measurement of the total DNA content of a cell nucleus to the complex and sophisticated enumeration of small segments of individual chromosomes.

This paper is limited to consideration of AQIC that measures the total DNA content of cell nuclei, along with features that describe the distribution of the DNA within the nucleus and the morphology of each nucleus. These cytometer systems compare the DNA content of each cell nucleus measured to the average DNA content of the measured normal cell population^[19]. Cervical “Pap” samples are predominantly comprised of normal cells, even when taken from a woman with invasive cervical cancer. This comparison identifies aneuploid cells even though the technique does not actually identify or count individual chromosomes. A cell with a DNA content 2.5 times that of a normal cell is reliably determined to be aneuploid, even though it is not possible to say that cell has, *e.g.*, 115 chromosomes. This technique is also known as “quantitative DNA Cytometry” or just “DNA Cytometry”.

When AQIC was first developed, it was common to apply the genetic language of “ploidy”. Unfortunately, this can be misleading, especially in today’s world of genomics, because one will be inclined to infer that individual chromosomes are identified and counted. The

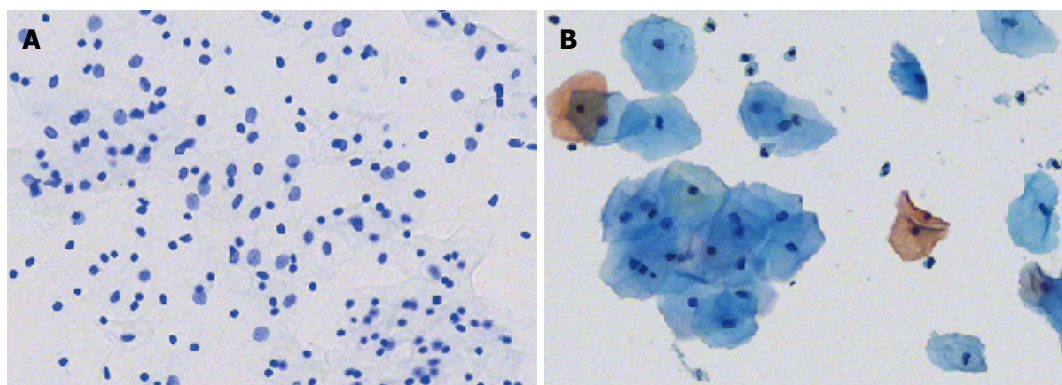


Figure 2 Liquid based cervical samples stained with A: Feulgen thionin and B: Pap stain.

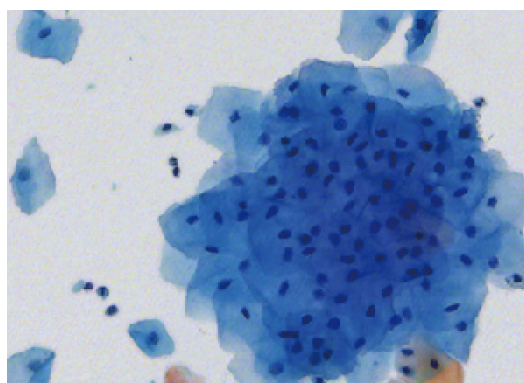


Figure 3 Liquid based cytology cervical sample example of a cell cluster obscured by cytoplasm.

term “DNA ploidy” was coined to be distinct from simply “ploidy” or “chromosomal ploidy” in an attempt to correct this sloppy language, with only middling success because today’s young life science students will certainly have encountered the terms of genetics and genomics, but are much less likely to have encountered DNA Cytometry. So it is important to understand that the AQIC technology discussed here does detect aneuploidy by cell measurement, but without explicitly enumerating chromosomes as a geneticist might expect^[20].

How DNA is measured

Automated quantitative image cytometers are comprised of a microscope fitted with a digital camera, motorized X-Y stage, robotic slide loader and automatic focus mechanism, all under computer control. The cytometers of this review operate on absorbance microscopy.

Most cytometry relies on staining particular molecules in the specimen and then quantifying the stain. For the automated quantitative image cytometry considered here, the Feulgen^[21] reaction stains the nuclear DNA specifically and proportionately to the amount of DNA present, everything else remains clear and unstained^[22]. For cervical samples, this greatly simplifies the scene on the slide because the cell cytoplasm is left unstained, as is any red blood (no DNA). Only the DNA contained in the nuclei of epithelial cells, white blood cells and, if pres-

ent, sperm cell nuclei are stained; the possibility of “false aneuploidy” arising from epithelial cells being overlaid by other epithelial cells, white blood cells or sperm cells is discussed later. Several microorganisms are commonly found in cervix samples, especially Döderlein’s bacillus which can completely cover the cervix epithelial cells; the staining protocol does not stain any of these microorganisms either.

Figures 2 are liquid based cytology (LBC) cervical samples with the blue DNA stain thionin (Figure 2A) and with the Pap stain used by conventional cytology (Figure 2B). The images were taken with the same optics (magnification, *etc.*) on the same cytometer. Notice that there are many more cells in the 2A image than in the 2B image and, even so, the scene is much simpler. Notice also that not all cells are in focus at the same time in either image due to the 3-dimensional stacking of the cells. Figure 3 shows a cluster of cells that is very difficult to examine due to the overlapping stained cytoplasm; this would not be a problem with only the DNA stain. Figure 4 shows “low grade abnormality” in the 3 Pap stained photos (arrows), which is especially difficult to see in Figure 4D. The large blue nucleus (Figure 4A) is aneuploid with a DNA content about 2.7 times normal. The key message is that the DNA stained slides are much simpler to measure and interpret than the conventional Pap stained slides.

Measurement of the amount of DNA with a cytometer is identical to the measurement of a chemical with a spectrometer. The basic idea of a spectrometer is shown in Figure 5—light is selected by a slit into a beam which directs it onto a monochromator. A particular color is selected by another slit, the beam is passed through the chemical sample which absorbs some of the light and any remaining light is detected with a photosensor. The concentration of the chemical in the sample can be precisely measured by applying a rule of physics known as Beer’s Law.

A quantitative cytometer is a very simplified spectrometer (Figure 6). The optimum light color is selected with color filter and passes through the glass microscope slide. Some of any light that passes through stained DNA is absorbed and the remaining light is detected by the pix-

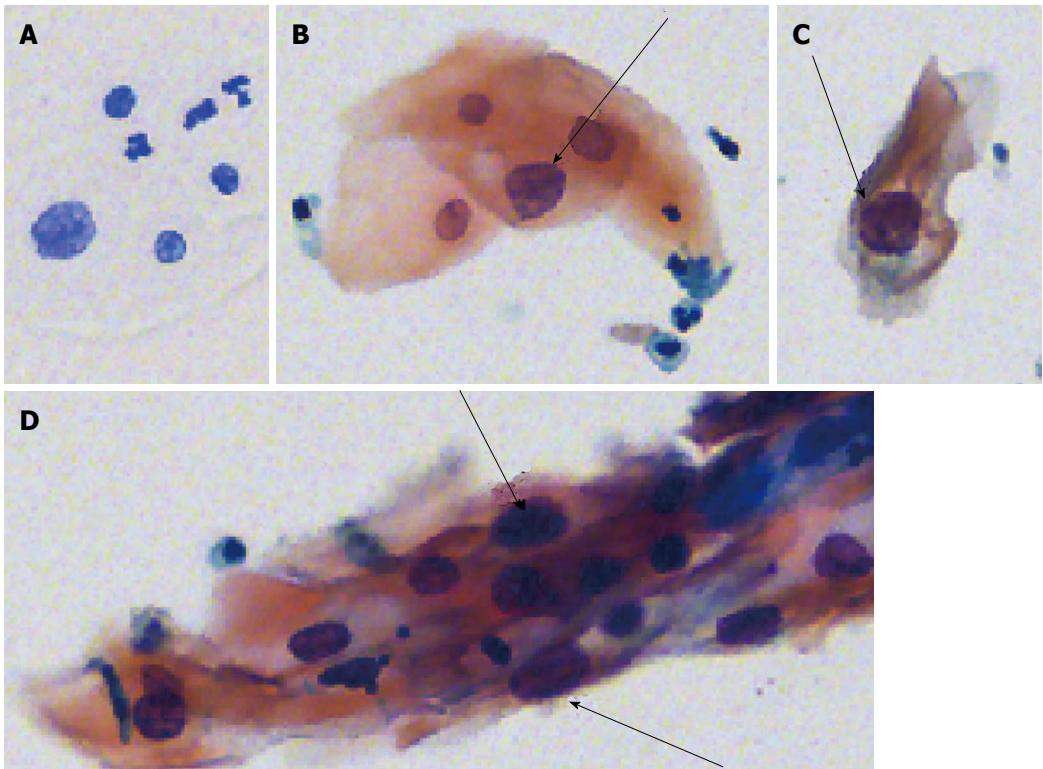


Figure 4 Abnormal cell nuclei. A: Feulgen thionin stained; B, C, D: Pap stained (arrows).

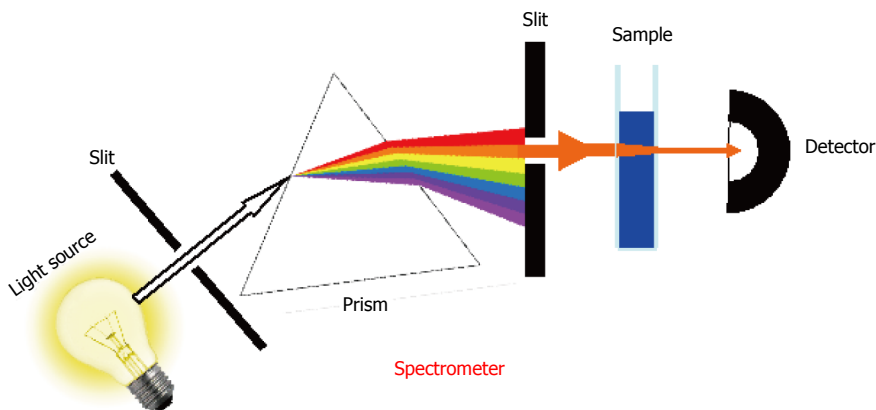


Figure 5 Basic principle of a spectrometer.

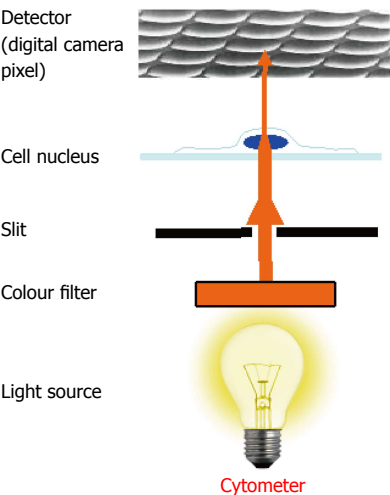


Figure 6 Basic principle of an image cytometer.

els of a digital camera. Beer’s law applies separately to every pixel of the camera-in effect, a cytometer is a “Mega-Micro-Spectrometer”-that is, millions of tiny, pixel-sized spectrometers. The total DNA present in a nucleus is obtained by adding up the DNA measured by each pixel of the image of that nucleus. A recent extensive review discusses all of the technical considerations for quantitative cytometry^[23].

AUTOMATED QUANTITATIVE IMAGE CYTOMETRY: OVERVIEW

Scanner operation

This review is focused on AQICs that feature “walk away automation” where barcoded stained slides are placed in a slide loader and the operator initiates scanning on a supervisor computer and the cytometer operates without

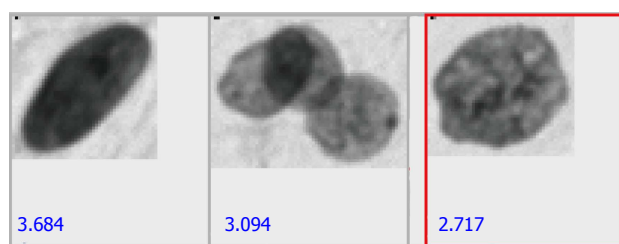


Figure 7 Gallery of potentially aneuploid cell nuclei.

further intervention. Although such machines can scan smears, they are generally employed with LBC samples; indeed, LBC was invented by Cytec Corp. (now Hologic) with the intended goal to simplify the task of automated imaging^[24]. All of the operations of focusing, image capture, segmentation of the scene into foreground and background, feature measurement *etc.* are performed without operator intervention.

Modern AQICs scan a LBC cervical slide in typically 5-10 min and are constantly getting faster, especially due to improvements in computer and digital camera speeds. While the scan rate is typically between 5 and 10 samples per hour, the throughput is generally defined by the slide loader capacity for overnight scanning and so, depending on the particular vendor's design, can range from 75 to 250 per cytometer per day or 15000 to more than 50000 slides per cytometer per year, based on 225 working days per year. If a large volume of samples is available, as is the case for tests in an organized screening program, automated quantitative image cytometry can be very efficient.

Scan review and report generation

Reporting with an automated quantitative image cytometer is done at the conclusion of an interactive review of the scan data for each slide which is comprised of stored images of the cell nuclei, counts of various cell types, and histograms and scatter plots of cell DNA content and other features of the cell nuclei. The reviewer follows a very simple checklist procedure to systematically examine the data, looking: (1) first to check that the DNA scale (normalization) is valid, then; (2) checking any images that could be of aneuploid cell nuclei, then; (3) looking for aneuploid "stemlines", then; (4) assessing cell proliferation; and (5) if none of these are present, then the case is negative and an assessment of scanning adequacy is made.

The idea that a scan has examined enough cells to be deemed to be a "satisfactory" or "adequate" assessment only applies to slides that are free of abnormality because slides deemed to be positive for abnormality are considered to be satisfactory regardless of how many cells were measured.

More than 90% of slides are clearly negative or clearly positive and so working through this checklist for those slides typically takes 1 min or less, including generating the report, once the technician has several days of train-

ing and experience. The remaining < 10% of cases are more problematic because their result hinges on the detailed assessment of typically 5 or fewer images of what could be aneuploid cells. The central issue is not "exactly what kind of cell is this an image of?", but the much simpler question of "is this the image of a single cell nucleus or an artifact caused by the overlap of two or more cell nuclei or white blood cells or sperm?"

Figure 7 shows a mini image gallery. The leftmost image is clearly a single nucleus and the next image is clearly of overlapping nuclei as evidenced by the distinct overlapping boundaries and dark overlap area. In fact, the scan computer has no difficulty identifying the second image as being of overlapping cells and automatically places it into a separate group, so in "real life" the reviewer would be unlikely to ever look at this second image.

The last image in the red box (previously shown in Figure 4A) is possibly more difficult because the shape suggests two overlapping ovals but the distinct boundaries and dark overlapping areas are absent. This is actually a single nucleus and the reviewer has the option of clicking on the image and the slide is automatically moved to place that cell into the center of the field of view of the review microscope and it is easily determined to be a single nucleus by panning slightly through focus.

Even when some images must be revisited with the review microscope, the overall task is simple and fast. Most revisit cases take less than 5 min each, so a typical reviewer can work through 40 cases per hour on a sustained basis (4 slides requiring revisiting plus 36 slides without revisiting). Some vendors provide review/revisit microscopes that are separate from the cytometer, while for other vendors the cytometer serves both scan and review/revisit functions.

The report that is generated summarizes the data showing a gallery of images of the most significant cell nuclei, a histogram of the DNA content, a scatter plot of area *vs* DNA content and a summary of cell counts in different DNA ranges.

In conventional cytology of the Pap test, most of the world reports according to TBS 2001 (The Bethesda System, revision of 2001^[25]) which is very rich, very nuanced, very complicated and with rather poor inter- and intra-observer agreement^[26]. From the viewpoint of patient management, there are really only 4 basic recommendations: (1) Return for another test at the routine screening interval (typically 3-5 years), the test is negative (no abnormality found); (2) Go to colposcopy within a few weeks for a detailed gynecological examination and possible biopsy, the test is positive (abnormality clearly found); (3) Return for another test at an interval shorter than the routine screening interval (typically 6 mo), the test was equivocal (too atypical to call "negative" but not enough to call "positive")-such cases are usually resolved by time; and (4) Return for another test at an interval shorter than the equivocal repeat interval (typically 1 mo), there was no valid test result due to some process failure.

The basic scheme for screening is shown in Figure 1

(recommendation #4 above is not shown). The key focus and goal of the DNA ploidy test is to provide the correct management recommendation from this set of 4 for each woman. A set of objective rules is applied to the data in each report to make the correct follow up patient management recommendation.

These rules can be adjusted to adapt to the reality of each local situation. For example, in a setting where a woman is unlikely to be tested more than once or twice in her lifetime, it may be decided to adjust the rules to make the “negative predictive value” (the chance that a woman who has a negative test result is actually free of cervical cancer or significant pre-cancer) higher than it would be where more testing is done per lifetime. Or, if there are very limited gynecology and colposcopy resources, it may be required that the “positive predictive value” (the chance that a woman who has a positive test result actually has cervical cancer or significant pre-cancer) be adjusted so as to not overwhelm the diagnostic and therapeutic resources available.

The key advantage of DNA Cytometry is that it takes typically only 2-4 wk to train a technician (good high school graduate with some work experience) to competently perform all tasks: cell deposition onto glass slides, staining, operation of the DNA scanner, scan data review and reporting. This contrasts with the 1-2 years of special training for cytotechnologist^[27,28] and 3-6 years of specialization training following receipt of an MD degree for a cytopathologist^[29-32]. This is not to suggest equivalence between a minimally trained technician and skill of well trained cytotechnologists and cytopathologists, but it is to suggest comparable cervical cancer screening test performance, as summarized later in this review. It is this key advantage that makes automated quantitative image cytometry a candidate for screening in low resource settings where the conventional cytology Pap test cannot be performed due to the lack of trained cytotechnologists or cytopathologists. In such settings, AQIC could mean the difference between screening and not screening.

AQIC DNA PLOIDY: CLINICAL CONSIDERATIONS

Scanner operation: What constitutes a scan?

Two different endpoints are used to define what constitutes an adequate scan for AQIC of liquid based cervix slides: (1) scan all cells deposited on the slide; and (2) scan a preset number of epithelial cells on the slide. Option (1) is the approach primarily motivated by litigation—if an abnormal cell is present on the slide there could be liability. Option (2) is based on science and is completely consistent with current international cervical cancer screening guidelines^[33].

What minimum number of epithelial cells must be measured for a scan to be satisfactory? A starting point to answer this question is to consider the guidelines for conventional cytology. TBS 2001^[25] stated “Minimal squamous cellularity requirements for a specimen to qualify as

“satisfactory” ... (is) 5000 squamous cells for liquid-based preparations.” It has been reported^[34] that this guideline was based on “...personal communications from the authors of two (subsequently published) papers^[35,36].” In fact, reference^[36] showed a threshold behavior of a jump in sensitivity for ASCUS+, LSIL+ and HSIL+ on Surepath (Becton Dickinson) LBC samples with < 5000 epithelial cells compared to those with > 5000 epithelial cells, supporting this guideline. However, reference^[35] concluded that “Cellularity does not provide assurance of adequacy. Any cellularity criterion should be based on measurement of the prevalence of abnormal cells on abnormal slides.” McQueen^[34] seems to be the only publication so far to take this approach with ThinPrep slides (Hologic) and yet they also conclude: “We have demonstrated that the range of ratios of dyskaryotic to total squamous cells in ThinPrep® preparations is such that it is not feasible to set a minimum acceptable total squamous cellularity so that there is an acceptable probability that all specimen vials containing dyskaryotic cells will be identified. In the light of this, a pragmatic approach should be adopted by deciding on an arbitrary minimum acceptable total squamous cellularity which ensures a rate of detection of abnormality that is at least as good as that for (smears) and which does not impose undue burdens on the users and providers of the screening program.” The 2008 American Society for Colposcopy and Cervical Pathology (ASCCP) committee review^[33] fell just short of endorsing this recommendation but noted that the TBS 2001 guidelines have been in wide use for many years and stated: “The Bethesda 2001 squamous cellularity criteria provide an acceptable threshold of unsatisfactory results for most patient populations and laboratory settings, although additional studies and data would be useful.”

McQueen^[34] estimated that for ThinPrep slides there must be at least 16 “targets” (scenes of abnormality) and comprised of at least 87 dyskaryotic cells in total in order to reduce the probability of a false negative result to 2%. This is for Pap stained samples screened visually by experienced cytotechnologists. What is the situation for DNA Cytometry?

Automated quantitative image cytometry is measurement-based and so the detection of even a single aneuploid cell is done with a high degree of confidence, as discussed more in the next section. If we assume that LBC mixes the sample so that the subsample of both physiological cell clusters and isolated cells deposited on the slide is statistically randomized and representative of what is in the vial, then it is possible to calculate from the Poisson distribution, the probability of a real false negative case, defined as having no aneuploid epithelial cell present among the epithelial cells measured. Figure 8 shows the probability of a real false negative occurring given that 5000 epithelial cells are measured. Note that the figure is a log-log plot. Figure 8 shows that when the ratio of normal to abnormal cells is less than about 1000:1, the probability of a real false negative is less than

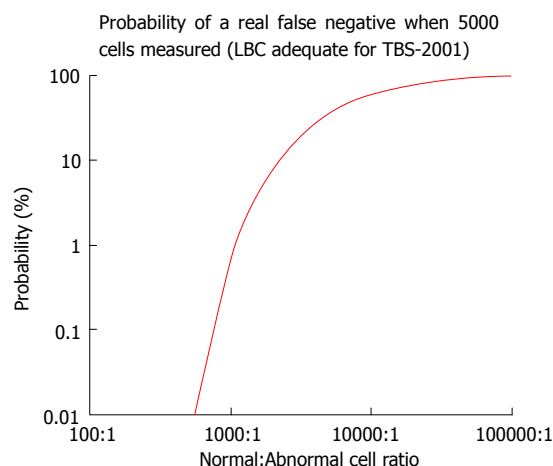


Figure 8 The probability of a real false negative occurring given that 5000 epithelial cells are measured.

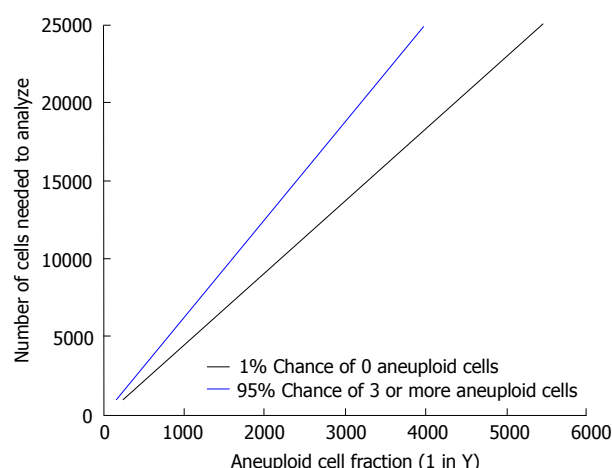


Figure 10 Given an aneuploid cell fraction, how many cells must be measured to get 1% chance of a real false negative or a 95% chance of a positive test result.

about 1% when 5000 epithelial cells are measured. McQueen *et al*^[34] counted the number of dyskaryotic cells and total squamous cells on 23 HSIL slides and found only 1 (4.3%) to have a normal:abnormal ratio of $> 1000:1$ -that case was 4600:1.

A more general way of looking at this is shown by the black line in Figure 9 (note the log-linear scale). The X-axis is the aneuploid:normal cell ratio times the number of epithelial cells measured, so it does not just apply to the case of 5000 cells as in Figure 8. The simple “rules of thumb” are: (1) if the aneuploid:normal cell ratio times the number of cells measured is about 5, then the probability of a real false negative is just under 1%; and (2) if this product is about 7, then the probability of a real false negative is approximately 0.1%, which is well into the realm of diminishing returns. That is, to get a $\leq 1\%$ chance of a real false negative case when the ratio of normal to abnormal cells is 1000:1 requires measuring 5000 epithelial cells; if the normal to abnormal ratio is 5000:1, then 25000 epithelial cells must be measured and

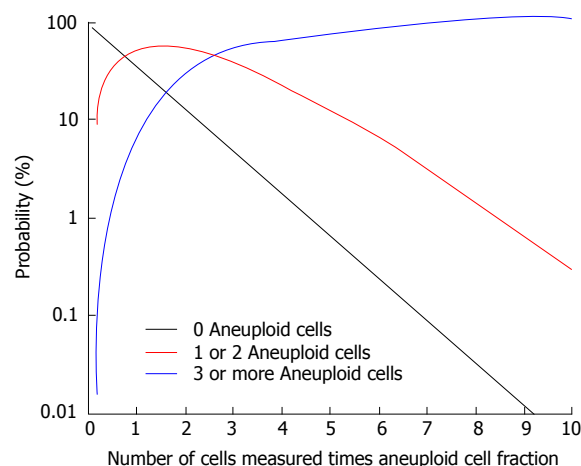


Figure 9 General relationship between number of cells measured and the probability of a real false negative. The red line is considered “equivocal”.

so on. Setting a goal of a real false negative rate of $\leq 1\%$ is probably very aggressive for screening.

A final way to look at this is Figure 10 which shows how many cells must be measured for a 1% chance of a real false negative case (black line) or for a 95% chance of the being 3 or more aneuploid cells measured (blue line). Most publications on AQIC applied to cervical cancer screening report measuring 6000 or 8000 epithelial cells and so theoretically have a $< 1\%$ probability of having a real false negative when the normal:aneuploid cell ratio is less than 1300:1 and 1700:1, respectively.

SCAN REVIEW AND REPORT GENERATION

Normalization and DNA scale

Previously, it was mentioned that Beer’s law is applied to measure DNA for each pixel of the image and that the DNA content of each cell is determined by simply adding up the DNA content of each pixel of the image of that cell nucleus. This procedure quantifies the DNA on an arbitrary scale, but it is more useful to convert this to a relative DNA scale by normalizing to the normal diploid cell population. Again, cervical “Pap” samples are predominantly comprised of normal diploid cells, even when taken from a woman with invasive cervical cancer, and so for Pap samples such an internal reference cell population always exists. The normalized scale described here is called “DNA Index” or “DI” in which diploid cells have a DI = 1, tetraploid have DI = 2, *etc*. Figure 11 is an example of such a histogram measured for a pig liver “touch” preparation. The green peak represents purely single diploid hepatocytes, but the other peaks include mixtures of single tetraploid cells and clusters of diploid and/or tetraploid cells used to check the linearity of the DNA measurement. Arguably, the DNA Index is the natural scale to use for cancer screening which is primarily concerned with deviations from diploid for somatic cells.

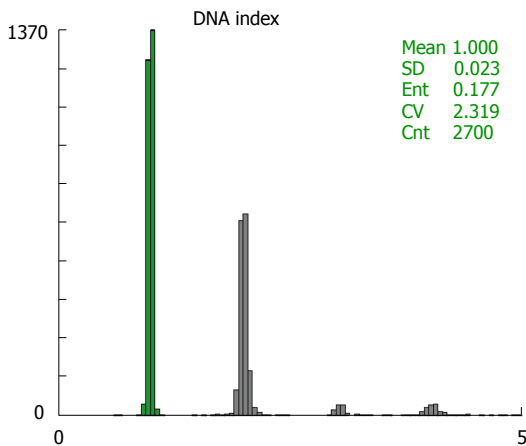


Figure 11 DNA index for pig hepatocytes for DNA linearity calibration.

However, the original DNA scale is the “C-index” scale in which $DI = 1$ is designated as “2C”, $DI = 2$ is “4C” *etc.* This scale was first used in 1950 by the American, Swift^[37], in a study employing DNA measurement by microphotometry of Feulgen stained cells of various plant species (basically the same technique as AQIC). Swift found that within the non-dividing cells of a species, the DNA content of cells tended to be in quanta that he labeled “C” for “classes”: “Non-Dividing Tissues: Photometric measurements made on tissues where mitoses were uncommon tended to fall in certain well marked classes. Means of these classes fit in the series 1:2:4:8:16:32.” On this scale, 1C is the quantum of DNA in a (plant or animal) sperm cell. The DNA amount corresponding to 1C varies from species to species. An earlier similar study of various animal species^[38] noted the ratio 1:2:4 for DNA in cells from rat liver, but did not describe this in terms of the C-index, which would have been 2C:4C:8C. Arguably, the C-index is the natural scale for biology, in general, as opposed to the narrow field of cancer.

The first step in the data review checklist is to determine that the DNA normalization is valid, meaning that the normal diploid cell population was correctly identified and that the normalization was correctly applied. Figure 12 is a typical, correctly normalized DI histogram of the cervix sample taken from a healthy woman. The scatter plot 12 shows almost all diploid epithelial cells to be in a tight distribution except for a very few hypo-diploid epithelial cells highlighted by the blue oval. This hypo-diploid skewing is a general feature of cervix samples and is comprised of dead epithelial cells in which the DNA has degenerated. The amount of skewing increases with the presence of infection, particularly, but not limited to, *trichomonas vaginalis*. The skewing is much more obvious in Figure 13 where it does not affect the normalization. The last example (Figure 14) is of a case of *trichomonas vaginalis* and shows a peak of cells with degenerated DNA that is bigger than the normal diploid peak that should be used to normalize and define the DNA scale. It is beyond the scope of this review to dis-

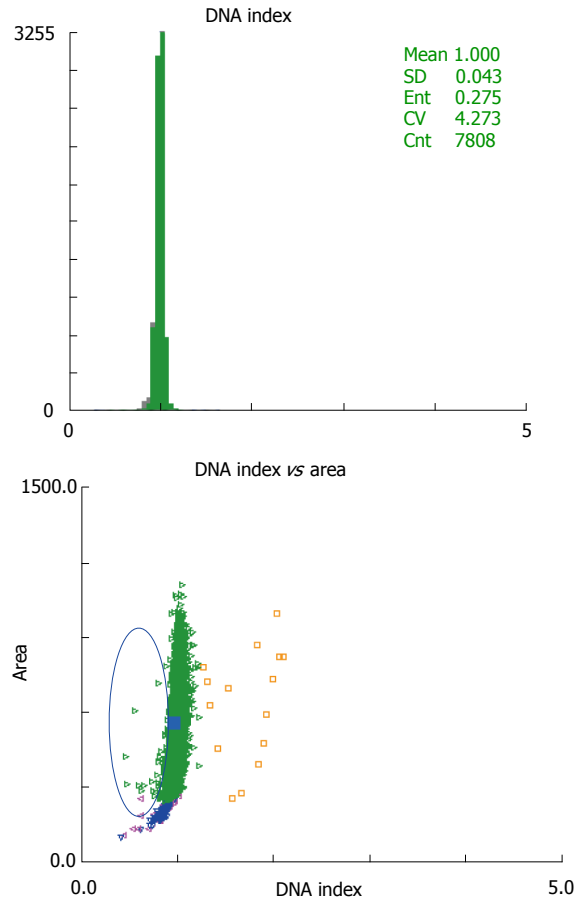


Figure 12 Typical DNA ploidy data for sample taken from a healthy cervix.

cuss automatic normalization algorithms and technician training strategies to deal with this. However, this problem is minimized if the sample taker cleans the cervix of excess discharge and secretions prior to taking the sample, as is called for by most sampling protocols^[33,39]. Some gynecologists believe this to compromise the sample, but an Randomized controlled trial (RCT) that involved taking two consecutive samples has shown the results to be independent of sampling sequence^[40].

High DI aneuploid cells and $DI > 2.5$ (or $> 5C$)

The second step in the data review checklist is to examine any images that could be of aneuploid cell nuclei, most usually defined as having $DI > 2.5$ or “5C exceeding”. Why is this the practical definition of aneuploidy?

Since AQIC does not identify and count individual chromosomes, it is usually not possible to discriminate normal cells in cell cycle (mitosis), for which the DNA content ranges from $1 \leq DI \leq 2$, from aneuploid cells with similar DI. Given that no measurements are exact, it is usual to apply a 10% uncertainty^[20] meaning that normal cell cycle ranges from $0.9 \leq DI \leq 2.2$ and hence the likelihood is that cells with $DI > 2.2$ are aneuploid. The convention of $DI > 2.5$ ($> 5C$) was probably a “padding” to the 10% DNA measurement uncertainty, for broad, conservative generalizability. However, Guillaud *et al*^[41] looked directly at the DNA ploidy sensitivity and speci-

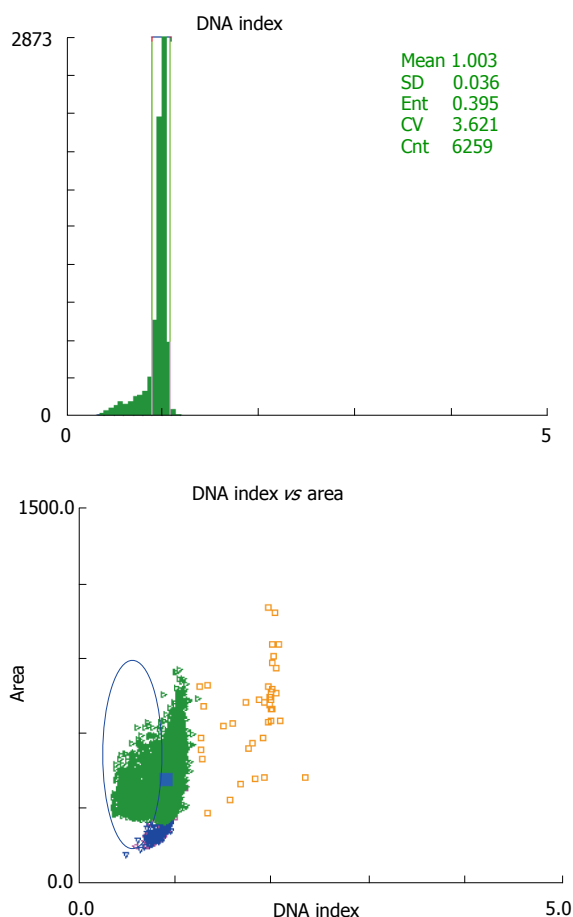


Figure 13 Typical DNA ploidy data for sample taken from a cervix without removing excess secretions or discharge.

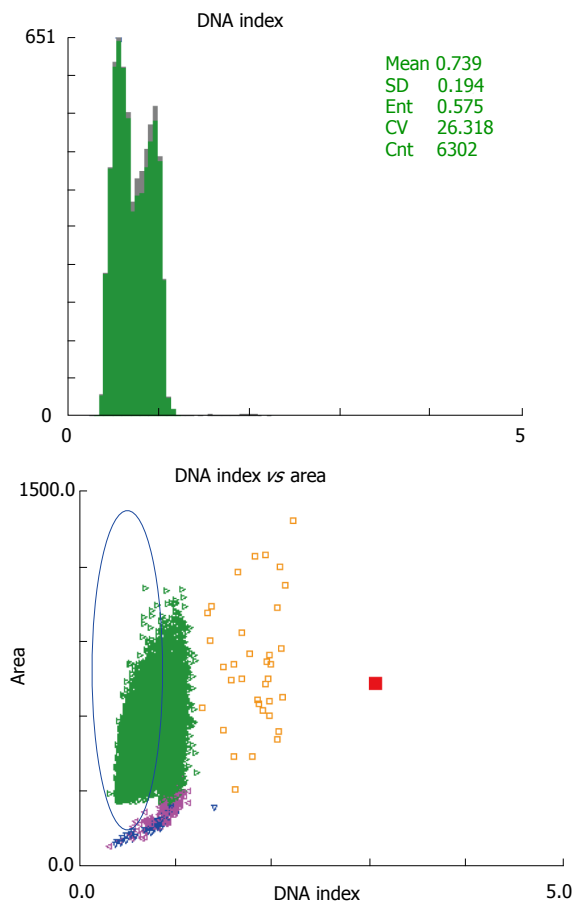


Figure 14 DNA ploidy data for sample taken from a cervix infected with *trichomonas vaginalis* without removing excess secretions or discharge.

ficiency for cervical cancer screening by AQIC as a function of DNA content of putative aneuploid cells. When 3 or more aneuploid cells were present, the threshold converged to $DI \geq 2.2$ (4.4C), but when only 2 or 1 aneuploid cells were present, the threshold rose to $DI \geq 2.3$ (4.6C) and $DI \geq 2.4$ (4.8C), respectively. These results may be specific to their cytometer and their particular laboratory operations standards; other labs may want to calibrate their performance if they choose to adjust the definition of high DI aneuploidy.

Another potential ambiguity is due to polyploidy which refers to euploid cells with $DI = 2, 4, 8, 16, \text{etc.}$ (4C, 8C, 16C, 32C...). As mentioned previously, this condition does occur normally in some tissues^[2] and, even when abnormal, does not necessarily indicate cancer. Concern for polyploidy induced by koilocytosis led Chatelain *et al.*^[42] to propose setting the threshold for aneuploidy in cervical cancer to $DI \geq 4.5$ ($\geq 9C$). However, for cervical cancer screening, such restrictively defined aneuploid cells are too rare to give the test reasonable sensitivity, even in high risk HPV (hrHPV) positive cases^[41,43-45] and no evidence of threshold behavior at $DI = 4.5$ was observed by Guillaud *et al.*^[41]. For the purposes of cervical cancer screening, the effective definition of an aneuploid cell is one with $DI \geq 2.5$ (5C). Although the 9C aneuploidy threshold is not useful for cervical cancer screening, it

may have utility in diagnosis.

Figure 15 shows the histogram, scatter plot and gallery of the 8 aneuploid cells found in this typical positive case.

Aneuploid stemlines

The third step in the data review checklist is to check aneuploid stemlines which appear as a peak in the DNA histogram, but not at $DI = 1$ or 2 (2C or 4C) where normal cycling cell peaks appear^[20]. The fact that stemline cells are in a DI peak means that they have more or less the same DNA content and so they must divide coherently and with a relatively minor amount of chromosomal instability. By definition, such stemlines are aneuploid. Stemline cells are usually in mitotic cell cycle and so a smaller G_2/M peak is usually present at twice the DI of the stemline G_1/G_0 , which is usually but not always found between $1 \leq DI \leq 2$. Figure 16 is an example of a stemline histogram with G_1/G_0 at $DI = 1.6$ and with cells consistent with G_2/M at $DI = 3.2$, highlighted in blue in the right histogram with the broken vertical scale. Figure 17 is the quite rare case where the stemline G_1/G_0 is at $DI = 2.3$ (≥ 2 is rare) and with cells consistent with G_2/M at $DI = 4.6$ also highlighted in blue in the right histogram with the broken vertical scale.

Although looking for aneuploid stemlines is a step in the review checklist, it is rare for a sample to have a stem-

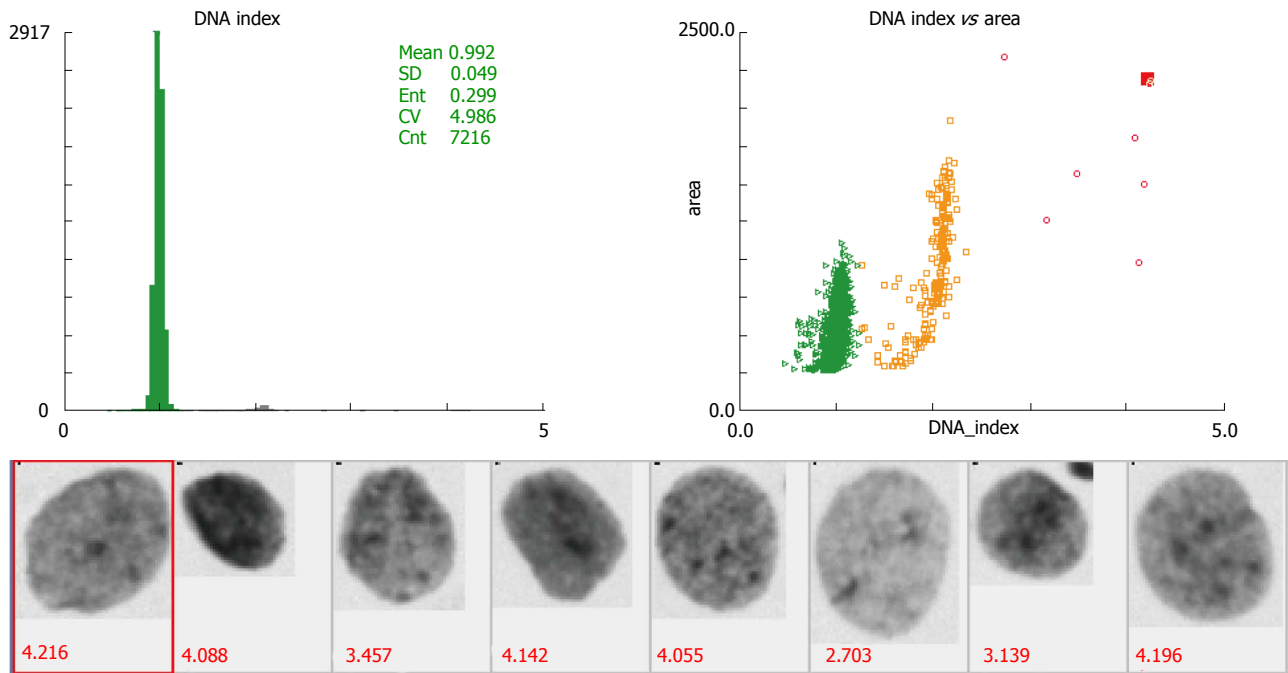


Figure 15 DNA ploidy data from a typical positive case with several aneuploid nuclei with DI > 2.5, the aneuploid nucleus gallery is shown with measured DI indicated in red.

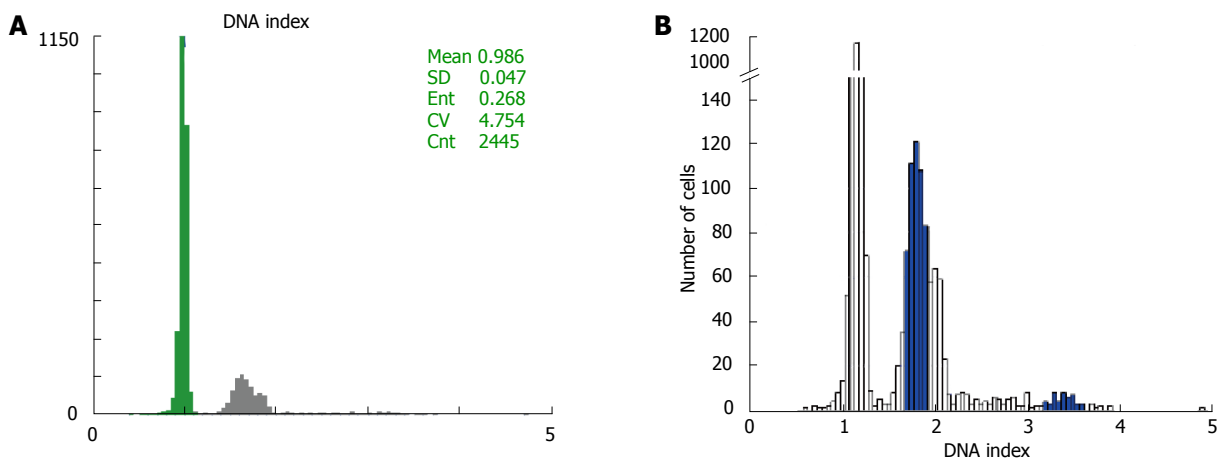


Figure 16 DNA ploidy histograms of aneuploid stemline at DI = 1.6. A: Full histogram; B: Histogram with broken vertical scale.

line without there also being aneuploid cells with $DI \geq 2.5$, so identifying stemlines is usually redundant.

The relative lack of chromosomal instability on an aneuploid stemline may mean that the cell morphology looks rather normal and a biopsy may be interpreted as displaying hyperplasia rather than neoplasia, causing the stemline to be seen as an apparent false positive. However, hyperplasia is genetically normal but stemlines identified by DNA Cytometry are genetically abnormal and so are neoplastic. Presumably, the presence of identifiable stemlines occurs early in the process of neoplastic transformation. This author is not aware of any publications on the malignant potential or aggressiveness of lesions of the uterine cervix that are primarily characterized by aneuploid stemlines.

Cell proliferation

The fourth step in the data review checklist is to determine if there is unusual cell proliferation. Epithelial cells in a properly taken sample from a healthy cervix should almost all be completely differentiated and so do not divide. As mentioned previously, in the range $0.9 \leq DI \leq 2.2$ it is not possible by simple DNA Cytometry to discriminate aneuploid cells from normal cells in mitotic cell cycle. Li *et al*^[40] added anti-Ki67 immunostain (marker of cells in mitosis) to Feulgen stained cervical samples, but found no improvement in sensitivity and specificity compared to DNA Cytometry alone.

Excessive cell proliferation could indicate, among other things: (1) repair of wounds or repair processes due to fungal, bacterial and other infections; (2) response

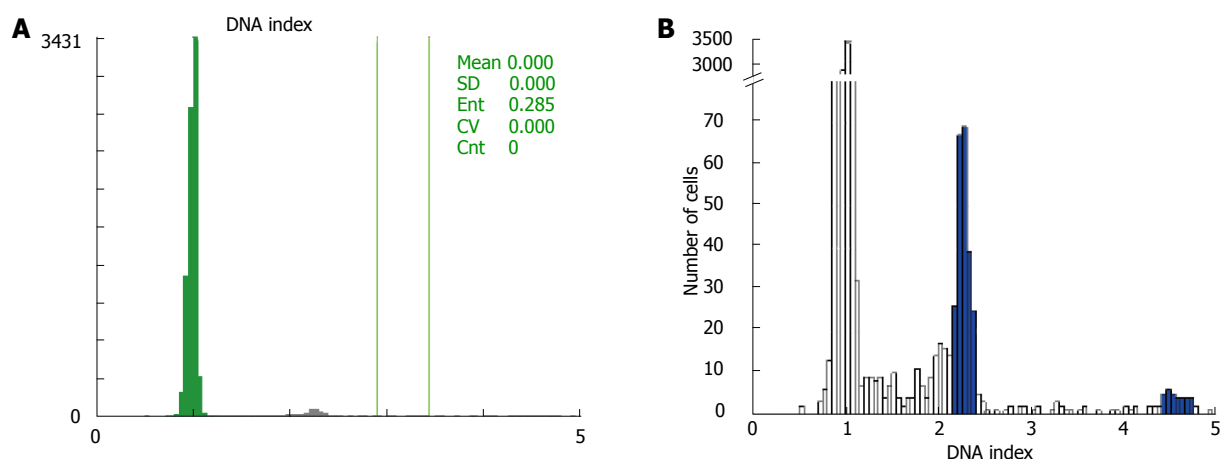


Figure 17 DNA ploidy histograms of a rare aneuploid stemline at DI = 2.3. A: Full histogram; B: Histogram with broken vertical scale.

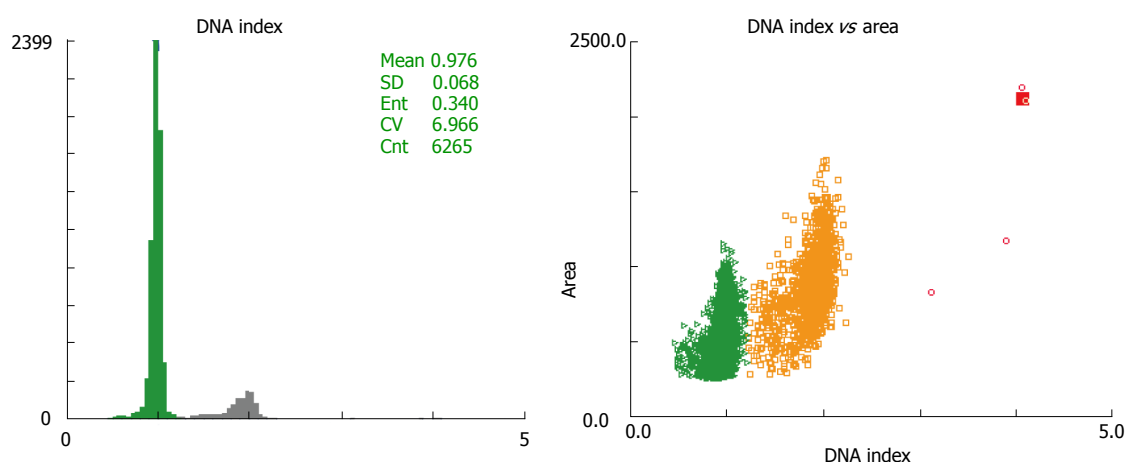


Figure 18 DNA ploidy data of a high proliferation case which also shows high DI aneuploid cells.

to hormones, drugs or radiation, especially in cancer treatment; (3) response to viral infections like HPV; (4) hyperplasia as an epigenetic response to some chemical, mechanical or other stress; and (5) neoplasia.

One expects that applying this criterion alone for cancer screening will have a relatively high false positive rate with so many non-neoplastic causes for cell proliferation. This criterion is usually applied by comparing the number of epithelial cells with $1.25 \leq DI \leq 2.5$ to the total epithelial cell count. Figure 18 is a typical example showing over 15% proliferation and, as is usually the case, there are 4 aneuploid cells also present, making the proliferation rate determination redundant. Figure 19 is a rare case without any measured aneuploid cells and has a proliferation fraction of 8.7%.

The original proliferation rule was derived from unpublished data of the author at the British Columbia Cancer Agency (BCCA) about 20 years ago (Figure 20), which set a threshold of 10% proliferation for calling a case positive, which is approximately the boundary between LSIL and HSIL. Most of the papers published in China in the past 10 years report using this value. A

second “equivocal” threshold is set at 5% proliferation which is approximately the value for ASCUS and is used as an “equivocal” diagnosis, discussed later.

No evidence of abnormality-sample adequacy

The fifth and last step in the data review checklist only applies when none of the preceding criteria for positivity are present. When no abnormality is found, the assessment of sample adequacy must be applied—were enough cells examined for a high probability that the case is actually negative? There is always some reluctance to declare a sample to be “unsatisfactory” with a recommendation of a return visit of the woman for another sample, which must balance the increased but unknown risk to the woman with an unsatisfactory test^[33] against “... undue burdens on the users and providers of the screening program”^[34], including costs. Although the background of this has already been discussed at length, none of the publications reviewed here have included a definition of what their laboratory uses for an adequacy standard. Guillaud *et al*^[41] determined 2000 or more epithelial cells on ThinPrep slides to optimize the sensitivity and speci-

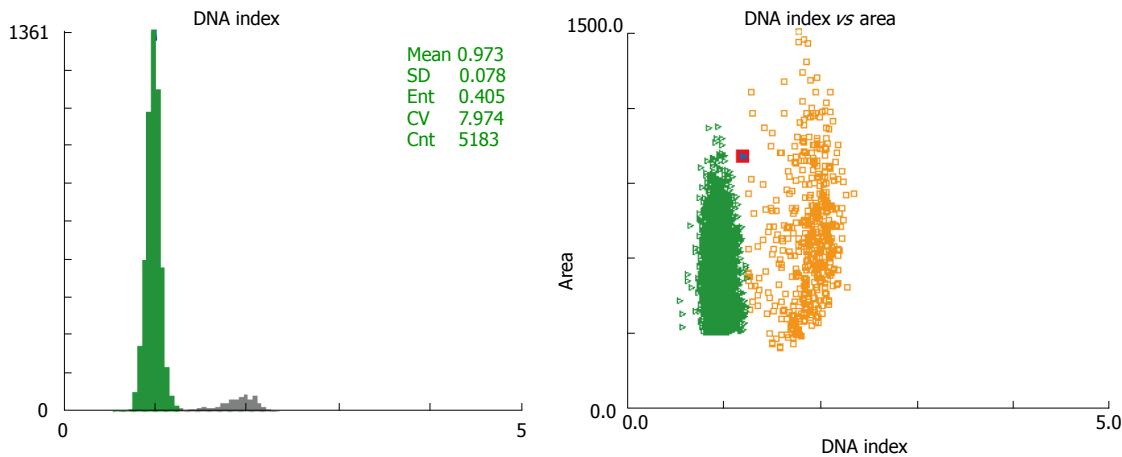


Figure 19 DNA ploidy data of a case with "equivocal" proliferation and no high DI aneuploid cells.

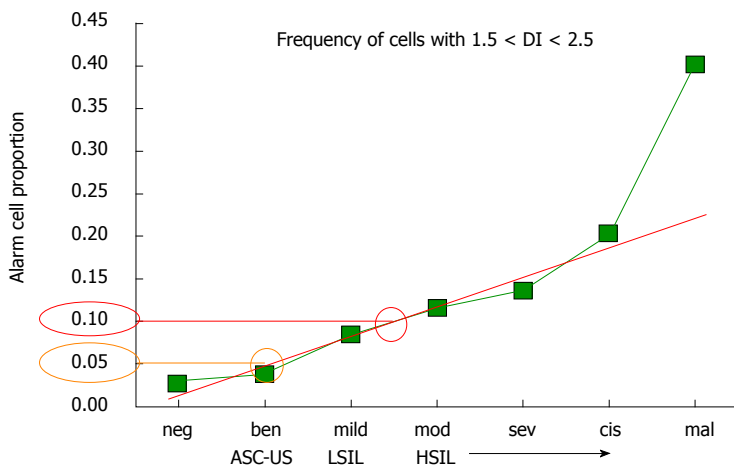


Figure 20 Original data from British Columbia Cancer Agency used to formulate the "proliferation" rules, based on measurement of > 500 samples.

ficity in a research setting.

FRAMEWORK FOR EVALUATING SCREENING TESTS

Arbyn *et al*^[47] and Adriaensen *et al*^[48] reviewed how to evaluate cervical cancer screening technologies through their various phases, in 2009 and updated in 2013, with extensive references.

However, most of the papers available for examination for this review are cross-sectional, observational studies that compare automated quantitative image cytometry to liquid based conventional cytology and the analysis centers on 2×2 contingency tables and the extraction of comparative sensitivity, specificity, positive and negative predictive values, usually also reported in the paper abstract. The literature has similar analyses for screening for other cancers with other technologies^[49]. In the opinion of this reviewer, these analyses are confusing at best and highly misleading at worst and most are singular in their absence of insight into genuine clinical utility of the tests. This section attempts to provide some suggestions to improve the data analysis with particular focus on the clinical meaning.

A typical study reviewed here is observational, ap-

plied to 10000 subjects screened with both Tests 1 and 2; the 600 who are positive by either test receive a recommendation to attend colposcopy; and the 250 available biopsy results become the exclusive focus of the analysis—the other 9750 cases of the study are ignored! In this analysis example there are "excluded" data (the $10000 - 600 = 9400$ cases that test negative by both Tests 1 and 2) and "missing" data (the $600 - 250 = 350$ cases who tested positive by at least one of Test 1 and 2 but with no follow-up result). Typically, there are 5 biopsy outcomes: negative (or cervicitis or inflammatory), CIN1, CIN2, CIN3 and invasive cancer. Tests 1 and 2 also typically have several diagnostic grades, such as NILM, ASCUS, LSIL, *etc*. The study applies some thresholds to collapse Test 1 *vs* histology and Test 2 *vs* histology to 2×2 contingency tables and the well-known formulae are applied to calculate sensitivity, specificity and predictive values. Typically, the reported specificities are very low as are the negative predictive values (NPVs), which is entirely an artifact of the failure to include the other 9400 negative cases in the analysis. Some statistics software even extracts the area under the receiver operator characteristics (ROC) curve from this single measured point, which is also often reported in the paper abstract. Finally, many papers also report the result of combining Tests 1 and

2 based on these same 2×2 tables, almost always as the logical “or” of positive results (the combined test is positive if either 1 or 2 is positive).

What is the interpretation of the results based solely on the 2×2 tables for the small subset of cases that have biopsy results available? For the moment, let us ignore the problems of verification bias, missing data and imperfect “gold standard” reference diagnosis—they are discussed later. Because the table is comprised only of cases testing positive by one or both screen tests, both the sensitivity and positive predictive value (PPV) are legitimate estimates of the screen test values and the PPV can be interpreted as the probability that a woman sent for biopsy will have a positive result. The specificity and negative predictive values are meaningless, however, because the “excluded” data is the vast majority of negative cases. In fact, the negative cases that are included in this 2×2 table are very strongly biased because the cases negative by Test 1 were positive by Test 2 and *vice versa*—otherwise colposcopy would not have been recommended (a few cases may be negative by both Test 1 and 2, but a biopsy resulted from other clinical evidence, which is not usually consistent with the notion of “screening”^[50]). In this fictional example, the vast majority of the 9400 subjects who tested negative by both tests are indeed negative and with less doubt than the highly biased negative cases included in the 2×2 table of biopsy results. The only value this “specificity” and “NPV” have is to show them to be higher in one test than the other, but the real magnitude of this difference cannot be estimated from these results. This reviewer believes that authors who perform such analysis should not report these as “specificity” and “NPV” but should find new terms, such as “reduced” or “biopsy biased” specificity and NPV, or better yet, not report them at all as they have no clinical meaning. It is ironic that for this kind of observational study the specificity and NPV are so grossly miscalculated and reported when, in fact, they can usually be quite reliably determined, as discussed later.

Given a 2×2 contingency table, the mathematical definitions of sensitivity, specificity, positive and negative predictive values are crystal clear, but the same is not true for their meaning. It is very common to have different definitions of “sensitivity”, “specificity” or “predictive” values”, even though the formulae by which they are extracted for the 2×2 tables are invariant; that is, the meaning depends on the table content. For example, in the world of HPV testing, there is the “analytic sensitivity” which refers to the ability of the test to detect the presence of HPV as distinct from “clinical sensitivity” which refers to the ability of the test to predict the presence of CIN^[51-53]; in general, too high analytic HPV sensitivity causes very low clinical specificity. As another example, in the screening mammography program of British Columbia^[54], the positive predictive value of a single screening mammogram ranges from about 2% to 20%, depending on the age of the woman, averaging to about 6.5% across all ages. This is the “test PPV”.

However, the screening program does not send women with a positive screen mammogram to biopsy but instead performs other tests, primarily diagnostic mammograms and/or ultrasound. Of the women actually sent for biopsy, about 33% are found to have cancer or DCIS—this is the “program PPV” and its clinical interpretation is “the probability that a woman sent for biopsy actually has breast cancer.”

Excluded data

While a screening test ideally should have both high sensitivity and specificity, high specificity is more critical. A screen test with a sensitivity of 50% and specificity of 95% would be inefficient but could be useful; the conventional Pap test has approximately this performance and it has reduced cervical cancer mortality by 70% in countries where it has been effectively applied. A screen test with a sensitivity of 95% and specificity of 50% would not be useful by itself because half of the screened population would have false positive test results. Depending on the follow-up consequences of a positive screen test result, most screen tests must have a specificity of at least 90% and probably many require specificity > 98%. In many of the papers reviewed here, the specificities are reported as “test” specificities in the abstract and are very low, often 40%-80% and even as low as 2%-3%^[55], entirely an artifact of failing to include most of the negative test data. On seeing these low values, those who understand screening might dismiss both Tests 1 and 2 as being completely useless.

These considerations show that the excluded cases that tested negative by both Test 1 and 2 should be included in the contingency tables (the effect of verification bias is discussed later) in order to obtain reasonable estimates of the specificity and NPV.

Screening does not have binary results

A key problem with 2×2 tables is the clinical reality that there are not two but three screening test results^[56]: positive, negative and equivocal, as shown in Figure 1, and defined as “too atypical to call ‘negative’ but not enough to call ‘positive’.” It is common to use time and re-testing to resolve equivocal cases, which is especially important for cervical cancer screening given that most “pre-cancers” resolve without medical intervention, especially in younger women^[57-64]. A recent study estimates that < 2% of CIN2/3 lesions progress to cancer within 10 years^[65]. At BCCA, ASCUS and LSIL are considered to be equivocal and the management recommendation is for repeat Pap testing at 6 mo intervals up to 4 times; if there is no resolution of the case by then, colposcopy is recommended^[50]. The most recent ASCCP management guidelines^[66] treat cytology ASCUS and histology CIN1 in all women and LSIL and CIN2 in young women as equivocal, with follow-up intermediate between how negative and positive cases are managed. While there is concern for loss of the patient to follow-up and undue burden on the patient, this must be balanced against the burdens

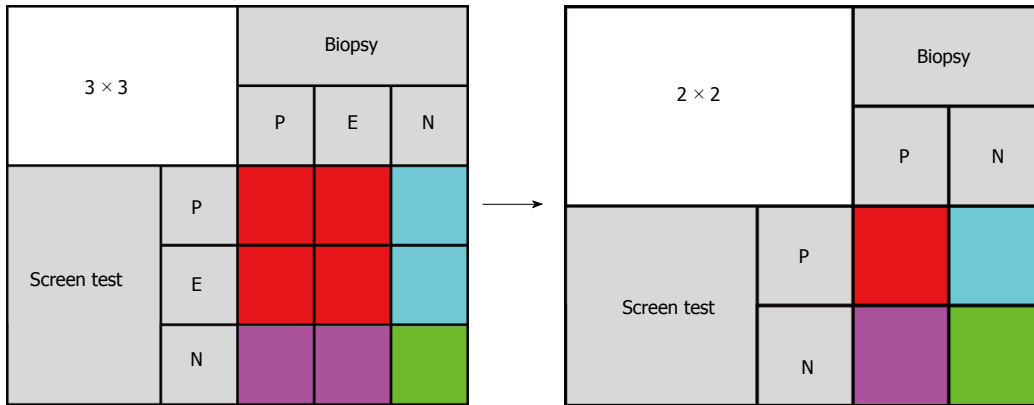


Figure 21 Mapping of 3×3 to 2×2 tables for sensitivity, specificity and negative predictive value.

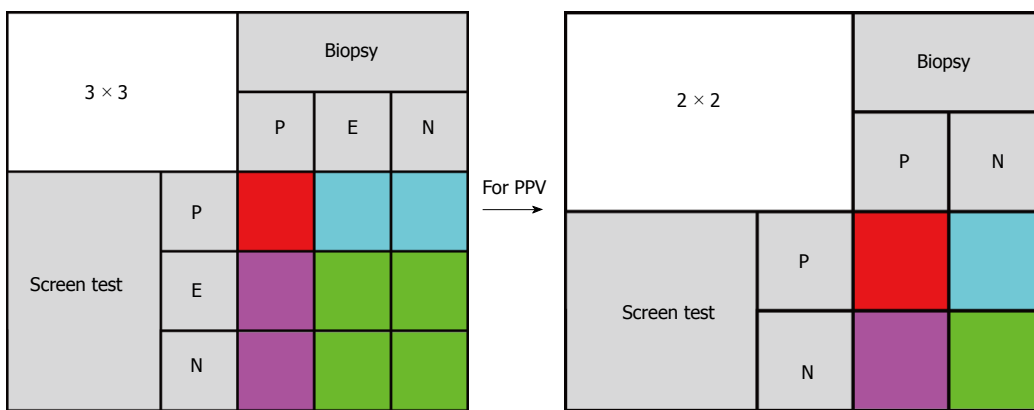


Figure 22 Mapping of 3×3 to 2×2 tables for positive predictive value.

and possible negative consequences of overtreatment, especially in younger women^[67-75]. Various strategies are possible to mitigate the burden of repeat testing.

For DNA Cytometry, most authors follow the recommendation that cases with the detection of 1 or 2 high DI aneuploid cells should be considered “equivocal” while ≥ 3 are considered positive (Figures 9, 10). Some laboratories also call cases equivocal that have between 5 and 10% proliferation fraction, as shown in Figure 20.

Unfortunately, there are no standard formulae for managing 3×3 tables, so some consideration should be given to the clinical meaning of what is calculated and reported. In collapsing the screen Test 1 or 2 and histology “gold standard” 3×3 tables to 2×2 contingency tables, one possible clinically relevant approach is to set the thresholds between “negative” and “equivocal” for sensitivity, specificity and NPV as shown in Figure 21.

Here the clinical interpretation of the screen test performance is: Sensitivity-the probability that positive gold standard cases had screen test results of positive or at least of equivocal. The notion is that equivocal screen cases cannot be called negative since they do carry a recommendation for follow-up; equivocal screen tests may represent delayed diagnosis cases but not missed diagnosis cases; Specificity-the probability that screen Test 1 negative cases are also screen Test 2 negative and biopsy

negative (when biopsy is available) cases; and Negative Predictive Value-its complement, (1-NPV), is the probability that a woman who is told she is disease free is actually not.

For PPV, the collapse of the 3×3 to 2×2 table follows a different scheme (Figure 22) because its usual clinical interpretation is: Positive Predictive Value-the probability that a patient sent for biopsy will have a positive biopsy result. Women with equivocal screen test results will not generally be recommended for colposcopy and possible biopsy.

A worked example

To illustrate the magnitude of the differences between these different approaches to analysis we work through an example based on data by Bao^[76] from 2009, selected because it is one of the larger studies published and because it has reasonably complete reporting of the data. Table 1 shows the overall results for cytology and DNA Cytometry for almost 20000 cases.

The screen test vs biopsy results are Table 2 for Cytology and Table 3 for DNA Cytometry.

The 2×2 tables used in the publication are shown in Table 4 using CIN2+, LSIL+ and DNA Positive as the thresholds for histology, cytology and DNA Cytometry, respectively.

Table 1 Cytology and DNA Cytometry results from Ref^[76]

Cytology ¹		DNA Cytometry	
NILM	18503	Negative	17855
ASCUS	720 (0.40)	Equivocal	1395
LSIL	296 (0.89)	Positive	371
HSIL	59 (1.00)		
Total	19621	Total	19621

¹Number in parenthesis is the fraction with at least 1 aneuploid cell by DNA Cytometry. NILM: Negative for intraepithelial lesion or malignancy; ASCUS: Atypical squamous cells of undetermined significance; LSIL: Low grade squamous intraepithelial lesion; HSIL: High grade squamous intraepithelial lesion.

Table 2 Biopsy results vs Cytology from Ref^[76]

Histology	Total	Cytology			
		NILM/inflam	ASCUS	LSIL	HSIL
Ca	15		6	5	4
CIN3	43	5	10	14	14
CIN2	66	12	15	23	16
CIN1	98	42	22	21	13
CC/Neg	401	285	86	18	12
Total	623	344	139	81	59

NILM: Negative for intraepithelial lesion or malignancy; ASCUS: Atypical squamous cells of undetermined significance; LSIL: Low grade squamous intraepithelial lesion; HSIL: High grade squamous intraepithelial lesion; Ca: Invasive cancer; CIN: Cervical intraepithelial neoplasia; CC: Cervicitis.

Table 3 Biopsy results vs DNA Cytometry from Ref^[76]

Histology	Total	DNA Cytometry		
		Neg	Equiv	Pos
Ca	15		2	13
CIN3	43	1	12	30
CIN2	66	3	19	44
CIN1	98	15	55	28
CC/Neg	401	87	280	34
Total	623	106	368	149

Ca: Invasive cancer; CIN: Cervical intraepithelial neoplasia; CC: Cervicitis.

The test performance results are given in Table 5. The authors did not report the NPV and PPV in their publication. Based on these numbers, the performance of the two tests is quite comparable, although cytology missed 6 of 15 invasive cancers using the LSIL+ threshold while DNA Cytometry missed 2.

Next are 3 × 3 tables that include the previously excluded negative cases, but also with the missing data still deleted (as with the author's analysis above); biopsy results are compared with Cytology in Table 6 and with DNA Cytometry in Table 7. The definitions of Positive, Negative and Equivocal are arbitrary and arguable but used for illustration.

Following the approach outlined above to collapse the 3 × 3 tables to 2 × 2 tables according to the clinical interpretation described above yields new test performance

Table 4 2 × 2 Contingency Tables for Biopsy results vs Cytology and vs DNA Cytometry from Ref^[76]

Histology	Total	Cytology		DNA Cytometry	
		LSIL +	NILM/ASCUS	Pos	Neg/Equiv
CIN2+	124	76	48	87	37
Neg/CIN1	499	64	435	62	437
Total	623	140	483	149	474

NILM: Negative for intraepithelial lesion or malignancy; ASCUS: Atypical squamous cells of undetermined significance; LSIL+: Low grade squamous intraepithelial lesion or higher; CIN: Cervical intraepithelial neoplasia.

Table 5 Cytology and Cytometry test performance based on data and analysis from Ref^[76]

	Cytology	DNA Cytometry
Sensitivity	61.3%	70.2%
Specificity	87.2%	87.6%
PPV	54.3%	58.4%
NPV	90.1%	92.2%

PPV: Positive predictive value; NPV: Negative predictive value.

Table 6 3 × 3 Contingency Table for Biopsy results vs Cytology from Ref^[76]

Histology	Total	Cytology		
		Positive (LSIL +)	Equivocal (ASCUS)	Negative
Positive (CIN2+)	124	76	31	17
Equivocal (CIN1)	98	34	22	42
Negative	18560	30	86	18444
Total	18782	140	139	18503

ASCUS: Atypical squamous cells of undetermined significance; LSIL+: Low grade squamous intraepithelial lesion or higher; CIN2+: Cervical intraepithelial neoplasia, grade 2 or higher; CIN1: Cervical intraepithelial neoplasia, grade 1.

Table 7 3 × 3 Contingency Table for Biopsy results vs DNA Cytometry from Ref^[76]

Histology	Total	DNA Cytometry		
		Positive	Equivocal	Negative
Positive (CIN2+)	124	87	33	4
Equivocal (CIN1)	98	28	55	15
Negative	18150	34	280	17836
Total	18372	149	368	17855

CIN2+: Cervical intraepithelial neoplasia, grade 2 or higher; CIN1: Cervical intraepithelial neoplasia, grade 1.

results shown in Table 8.

The increase in sensitivities is due to considering equivocal cases as positive since they include a recommendation for follow-up. The PPVs are unchanged. The specificities and NPVs are much higher when the negative cases are included and are consistent with the

Table 8 Cytology and Cytometry test performance based on data from Ref^[76] and revised analysis

	Cytology	DNA Cytometry
Sensitivity	73.4%	91.4%
Specificity	99.4%	98.3%
PPV	54.3%	58.4%
NPV	99.68%	99.89%

PPV: Positive predictive value; NPV: Negative predictive value.

requirements for screening test performance.

Many authors interpret the high NPVs as being almost equal and so to be uninteresting. In fact, the clinical significance of the NPV is seen from its complement (1-NPV), which relates to “false negative” cases. In this example of 10000 women who are advised that they are disease free, in fact, 32 or 11 are not, based on cytology or DNA Cytometry, respectively. In this regard, the performance is quite different between the two tests. This is especially important when routine screening is infrequent, say once or twice during a woman’s lifetime.

An alternative approach to compare the tests is to simply tabulate the results descriptively as in Table 9.

This study was primarily of rural women who had not previously been screened.

The impact of uncorrected verification bias

Most observational screening studies suffer from verification bias because the “gold standard” of colposcopy guided biopsy is only applied to the cases that test positive by the screen test, so the true disease state of those who test negative is not verified. There are statistical methods to correct for this kind of bias (e.g.^[77]). RCTs sometimes manage this by performing colposcopy and biopsy on a random sample of the negative cases, under proper informed consent and ethics board approval (e.g.^[78]), although this also has its pitfalls^[47].

It may be instructive to look at the impact when this kind of verification bias is left uncorrected. Figure 23 plots the “DNA Cytometry” data of the previous example (Table 8) as a function of the fraction of positive and equivocal cases detected in the study; the left axis is where all positive and equivocal cases were detected and it declines to where only 50% were detected at the right axis. PPV is completely unaffected because it relates only to ratio of positives that the screen correctly identified to the total that it tested positive; hence, any false negatives do not affect this ratio. NPV and specificity decline only slightly over the range plotted. It is the sensitivity, of course, that is potentially most impacted by this kind of verification bias, with an inverse proportional linear dependence; that is, if only half of the positive cases were detected in the study, then the measured sensitivity is double the correct value.

In summary, when this kind of verification bias is left uncorrected it has no impact or almost no impact on predictive values or specificity but it can significantly inflate the measured sensitivities. However, in studies such

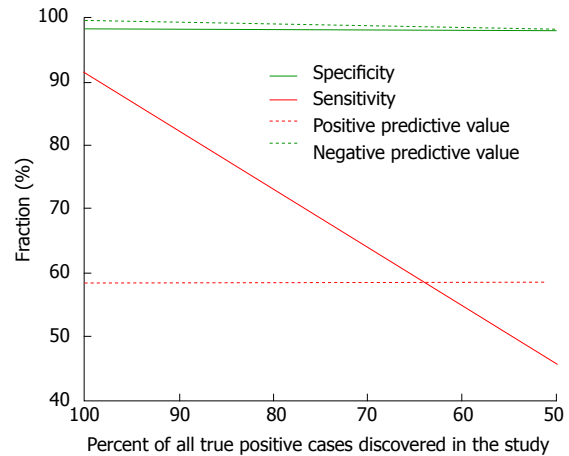


Figure 23 Effect of uncorrected verification bias on test performance indicators.

as those described above, it is likely that well over 90% of the true positive cases were detected by one or both of the screening tests^[47,79], so the impact of uncorrected verification bias in this type of study is unlikely to be very large.

Unbiased comparisons of test performance

It has been shown that the ratio of (Test 2) : (Test 1) for true positive rate and for false positive rate determined just from the complete biopsy results (with no missing data) does not suffer verification bias of the type discussed above (not subjecting negative cases to the “gold standard” test)^[80,81]. This may be enough to rank Test 1 *vs* Test 2.

Table 10^[80] summarizes the biopsy data required and the relative true positive rate, *relTPR*, and relative false positive rate, *relFPR*, are simply^[81]:

$$relFPR (\text{Test 2/Test 1}) = \{(a + b)/[n]\} / \{(a + c)/[N]\} = (a + b)/(a + c)$$

$$relTPR (\text{Test 2/Test 1}) = \{(A + B)/[N]\} / \{(A + C)/[N]\} = (A + B)/(A + C)$$

The key point is that the unknown [n] and [N] are eliminated, leaving only known values from the biopsy result table. If *relTPR* increases and *relFPR* decreases, then Test 2 is easily judged to be the better test, although as Arbyn *et al*^[47] points out, such cross-sectional results do not necessarily translate into longitudinal improvement in the screening program. It is more generally the situation that both *relTPR* and *relFPR* increase at the same time, in which case it is necessary to consider the disease prevalence in the target screen population to determine if the cost of increased false positives justifies the benefit of the increased true positive detection. In the special case where the target population and the study population have the same prevalence (this is generally true for observational studies of routine clinical data), then the *FP/TP* ratio is given by: $FP/TP = (B - C)/(b - c)$. This ratio is one “figure of merit” in deciding if Test 2 is better than Test

Table 9 Descriptive summary of the data of Ref [76] as re-analyzed *n* (%)

Clinical result		Cytology number of cases	DNA Cytometry number of cases	Cytometry-Cytology number (%)
1	Number of women immediately returned to routine screening	18503	17855	-648 (-3.5)
2	Number of CIN1+ cases (false negative cases) per 10000 women returned to routine screening	32	11	-21 (-66)
3	Number of women referred to immediate colposcopy	140	149	9 (6)
4	Number of cases of invasive cancer immediately diagnosed	9	13	4 (44)
5	Number of CIN3+ cases immediately diagnosed	37	43	6 (16)
6	Number of clinically positive cases (CIN2+) immediately diagnosed by colposcopy	76	87	11 (15)
7	Number of women requiring 6 mo follow-up due to equivocal result	139	368	229 (165)
8	Number of women with potentially delayed clinically positive (CIN2+) diagnosis	31	33	2 (6)
9	Number of CIN3+ cases missed	5	1	4 (80)
10	Number of CIN2+ cases missed	17	4	13 (76)

Table 10 Definitions of the biopsy data required for comparison of two screening tests independent of simple verification bias, Ref^[80]

	Gold standard positive			Gold standard negative		
	Test 1+	Test 1-	Total	Test 1+	Test 1-	Total
Test 2+	a	b	a + b	A	B	A + B
Test 2-	c	[d]	[c + d]	C	[D]	[C + D]
Total	a + c	[b + d]	[n]	A + C	[B + D]	[N]

Variables in square brackets have unknown values.

Table 11 Biopsy data from Ref^[82] for simple verification bias free comparison of Cytology vs DNA cytometry

	Biopsy +			Biopsy -		
	Cytology +	Cytology -	Total	Cytology +	Cytology -	Total
AQIC+	130	37	167	30	23	53
AQIC-	1	0	1	0	0	0
Total	131	37	168	30	23	53

AQIC+: Positive by automated quantitative image cytometry; AQIC-: Negative by automated quantitative image cytometry.

1 and comes down to the judgment of “how many extra false positive cases are acceptable for each additional true positive case detected?” The value of this ratio will depend upon the undiagnosed disease prevalence.

Exactly the same results are obtained by inspection of the appropriate 2×2 tables for each test *vs* the biopsy results; both calculation methods are illustrated next.

Tian *et al*^[82], provided the case by case data for that paper to allow illustration of the method here. The first look at this is based on “positive” defined as ASCUS+, Equivocal+ and CIN1+ for cytology, DNA Cytometry (AQIC) and pathology, respectively, summarized in Table 11. The *re*TPR = 1.3 and *re*FPR = 1.8, both increasing as expected. However, the *FP*/*TP* ratio is just 0.64, meaning that for every extra true positive detected by AQIC, 2/3 additional false positive cases occur, which is probably an acceptable trade-off.

The same result is obtained from the published^[82] 2×2 tables, using the same thresholds for positive, as shown in Table 12. By inspection *re*TPR = $167/131 = 1.3$ and *re*FPR = $53/30 = 1.8$ and *FP*/*TP* = $(53-30)/(167-131) = 23/36 = 0.64$.

These ratios may provide a useful, unbiased “figure of merit” with which to compare one test to another. The key message is that these ratios-the relative true positive rate, *re*TPR, relative false positive rate, *re*FPR and *FP*/*TP*-are not subject to verification bias of the type due to failing to perform the “gold standard” reference test on the large number for cases that test negative by both Test 1 and Test 2. However, they may be subject to bias due to missing data, discussed next.

Missing data

But what about the missing data-the cases recommended for colposcopy for whom there is no follow-up information? This is a particular kind of verification bias and there are formal methods to correct for it (for example^[77,83-87]) that involve using statistical methods to impute the missing data with minimal bias, but they are beyond the scope of this paper. The simplest method is to delete these cases from the analysis, as was done in the example above and in all of the DNA Cytometry papers reviewed here. This is approximately equivalent to assuming that the missing data is the same as the known data. Because the missing data is all from cases that tested positive by either or both Tests 1 and 2, this has no effect on the NPV, for reasons analogous to why the PPV was unaffected in Figure 23. There is no simple, general statement one can make about the impact of the missing data on the sensitivity, specificity or PPV except that the range of possible deviation between the uncorrected measurements and the true values is proportional to the amount of missing data. Each of the three performance measures could rise, fall or stay the same if the missing data were not missing. The key message is that it is extremely important to organize studies so as to minimize the amount of missing data. In the Bao paper, they did not report the total number of colposcopy follow up recommendations but, depending on the correlations between cytology and cytometry, at least 65% of the biopsy follow up data is missing, which is very typical for this series of papers from China.

Table 12 Alternative presentation of Biopsy data from Ref^[82] for simple verification bias free comparison of Cytology vs DNA Cytometry

Histology	Cytology			Histology	DNA Cytometry		
	Total	ASCUS+	NILM		Total	Pos/equiv	Neg
CIN1+	168	131	37	CIN1+	127	167	1
Neg	53	30	23	Neg	121	53	0
Total	221	161	60	Total	221	220	1

NILM: Negative for intraepithelial lesion or malignancy; ASCUS: Atypical squamous cells of undetermined significance; CIN1+: Cervical intraepithelial neoplasia, grade 1 or higher.

Effects of an imperfect “gold standard” reference

The “gold standard” reference diagnosis for cervical cancer studies is usually colposcopy directed biopsy, which a number of studies over the past decade have shown to be imperfect^[88-92]. More worrying were studies that showed that the measured sensitivity of Visual Inspection with Acetic Acid (VIA) were substantially inflated (by 1/6 to 1/3) due to use of colposcopy directed biopsy as the “gold standard” reference^[93-95]. Theoretical studies^[96] of such use of imperfect references have shown that if the screen and reference test errors are statistically correlated, then the test accuracy (sensitivity and specificity) measurements are over-estimated; conversely, if the screen and reference test errors are statistically independent, then the test accuracy measurements are underestimated. VIA is essentially a variation of colposcopy and so is highly correlated.

It has also become clear over the past decade that colposcopy sensitivity improves the more biopsies that are taken^[88,93,95,97,98], independent of the skill^[99] or medical training^[90] of the person performing the colposcopy, ranging from nurse-practitioner to gynecological oncologists. Many propose supplementing any lesion biopsies with a random biopsy from any quadrant without visible lesions^[97,99]. However, while this increases the rate of detection of CIN2+, it does not appear to affect the measured sensitivity of cytology, although it may improve the specificity slightly^[94]. It is unknown if taking more biopsies has any effect on the DNA Cytometry accuracy measurements but one might expect any effect to be small based on the cytology situation.

Combining binary test results

A great many papers, including several reviewed here, look at combining the results of two binary (or dichotomous) tests with the hope that the combined test result is an improvement over either of the component Tests 1 and 2. This is also applied to tests like Hybrid Capture 2 (HC2) HPV test that measures continuously valued viral load but which is made binary by applying a threshold, ≥ 1 RLU/Cutoff is positive, otherwise it is negative^[100].

It can be proven that there are only two non-trivial ways to combine 2 binary tests: as the logical “and” of positive cases, in which the result is positive only if both Tests 1 and 2 are positive, and as the logical “or” of posi-

Table 13 The test performance limits for the combination of two binary valued tests

	Sensitivity		Specificity	
	Minimum	Maximum	Minimum	Maximum
Logical “AND”	$(Se_1 + Se_2) - 1^1$	$\text{Min}(Se_1, Se_2)$	$\text{Max}(Sp_1, Sp_2)$	$(Sp_1 + Sp_2)^3$
Logical “OR”	$\text{Max}(Se_1, Se_2)$	$(Se_1 + Se_2)^4$	$(Sp_1 + Sp_2) - 1^2$	$\text{Min}(Sp_1, Sp_2)$

¹0% if $(Se_1 + Se_2) \leq 100\%$; ²0% if $(Sp_1 + Sp_2) \leq 100\%$; ³100% if $(Sp_1 + Sp_2) \geq 100\%$; ⁴100% if $(Se_1 + Se_2) \geq 100\%$. Se: Sensitivity; Sp: Specificity.

tive cases, in which the result is positive if either or both Tests 1 and 2 are positive. It is also possible to prove that the combined test results will conform to the limits in Table 13, where “Min” and “Max” are the minimum or maximum of the two entries in parenthesis and where Sp = specificity and Se = sensitivity.

For the logical “and” of positive results, the specificity of the combined test will be at least as high as the specificity of the most specific component test and could be 100%; however, the sensitivity will be no better than the sensitivity of the least sensitive component test and could be zero.

Conversely, for the logical “or” of positive results, the sensitivity of the combined test will be at least as high as the sensitivity of the most sensitive component test and could be 100%; however, the specificity will be no better than the specificity of the least specific component test and could be zero.

Examples of “and” and “or” combinations of binary tests and their compliance to these limits can be found in refs^[101-103].

Furthermore, it can also be proven that the result of combining these tests does not depend on the test order (they are commutative).

In either combination, it is not necessary to perform both tests on all subjects^[104]. For the logical “and” of positive test results, if the first test result is negative then the combined test result will also be negative, regardless of the second test result; similarly, for the logical “or” of positive test results, if the first test result is positive, it does not matter what the second test result is, the combined test result will be positive. This, plus the fact that the test order does not affect the results, means that a test strategy can be adapted to minimize costs in clinical practice (both must be done for comparative performance studies, of course).

There is one caveat with these rules: they apply for analytical tests that are independent in the sense that one test does not impact the other. For example, if one test compromised the sample for the other test, these rules might not apply. It is not so clear that cytology is a test that is independent of DNA Cytometry or HPV status, if the cytologist is aware of the DNA Cytometry or HPV results. Since most of the papers reviewed here are observational studies looking at both tests in routine use, it would be expected that the cytologist would, in general,

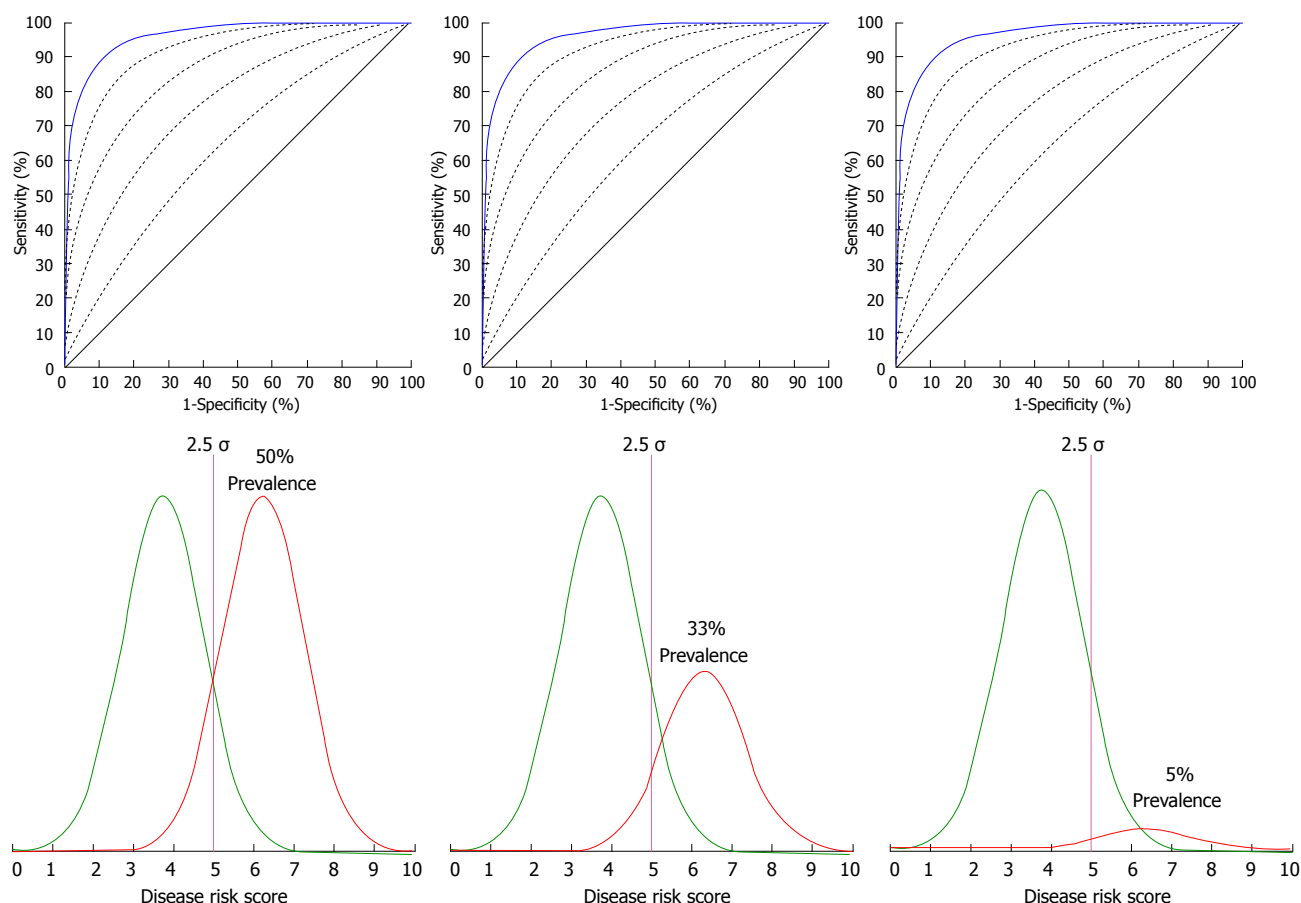


Figure 24 Sensitivity and specificity are independent of disease prevalence when the change in prevalence is only a scalar multiplier that does not change position, width or shape of the population distribution.

be aware of the results of the other tests since the goal is to get the correct answer for the patient, not to do a strict clinical study.

It does not seem to be appreciated by many authors that the limits in Table 13 seriously constrain any potential benefits from combining binary tests. Since one of sensitivity and specificity will rise as the other falls when the tests are combined, it usually only makes sense to combine tests that have very similar and high values for the one that will fall, so that it does not fall much and stays in range of what is useful. For example, cytology has poor sensitivity, but excellent specificity; hrHPV testing has excellent sensitivity, but only good specificity. Combining these tests will result in either excellent sensitivity and less than good specificity, or excellent specificity and poor sensitivity. There is no simple combination of HPV testing and cytology that works well. The work around for this until a better test or test combination is found is in the patient management algorithms, which are becoming increasingly complex^[6]. These algorithms manage “equivocal” cases with a combination of time and re-testing, in recognition that screen tests, even in combination, have 3 outcomes, not 2, as already discussed.

Impact of disease prevalence on estimates of test performance

A key issue in appreciating published study results is

to determine their degree of generalizability. A basic concept of epidemiology is that while predictive values explicitly depend strongly on disease prevalence, sensitivity and specificity are completely independent of it^[105]. Although this prevalence independence of sensitivity and specificity is certainly true, it is a fragile truth that may not survive test generalization. That is, if a published study demonstrates that Test A has sensitivity X and specificity Y with a particular disease prevalence Z, it does not mean that when applied to a different population with a disease prevalence of $1.5 \times Z$, Test A will have the same sensitivity and specificity found in the study, except if the mean, width and shape of the underlying distributions of positive and negative populations is also the same as in the study. Figure 24 illustrates ROC curves, which should be independent of disease prevalence since they essentially plot sensitivity *vs* specificity, for the case where the mean, width and shape of both positive and negative populations remains the same and only the positive distribution is multiplied by a scalar to reduce the distribution area without distortion or shifting of the peak position. As expected, the change in prevalence has no effect on the ROC curve or sensitivity and specificity, indicated by the blue line.

However, as a practical matter, “real life” changes in disease prevalence rarely occur this way. If the prevalence increases, and especially if it is a large increase, it also

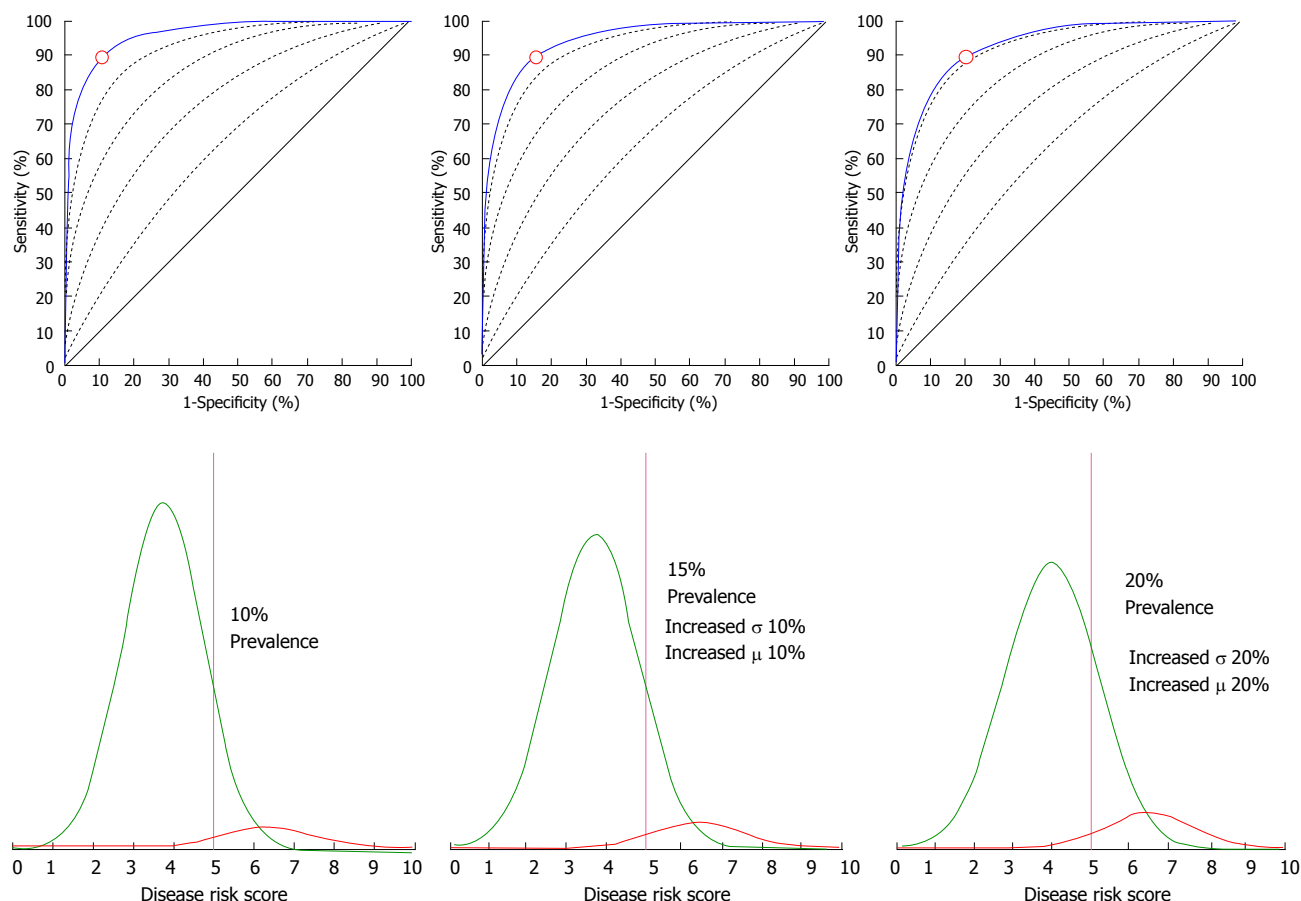


Figure 25 “Real life” changes in disease prevalence are usually accompanied by changes in both positive and negative population distribution positions, widths and shapes which do change the sensitivity and specificity.

probably causes changes in the mean, width and overall shape of both positive and negative distributions as the whole population is somewhat sicker, as illustrated in Figure 25. In scanning the figure from left to right, the prevalence increases, but the positions (μ) and widths (σ) of both distributions also increases. In this example, both distribution means (μ) shifted to the right the same amount-this would not cause any change in the ROC curve itself-the change in the ROC curve is caused by the change in widths (σ) of the distributions. The magenta line represents the Test threshold and the red point on the ROC curve corresponds to test performance at that threshold. Unless the test threshold is adjusted by re-calibrating the test to the new populations, then the test performance moves from optimal, as indicated by the shift of this operating point. This figure is based on symmetric analytic distributions for ease of calculation and drawing-there is no requirement that the underlying distributions for ROC curves are symmetric, analytic or even single peaked; hence, “real life” shifts in disease prevalence may cause different shifts in test performance than the simple ones illustrated here.

This result is technology independent and will apply not just to AQIC but also to hrHPV testing, for example. One situation where there is a large difference in undiagnosed disease prevalence is when a test is applied for

screening (testing people without symptoms) *vs* diagnosis (testing people with positive screen test results or with symptoms). Several recent publications have experimentally verified this for colposcopy^[106], hrHPV testing^[107], the conventional Pap test^[108], as well as a recent review of several meta-analyses of various diseases^[109]. The previous discussion of BCCA screening mammography *vs* diagnostic mammography is another example. Many physicians erroneously think that test sensitivity and specificity are invariant properties of the test and therefore independent of whom the test is applied to; this is not necessarily the case.

Although it is well understood that predictive values explicitly depend upon undiagnosed disease prevalence, it is less widely appreciated that this imposes significant limits on the positive predictive value. Why is the PPV not 100%? For a low prevalence disease, such as cancer in a screening setting, PPV is determined mostly by the false positives (or 1- specificity); PPV is only weakly dependent on sensitivity. Figure 26 shows the maximum possible PPV (under the condition that sensitivity is 100%) as a function of undiagnosed disease prevalence for various very high values of specificity. Even at a disease prevalence of 1% (1000/100000 persons) and with a test specificity of 99%, the PPV will only be 50%; if the test specificity falls to 98%, the PPV drops to about 33%.

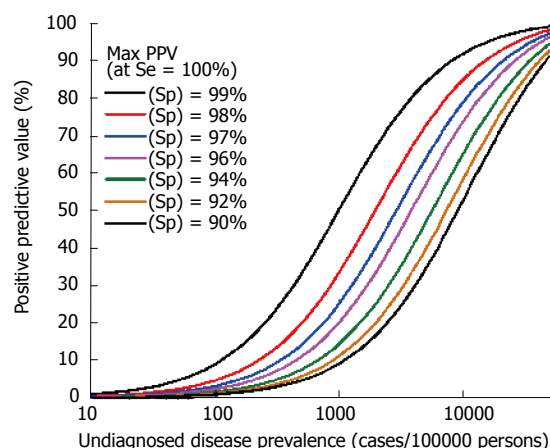


Figure 26 Positive predictive value at 100% test sensitivity as a function of undiagnosed disease prevalence for various high values of test specificity.

This is another demonstration of the crucial importance of high specificity for screening tests. It also illustrates that cancer screening is more than a test—it is a process by which negative cases are progressively removed from the population under surveillance until the disease prevalence of the remainder is increased to levels that allow acceptably high PPVs.

In summary, undiagnosed disease prevalence has a big impact on screening test performance, firstly by affecting the real world generalizability of sensitivity and specificity and secondly by limiting the maximum possible test PPV.

The large Table 14 summarizes the observed crude disease prevalence in several automated quantitative image cytometry studies from China from 2005–2013. The studies were selected as having the word “screening” in the title, abstract or introduction; studies with only the word “diagnosis” were excluded. As previously discussed, there is often substantial “missing” biopsy result data in studies from China. The column “follow-up ratio” is an estimate made by this reviewer of the extent to which high grade cytology had corresponding biopsy data—the blank entries indicate that it was not possible to estimate this. With few exceptions, the listed disease prevalences are underestimated, in many cases by a factor of 2 or more. The heterogeneity of the results is quite alarming and certainly justifies the warnings just made concerning generalizing the study test results for sensitivity and specificity. The very high disease prevalence in some studies that are claimed to be “screening” is quite vexing.

PUBLICATIONS ON THE APPLICATION OF DNA PLOIDY TO CERVICAL CANCER SCREENING

Since 2005, the use of AQIC testing for cervical and other cancers has continuously expanded to about 1 million tests per year in 2013. At least 60 publications on various comparisons for AQIC performance to other means of

screening or diagnosing cervical cancer have appeared since 2005, mostly in Chinese language journals. This section reviews some of these papers.

Disease prevalence

Perhaps a good starting point is Table 14 on measured crude prevalence rates of invasive cervical cancer and CIN. As shown previously, disease prevalence is a very important epidemiological quantity that impacts the performance and deployment of tests, especially screening tests. However, prevalence is very difficult to measure and there are two very different flavors of prevalence that can cause confusion. Some cancer monitoring agencies report cancer prevalence meaning as “How many people diagnosed with cancer are alive today?” These are estimated either as “limited-duration” prevalence (limited to cases diagnosed over, say, the past 10 years)^[143] or “complete” prevalence which is independent of when the cancer was diagnosed, as is done by SEER^[144]. Neither of these is relevant to cancer screening which is focused on and influenced by “undiagnosed” cancer prevalence. From here on, the term “prevalence” will be understood to mean “undiagnosed prevalence,” unless explicitly modified.

If the cancer is undiagnosed, how do we know how much there is? In a well screened population, the disease prevalence is less important than it is in poorly screened populations. An effective screening program will detect the “prevalent” cases of cancer within several years of its introduction (depending on the screening program effectiveness and deployment details) and will remove them from the pool of undiagnosed cancers, leaving mostly what are known as new “incident” cases, approximately equal to the incident rate for that cancer among that population times the screening interval: P (cases/100,000 people) is approximately equal to I (cases/100,000 people/year) \times Screen Interval (years). In an unscreened population, the introduction of screening is usually the time when the disease prevalence can best be estimated by measurement; otherwise, it can only be estimated from cancer natural history and population models. In the absence of screening, cancer is diagnosed either by investigation of symptoms or incidentally during some other medical investigation. There is usually a delay between the onset of symptoms and diagnosis because the patient, physician or both ignore the symptoms for some time, as they are rarely specific. A crude “back of the envelope” estimate of undiagnosed invasive cancer in an unscreened population would be the incidence rate times the delay between invasion and onset of threshold symptoms—that is, symptoms strong enough to lead to diagnostic investigation: P (cases/100,000 people) is approximately equal to I (cases/100,000 people/year) \times Symptom Threshold Interval (years). What is the incidence rate of cervical cancer in China? The official rate from the 2004–2005 “Third National Survey” is 3.2/100,000/year and the mortality is 2.4/100,000/year, age standardized to the world population^[145]. If it takes on average 10 years between the onset on invasive cervical cancer and the onset of symptoms significant enough to seek

Table 14 Crude cancer prevalence and other data from 36 published DNA Cytometry studies from China, 2005-2013

Ref.	Invasive cancer cases	CIN2+ cases	Total screened	Crude cancer prevalence (per 100000 persons)	Crude CIN2+ prevalence (%)	Follow-up ratio	Ca/CIN2+ (× 10)
[110]	3	14	500	600	2.8		2.14
[82]	7	100	23698	29.5	0.42	0.38	0.7
[111]	48	103	4020	1194	2.56	0.78	4.66
[112]	1	22	1200	83.3	1.83		0.45
[113]	2	17	673	297.2	2.53		1.18
[114]	4	12	3551	112.6	0.34		3.33
[115]	2	15	3162	63.3	0.47	0.39	1.33
[116]	14	78	4109	340.7	1.9	0.93	1.79
[117]	4	84	9261	43.2	0.91	0.36	0.48
[118]	2	30	2599	77	1.15		0.67
[119]	3	65	1200	250	5.42		0.46
[120]	9	111	6793	132.5	1.63	1	0.81
[121]	0	16	2003	0	0.8		0
[122]	4	87	2153	185.8	4.04		0.46
[123]	3	56	12079	24.8	0.46		0.54
[124]	5	26	3000	166.7	0.87		1.92
[125]	8	142	5886	135.9	2.41	1	0.56
[126]	11	147	7735	142.2	1.9	1	0.75
[127]	1	41	4598	21.7	0.89		0.24
[128]	4	34	12278	32.6	0.28		1.18
[129]	2	11	3589	55.7	0.31		1.82
[130]	3	17	1806	166.1	0.94		1.76
[131]	0	10	1206	0	0.83		0
[132]	8	51	3603	222	1.42	1	1.57
[133]	106	168	23993	441.8	0.7		6.31
[134]	6	36	1220	491.8	2.95		1.67
[76]	15	124	19621	76.4	0.63		1.21
[55]	2	21	3070	65.1	0.68		0.95
[135]	1	15	2000	50	0.75		0.67
[136]	0	5	1256	0	0.4		0
[137]	30	172	18097	165.8	0.95		1.74
[138]	1	7	451	221.7	1.55		1.43
[139]	10	53	430	2325.6	12.33		1.89
[140]	6	12	2832	211.9	0.42		5
[141]	15	95	8670	173	1.1		1.58
[142]	30	187	22169	135.3	0.84		1.6

CIN2+: Cervical intraepithelial neoplasia, grade 2 or higher.

medical attention, then this would predict invasive cervical cancer prevalence of 30-60/100000, allowing for differences in age standardized rates. This is a factor 10-40 less than what is reported in several entries in Table 14.

Although the Chinese registries consistently report such cervical cancer incidence rates^[146-150] and the international reports (based on the same registries) only slightly inflate them presumably due to different age standardization^[151,152], there are good reasons for skepticism, including the fact that the registries span only 2%-6% of the population of China^[145]. Pooled and population based studies have shown prevalence rates of CIN2+ and hrHPV infection to be similar to or higher than in other Asian and south Asian countries with much higher cervical cancer incidence rates^[145,153-156]. Although the official mortality rate is slightly higher than in countries like the United States, United Kingdom or Canada with long established, effective cervical cancer screening programs, the official incidence rate in China is only 1/3-1/2 of that in these countries, even though a great many women in China have never been screened^[145,157]. In Hong Kong,

opportunistic cervical screening was introduced in 1970 at which time the age standardized incidence rate was measured to be 25-35/100000/year^[158] and even today, with a comprehensive cervical cancer screening program, has a crude rate of 9.7 and age standardized rate 6.9/100000/year^[159]. One of the requirements of screening is that the disease must be common enough to justify the expenditure of resources and it has been suggested that a crude rate of about 3/100000/year is a reasonable threshold^[160]; below this incidence rate, screening is not justified. This is likely not the situation with cervical cancer in China, but only if cervical cancer is being seriously under-reported by the registries, putting the official incidence rate in serious error. There is a clear need to resolve if the surprisingly low official incidence rate for cervical cancer in China is correct or not.

Two pooled population based analyses^[154,161] found crude prevalence for invasive cervical cancer of 170/100000 and for CIN2+ of 2.6% for combined rural and urban populations not screened within the past 5 years, including a significant proportion of women who had never been

screened. These data are consistent with many of those in Table 14.

In Table 14, in which most of the prevalences are lower limits due to missing biopsy follow-up data, 8 of 36 studies found invasive cancer prevalence $> 250/100000$; these tended to be small hospital-based studies that were possibly contaminated by diagnosis cases. In the author's experience in China, the Pap test is widely used for diagnosis rather than for screening, even though there is evidence that samples from symptomatic women are often unsatisfactory due to blood and other reasons and have a higher false negative rate than from asymptomatic women^[39]. As previously discussed, test performance can vary considerably due to differences in disease prevalence in the population tested. Laboratories that routinely use the automated quantitative image cytometry method for differential diagnosis may require re-calibration of the operating point for their instrument.

Comparisons of AQIC with LBC

The diffusion of the automated quantitative image cytometry technology in China has primarily been through existing pathology laboratories which are generally hospital based. Although the cytometers of the various vendors have regulatory approvals^[162,163] and endorsement by various medical societies and expert groups, it is natural that each lab would compare performance with existing conventional liquid-based cytology. Consequently, most of the publications from China are cross-sectional, observational studies from routine clinical practice in which both liquid based conventional cytology and AQIC are done on the same sample and the test performances compared, as detailed previously. Most of these studies found substantially increased sensitivity (up to a factor of 2)^[76,82,111,112,117,119,120,125,126,129-131,133,134,136,137,140-142], although some found essentially equal^[55,115,116,118,120,121,124,132,135,138,139] for AQIC at a slight loss of specificity compared to conventional LBC. These reports are not summarized in a table due to the great variability in analyses and reporting as outlined previously. There would be great benefit if future publications followed a more clinically meaningful standard analysis and reporting.

One RCT conducted in China spanning some 23000 women was published and claimed substantial superiority in sensitivity and PPV comparing AQIC to conventional LBC^[133]. Unfortunately, this reviewer cannot understand the analysis done in this paper^[164] and cannot even say with confidence how many cases of cancer were detected in the study, even following clarification^[165]. The study also had some disturbing longitudinal results that were not explained. On face value, the claims made in the publication of this trial are consistent with those of the observational studies listed above.

These papers, to varying degrees, speak to three intertwined issues: (1) that AQIC is simple and effective and could be applied in low resource settings where skilled cytologists are in short supply; (2) that AQIC is a good "second opinion" or "adjunct" to add on to the conven-

tional LBC Pap test; or (3) that the combined test (AQIC positive or LBC cytology positive = case positive) is best because of the increased sensitivity. To this reviewer's knowledge, issue (1) has really not been deployed in practice in China, although there seems to be a consensus belief that it would work well; most AQIC in China is deployed in hospitals with trained cytologists. Issue (3) is connected to issue (2) but generally ignores the performance cost in combined test specificity and, as previously discussed in detail, most of these papers incorrectly calculate the test specificity. Also as discussed previously, combination tests have the potential to work best when both have very high and very similar values for, say, specificity, so that the combination will greatly improve the sensitivity but only negligibly reduce specificity, or *vice versa*. This is generally not the case for conventional cytology and AQIC, so it is more likely that it is optimal to only use the best test.

Is it necessary to do both conventional cytology and automated quantitative image cytometry? First, here is some evidence that it is not necessary to do both. The biopsy data of Tian *et al*^[82] was previously used to illustrate the unbiased method to compare two tests^[80,81] and demonstrated that AQIC compared to conventional LBC increased the true positive (TP) count at the cost of less than 1 false positive (FP) case each, which is excellent for screening. When both tests are combined as the "or" of positive results, depending on what thresholds are used, the number of TPs increased by at most 1 case (a CIN1) while the number of increased FPs range from 0-19, indicating that even the combination test provides no added value. Similarly, an earlier paper by Yu *et al*^[126] spanning almost 8000 cases, found that conventional LBC found one case (CIN2) missed by AQIC, but AQIC found 29 cases of CIN2+ missed by cytology using the ASCUS+ threshold. As expected, combining the two tests also added nothing.

The results of other papers show that doing both tests is beneficial. For example, the paper by Bao^[76], already discussed, spanned almost 20000 women and conventional LBC found 4 cases (1 CIN3 and 3 CIN2) missed by AQIC, a marginal improvement. This comes down to a question of resource availability. As mentioned in the introduction, the conclusion of our 2005 paper^[1] was that AQIC made it possible to do large scale screening inexpensively and accurately, even when skilled cytologists are not available. In that situation, doing conventional LBC is not an option.

However, when conventional LBC is an option and both tests can be performed, then the choice comes down to a mixture of the medical consequences of marginally improved sensitivity at the cost of worse specificity, combined with the business consequences, such as customer perception of value, marketing, cost, price, profit, acceptability to the payer, and so on. One Chinese vendor^[166] has developed a cytometer designed to scan counterstained slides, so that the blue thionin/counterstain amounts to a fake Papanicolaou stain. This cytometer conceptually scans the slides twice-once with

Table 15 Biopsy data for DNA Cytometry and hrHPV test results for 294 cases of Cytology ASCUS, from Ref^[169]

	hrHPV	Histology		
		Neg	CIN1	CIN2 +
hrHPV Pos	216			
Ploidy Neg		90	10	1
Ploidy Equ		22	25	8
Ploidy Pos		5	12	43
hrHPV Neg	78			
Ploidy Neg		35	0	1
Ploidy Equ		12	0	0
Ploidy Pos		14	16	0

hrHPV: High risk HPV; CIN2+: Cervical intraepithelial neoplasia, grade 2 or higher; CIN1: Cervical intraepithelial neoplasia, grade 1.

the optics set to minimize the visibility and interference of the counterstain to allow reasonably good DNA measurement, and once in full color for qualitative visual assessment by the reviewer. This system, in principle, has significant advantages over, for example, Hologic's Thin-Prep Imager which uses a similar approach^[167] to staining and imaging slides but fails to present any DNA quantitative information to the cytologist, operating instead only as qualitative assessment aid.

In summary, in most settings in China, automated quantitative image cytometry provides substantial increase in sensitivity and relatively slightly decreased specificity compared to conventional LBC. However, most labs that offer AQIC also perform conventional cytology on the same liquid based sample with the consequence of an incremental increase in sensitivity accompanied by an incremental decrease in specificity.

AQIC, cytology and hrHPV testing

Management of women with ASCUS, an equivocal cytology result and the most common non-negative result, has always been problematic. The ASCCP guidelines of 2006^[168] for the management of women with ASCUS cervical cancer screening tests called for: (1) immediate colposcopy; or (2) repeat cytology testing at 6 mo intervals until two consecutive negative follow-up Pap tests; or (3) "reflex" hrHPV testing, in which case women with hrHPV positive result are managed as if they have LSIL, or with hrHPV negative result have repeat cytology testing in 1 year.

However, these guidelines were revised in 2012^[66]. The new guidelines for management of women aged ≥ 25 with ASCUS cytology are: (1) immediate colposcopy is not recommended for women of any age; (2) hrHPV testing is preferred, in which case women: (a) with hrHPV positive result are managed as if they have LSIL, or (b) with hrHPV negative result are repeat co-tested (cytology and hrHPV) in 3 years; or (3) repeat cytology testing (not preferred) in one year, and (a) if the cytology is NILM, return to routine screening interval (3 years), or (b) if the repeat cytology is ASCUS+, attend colposcopy.

For women aged 21-24 with ASCUS, the management

is much more conservative: (1) immediate colposcopy is not recommended for women of any age; (2) repeat cytology testing in one year is preferred, and (a) if the first repeat cytology is ASC-H+, attend colposcopy, but (b) if the first repeat cytology is LSIL, ASCUS or NILM, repeat cytology in 1 year, and (i) if the 2nd repeat cytology is ASCUS+, attend colposcopy, or (ii) return to routine screening interval (3 years) when 2 consecutive annual repeat cytology test results are NILM; (3) hrHPV testing is not preferred, but women: (a) with hrHPV positive, repeat cytology in 1 year-immediate colposcopy or repeat hrHPV testing are not recommended, and (b) with hrHPV negative result are returned to the routine screening (cytology only) interval of 3 years.

A recent cross-sectional study by Zhang *et al*^[169] looked at DNA ploidy in 875 cases of ASCUS who had biopsies (of which 157 were CIN2+) and a subset of 294 of these cases (53 of which were CIN2+) were also tested for hrHPV. The raw results of the 294 cases are given in Table 15.

The performance indicators (prefaced with "b" to indicate "biopsy") for DNA ploidy for the whole data set and for hrHPV, DNA ploidy and hrHPV combined with DNA ploidy as an "and" of positive test results are given in Table 16 (the combined test result was not included in the Zhang paper). In general, DNA ploidy had better specificity and hence better PPV than hrHPV testing and both had similar NPV complement. As the authors concluded, AQIC is as effective as hrHPV testing for managing ASCUS patients, while being cheaper and easier to use.

As discussed previously, for the logical "and" of positive test results the specificity of the combined test will be at least as high as the specificity of the most specific component test and could be 100%; however, the sensitivity will be no better than the sensitivity of the least sensitive component test and could be zero. In fact, the combined test sensitivity did not fall below the least sensitive test which indicates that for detecting positive cases, DNA ploidy and HPV testing are very highly correlated. This is also an example of why it is generally the case that combining binary tests only works well if both have similarly high values for one performance indicator (either sensitivity or specificity); the idea is that this high valued performance indicator will drop only minimally when the tests are combined, while the lower valued other performance indicator will rise substantially. That is exactly what happened in this example. For reference, the PPV of cytology for all 875 ASCUS cases is only 18%.

If it is accepted that management of ASCUS will inevitably involve some equivocal cases who must be monitored over time, similar to the ASCCP guidelines above, then the 3×3 table analysis previously discussed could be applied. In this situation, only the "biopsy Positive Predictive Values" (bPPV) are revised upwards for each test, as shown in Table 17.

In the case of the combined test: (1) 60 cases would be identified as positive of which 43 will have CIN2+,

Table 16 Test performance indicators for DNA Cytometry, hrHPV tests and combined DNA Cytometry and hrHPV tests, for Cytology ASCUS cases, Ref^[169]

Performance indicator for CIN2+ (%)	875 Cases (157 CIN2+)	294 Cases (53 CIN2+)		
	DNA Ploidy	hrHPV	DNA Ploidy	DNA Ploidy and hrHPV
bSensitivity	98.8	98.1	96.2	96.2
bSpecificity	47.5	32.0	56.0	73.4
bPPV	29.1	24.1	32.5	44.3
bNPV	99.4	98.7	98.5	98.9

hrHPV: High risk HPV; CIN2+: Cervical intraepithelial neoplasia, grade 2 or higher; PPV: Positive predictive value; NPV: Negative predictive value. Prefix "b": From among biopsy cases only.

Table 17 Test performance indicators following re-analysis of the data of Table 16 from Ref^[169]

Performance indicator for CIN2+ (%)	875 Cases (157 CIN2+)	294 Cases (53 CIN2+)		
	DNA Ploidy	hrHPV	DNA Ploidy	DNA Ploidy and hrHPV
bSensitivity	98.8	98.1	96.2	96.2
bSpecificity	47.5	32.0	56.0	73.4
bPPV	40.8	24.1	47.8	71.7
bNPV	99.4	98.7	98.5	98.9

hrHPV: High risk HPV; CIN2+: Cervical intraepithelial neoplasia, grade 2 or higher; PPV: Positive predictive value; NPV: Negative predictive value. Prefix "b": From among biopsy cases only.

including 100% of the cancer cases, 88% of the CIN3 cases and 70% of the CIN2 cases, (2) 179 cases would be identified as negative, of which 2 would have CIN2+, and (3) 55 cases would be identified as being equivocal of which 8 would have CIN2+.

For comparison, if the hrHPV test was not done, the 3 state analysis outcomes would be: (1) 90 cases would be identified as positive of which 43 will have CIN2+, exactly as in the combined case above, (2) 137 cases would be identified as negative, of which 2 would have CIN2+, and (3) 67 cases would be identified as being equivocal of which 8 would have CIN2+.

The addition of the hrHPV test correctly removed 30 negative cases from the positive group and 12 from the equivocal group into the negative group.

The same research group more recently published a smaller prospective study^[170] of 327 cases of ASCUS, 193 of whom had the AQIC test and the remaining 134 had the hrHPV test. The performance indicators were essentially the same as the previous study and no statistically significant difference was detected between AQIC and hrHPV. Again, if the patient management followed the 3 × 3 table guidelines discussed previously, the PPV of DNA ploidy would have been boosted from 32% to 41%.

A tiny study from France using interactive (as opposed to automated) image analysis found similar concordance between DNA ploidy and hrHPV for ASCUS cases^[171].

A few additional studies comparing hrHPV with AQIC, but not focused on ASCUS, have been published in China^[172-174] and generally they show reasonable concordance between the two techniques but are not large

enough to draw more conclusions.

The previously mentioned study of Guillaud and co-workers^[41] compared conventional LBC (ThinPrep) with DNA ploidy and with hrHPV testing by QiaGen HC2 on more than 1500 high grade samples and concluded: "DNA ploidy shows comparable sensitivity, specificity, PPV and NPV values to conventional cytology and HC 2."

In summary, there is evidence that AQIC is at least as useful as hrHPV testing for managing ASCUS cases and probably for other abnormal cytology grades and is simpler and cheaper to perform.

CONCLUSION

Like all countries, China has low-, mid- and high-resource settings. Referring to the 2005 study objective recounted in introduction, the key resource under discussion here is the availability of experienced and highly trained cyto-technologists and cytopathologists-if they do not exist, then the conventional Pap test cannot be performed. The other key resource, of course, is money which constrains public health initiatives in all countries. This is not a discussion about the availability of electricity, water or similar resources. The objective cited in the introduction was related to this definition of low resource settings and not just in China. This review will look separately at the low resource and mid-/high-resource settings.

Training

A key element of AQIC technology is that it can be taught much more quickly than cytology and in the hands of the trainees, can perform comparably to conventional

LBC, performed by experienced and highly trained cytologists. The record in China has demonstrated that it is routinely possible to teach the technology from slide preparation and staining, to operation of the cytometer, review of the DNA ploidy data and report generation in 10 working days, especially when dedicated training facilities are available, as is the case with vendors in China. It is somewhat more difficult and inefficient to teach the technology in the end-user facility because the trainees usually have other routine work to do and so their attention is divided. Even so, this reviewer did have the opportunity to teach this technology to technicians in Morocco about 1 year ago at their facility; competition with their routine work was a big challenge, as were language and other issues, but the training was successful in a relatively short period of time (3 wk total). In this case, the trainees were experienced pathology lab technicians, so they were quick studies for sample preparation and staining. However, they had absolutely no experience in microscopy which is only done by the pathologists there and so all aspects of data and image review had to be mastered by them.

Various AQIC products from different vendors are in wide use in China in the hands of many hundreds of different operators and yet the review of publications in the previous section indicates that reasonably consistent results are being obtained under widely disparate conditions. This speaks to the inherent robustness of the DNA ploidy technology.

Low resource setting deployment

In every country, public health programs are first and foremost political initiatives, a fact that strongly shapes what is done and how. China started a “Two Cancer” screening program (cervical and breast) targeted at rural women in central and western China in 2009 that will continue until at least 2015^[175]. This is apparently a follow-on to a 2002-2010 cervical cancer screening program^[150], possibly to enjoy the anticipated high popularity and expected efficiencies of combined *vs* single disease screening^[176]. One curiosity with this initiative is that the target population is aged 30-59 for cervical cancer screening, which is appropriate, but 35-69 for breast screening by clinical breast exam^[177]; I have direct knowledge of the target age being 25-59 for breast cancer screening in Ordos under this program. Few outside of China advocate screening for breast cancer before age 50^[178] (although some, such as BCCA, initiate breast cancer screening starting at age 40^[54]) which, combined with the fact that Chinese women both inside and outside China have 1/3-1/2 the breast cancer incidence rates as other women^[179,180], has led a Hong Kong expert group, who have twice reviewed the evidence recently^[181,182], to conclude that there is insufficient evidence to recommend for or against breast cancer screening.

This reviewer has personal experience that AQIC was implemented for this program in the city of Ordos (metropolitan population 600000) in Inner Mongolia but it was required that a cytometer and support staff be

provided to each of the 8 or 10 participating hospitals rather than setting up a central lab with two technicians and only two cytometers, which would not only make QA easier to establish and maintain, but would enjoy the economy of scale that comes from a high volume, high throughput operation. The point is that the concepts of efficiency can be mixed and contradictory when public health programs are implemented because of many competing interests.

This is a point of great disappointment for this reviewer because the opportunity has so far been lost to learn in real practice how affordable cervical cancer screening could be in China. The 2012 study by Li *et al*^[176] determined the affordability limit for rural women for the “two cancer” screening to be 50 RMB, which is probably achievable with an efficient program implementation. Money remains a major constraint to this Two Cancer public health initiative^[177].

So far, China is the center of DNA ploidy application to cervical cancer screening. However, a pilot project is underway in Morocco. The first phase was to demonstrate that the technology could be taught and learned, the second phase was to compare the results with split samples and conventional LBC to determine if the technology had enough merit to move forward into phase three: the actual screening project, which is now underway. A similar project is being conducted in the Philippines^[183] but no reports on it are yet available.

Mid-and high-resource setting deployment

Most of the preceding papers reviewed are from laboratories that also perform cytology and so do not lack the key resource of trained and experienced cytologists. However, there does not seem to be the profession of “cytotechnologist” in China, except in Hong Kong^[184]. In many countries, screening by cytology is divided into 2 tasks: (1) the locator function, performed by a cytotechnologist who can sign out negative cases; and (2) the interpreter function performed by a cytopathologist who is the only one who can sign out positive cases. The locator skills of cytotechnologists are generally superior to those of cytopathologists^[185]. In China, normally these two tasks are performed by a cytopathologist. Although it is very rapidly improving, as recently as 2005 only 1/3 of licensed doctors in China held the equivalent of a bachelor's degree (only 1/8 in rural China)^[186], so there is a case to be made for AQIC technology even where cytologists are available. This is confirmed by the wide acceptance of the technology there to date.

One frustration facing the authors of most published studies in China is the previously discussed “missing data”-the inability to “close the loop” and gain access to biopsy and treatment data necessary to gauge relative success of the screening. All medical databases suffer from some “persons lost to follow-up” but there is a big difference between 60% and 6% missing data. There is no effective coordination and cooperation between the various medical providers and the various professional medical

societies and regulators seem to lack the clout to make such cooperation happen, even at the city district level, let alone the city, county, province or national level in China. There are undoubtedly many factors contributing to this situation. It is impossible to know how well any technology or program is doing if it cannot be measured. Missing data makes even cross-sectional studies, such as those reviewed here, very difficult. This situation will make longitudinal studies virtually impossible and longitudinal studies are a crucial element in the pathway to evaluating a screening strategy^[48]. Other countries, even the United States, have found ways to measure and share outcome data while still respecting patient autonomy and confidentiality. A great opportunity is being lost in China due to this lack of coordination and cooperation.

The revised ASCCP patient management guidelines^[66] were substantially informed by the remarkable database of 1.4 million women managed by Kaiser Permanente Northern California (KPNC) with both LBC and hrHPV testing from 2003 to 2010^[187-194]. This substantial longitudinal database allowed the formulation of “risk adapted” patient management guidelines. KPNC is a not-for-profit, private Health Maintenance Organization—essentially an activist insurance company that is also involved in the medical service delivery. Screening for cancer is a very easy way to spend lots of money because it involves testing people, of whom 90+% are healthy. So KPNC is highly motivated to evaluate what it does and to consider what it could do by adapting to maximize the health of its clients and minimize the cost. Perhaps, this is generalizable—that the insurer is the most motivated to effect the kind of change required to measure screening on the required scale.

FUTURE WORK

Much remains to be done, according to the framework for evaluating screening strategies outlined by Arbyn and co-workers^[48].

To this reviewer, the most exciting results to emerge from this review are the comparisons with hrHPV testing with DNA ploidy that seem to show that

DNA ploidy = LBC + hrHPV testing

The work on ASCUS needs to be repeated and expanded to all of the other categories of cytological abnormality. It would be especially interesting to conduct a study involving CareHPV^[195], the low priced hrHPV test from QiaGen for low resource settings. Combinations of DNA ploidy with HC 2 or other widely used hrHPV tests in “non-low resource” settings would also be valuable.

Much of the world has started vaccinating girls and in some cases, boys, against HPV and this is expected to expand throughout the world, including low resource countries^[196]. As the vaccinated cohorts reach screening age, existing well established cytology based screening

programs will become stressed because the frequency of abnormality will drop significantly (nominally 70% with current vaccines) which will make it increasingly more difficult for cytologists to maintain both competence and vigilance. DNA Ploidy by automated quantitative image cytometry would greatly relieve this stress.

hrHPV testing requires an accompanying “triage” test to manage the 10%-15% of women who will test positive^[197]. The ASCUS hrHPV/DNA ploidy data of Zhang *et al*^[169], reviewed above, seemed to be the ideal case for combining tests because both have comparably high sensitivity. However, in this example, both tests were made into binary tests which are constrained to increasing either sensitivity or specificity, while decreasing the other, even though both are inherently continuously valued tests. It would also be interesting to look at what happens when hrHPV and DNA ploidy are combined, not as binary tests but as continuously valued tests, for example, using Bayes’ theorem. It may then be possible to find conditions where both sensitivity and specificity increase at the same time. Nothing is free in this world, so it is unrealistic to expect that the joint improvement would exceed that of the binary test, but it might be enough to optimize the test combination for some screening applications.

The value of cervical cancer screening has been essentially limited to squamous cell carcinoma and has done little to reduce the mortality and morbidity from adenocarcinoma, which is increasing in incidence^[198-200]. It is unclear how much sampling contributes to the low sensitivity of cytology to adenocarcinoma, but studies using hrHPV testing suggest that many cases of adenocarcinoma are hrHPV positive and cytology negative, indicating that the samples are adequate^[191,201,202]. It would be interesting to compare the sensitivity for detection of adenocarcinoma for DNA Cytometry with that of conventional LBC and hrHPV testing.

A number of other issues and opportunities for further research have been brushed against in this review. The important issue of official cervical cancer incidence rates needs to be reconciled with those of the rest of the world and with the very high prevalence rates seen in many studies in China. To this end, it would be beneficial if papers more clearly defined their test populations, especially if they are an admixture of screening and diagnosis cases.

The question of whether conventional LBC done in addition to AQIC provides a net benefit remains unresolved, although it may be both vendor product and laboratory staff skill dependent.

The problem of the imperfect “gold standard” test and the possible impact, if any, that has on DNA ploidy results would be useful to know. Is there a difference in measured DNA ploidy sensitivity when the gold standard is colposcopy directed biopsy *vs* random biopsy?

Much was written here about a definition of sample adequacy but the question is not resolved. What is the best number of cells to measure to get a reliable nega-

tive test result, where “best” is a trade-off between scan time and false negative rate? How much is this influenced by sample taking? If the adequacy number is increased to, say, 50000 epithelial cells, what does this do to the diagnostic rules—that is, will 1 or 2 aneuploid cells still be “equivocal” and 3 positive? In fact, at any epithelial cell count threshold, it would be good to review all of the diagnostic rules including the proliferation rules for positive, negative and equivocal. While they seem to be working reasonably well now over a huge range of undiagnosed disease prevalence, are they optimal?

In a related matter, the concern expressed by Chatelain *et al*^[42] on HPV infection induced polyploidy may be worth looking at. That is, should cells with DI of 2, 4 or 8 be grouped separately from other DI > 2.5 cells to determine if they have different value in screening? This could be coupled to any HPV testing project.

Another similar issue is the malignant potential of aneuploid stemlines. This requires good longitudinal data and also poses possible problems with ethical approval. However, if such stemlines routinely result in negative colposcopy and/or biopsy (seen as hyperplasia), it would be interesting to follow such patients over 5 years to see how much it progresses.

Finally, one of the weaknesses in DNA ploidy by the methods described here comes from Feulgen staining which is very slow (3 to 4 h procedure) and can also be somewhat finicky. Guillaud *et al*^[203] developed a modified Feulgen process for the dye Azure A (a thiazine like thionin, methylene blue, Azure B *etc.*) that takes 30 min but produces a slightly wider diploid peak. Another rapid Feulgen reaction was reported from China^[204] but it seemed to be partly a re-discovery of acid hydrolysis at a temperature of 60°C, which was used by Feulgen^[22] originally and has been not recommended for routine work due to the need for precise control of all staining conditions. Claims are made from time to time that variations of the hematoxylin staining are quantitative, but this has been rejected experimentally by Biesterfeld^[205]. The need for an improved (faster) staining protocol remains.

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WJCO 5th Anniversary Special Issues (4): Head and neck cancer

Chemotherapy advances in locally advanced head and neck cancer

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Core tip: For select patient subsets the addition of chemotherapy to radiation in head and neck squamous cell cancer improves outcome. Most data is for concurrent cisplatin although other agents are also being explored. There has recently been interest in induction chemotherapy, the induction studies although heterogeneous have failed to show an improvement in overall survival. In this article we discuss the data regarding concurrent chemotherapy and also the data regarding induction therapy and which patient subsets we feel are best suited for induction chemotherapy (patients with N3 disease and those expected to have a delay in starting concurrent concurrent chemoradiotherapy).

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Abstract

The management of locally advanced unresectable head and neck squamous cell cancer (HNSCC) continues to improve. One of the major advances in the treatment of HNSCC was the addition of chemotherapy to radiation in the treatment of non-surgical patients. The majority of the data regarding chemotherapy in HNSCC involve cisplatin chemotherapy with concurrent radiation. However, several new approaches have included targeted therapy against epidermal growth factor receptor and several recent studies have explored the role of induction chemotherapy in the treatment of HNSCC. The purpose of this article is to provide an overview of the role of chemotherapy in the treatment of locally advanced HNSCC.

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Key words: Head and neck cancer; Chemotherapy; Induction

SOURCES AND SELECTION CRITERIA

We performed a Pubmed search for manuscripts published between 1995 and November 2013 using the following search keywords: “head and neck cancer and chemotherapy”, “head and neck cancer and radiation”, “head and neck cancer and chemoradiation”, “head and neck cancer and induction chemotherapy”, and “head and neck cancer and epidermal growth factor receptor (EGFR) targeted therapy”. The search was limited to the English language to and humans. In addition, we reviewed the references from the National Comprehensive Cancer Network (NCCN) guidelines and those from the selected publications to include landmark articles. Manuscripts were selected for inclusion based on the author’s assessment of the paper’s relevance to the topics included

in this review.

CONCURRENT CHEMORADIOTHERAPY

Head and neck squamous cell carcinoma (HNSCC) is a challenging cancer to treat and cure. Surgical management continues to be the standard of care for many HNSCC including most cancers in the oral cavity. For patients with locally advanced disease not amenable to surgical resection, concurrent chemoradiotherapy (CRT) is now recognized worldwide as a standard treatment option^[1]. Evidence has largely supported the use of radiation treatment concurrent with three cycles of bolus cisplatin^[2], although several other agents have also been studied^[3-5]. Despite improved outcomes with CRT, disease recurrence and treatment toxicity continue to be challenges with this treatment paradigm^[6]. To obtain improved outcomes and mitigate disease recurrence and treatment toxicity, new agents such as cetuximab^[7] and induction chemotherapy^[8] have been explored.

Prior to 2000, radiation alone was the predominant non-surgical treatment modality offered to patients with HNSCC. The introduction of CRT was based on several phase III trials showing a survival benefit of adding chemotherapy to radiation *vs* radiation alone in locally advanced HNSCC^[1,3-5].

A meta-analysis of 87 trials conducted by Pignon *et al*^[9] from 1965 to 2000, which included 16485 patients, found an absolute survival benefit for chemotherapy of 4.5% at 5 years and an absolute benefit for concurrent CRT of 6.5%. The hazard ratio (HR) of death was 0.81 (0.78-0.86, $P < 0.0001$). In this meta-analysis, there was a statistically insignificant benefit for induction chemotherapy with an absolute benefit of 2.4% at 5 years with a HR of death of 0.96 (0.9-1.02, $P = 0.18$)^[9]. Another important finding of this meta-analysis is that adding chemotherapy to radiation was not beneficial in certain patient subsets, an observation that would have been impossible to establish in smaller studies due to limited sample size in individual trials. These subsets included patients with Eastern Cooperative Oncology Group (ECOG) performance status 2 and 3, stage I - II tumors, and "orphan cancers", which included HNSCC outside the oral cavity, oropharynx, larynx, hypopharynx, and nasopharynx. Additionally, no survival advantage was seen in patients over age 70 when concurrent platinum based chemotherapy was administered concurrently with radiation. Analysis of various chemotherapeutic regimens showed that single agent platin was the most efficacious.

Although chemotherapy in combination with radiation improves survival in patients with locally advanced HNSCC, it does increase toxicity. Adelstein *et al*^[1] showed increased treatment related toxicities such as nausea/vomiting, leukopenia, anemia and kidney injury. In the CRT arm 77% of patients exhibited grade 3 or higher toxicity when given CRT using 70 Gray (Gy) five days per week and cisplatin *vs* 52% of patients who received radiation alone^[1].

ROLE OF EPIDERMAL GROWTH FACTOR RECEPTOR TARGETED THERAPY

EGFR is overexpressed in almost all HNSCC tumors, and overexpression of EGFR correlates with higher disease stage, lymph node metastasis, and poorer survival^[10,11]. An important breakthrough has been molecular targeted therapies to target EGFR^[12,13]. Cetuximab, a chimeric humanized monoclonal antibody against EGFR, has lead the way in targeted therapy after first receiving Food and Drug Administration (FDA) approval in 2011 in HNSCC for recurrent and metastatic HNSCC and subsequently in 2006 for locally advanced HNSCC, which we discuss below in the Bonner *et al*^[14] study. It has also received FDA approval in the first line setting for metastatic colorectal cancer in 2012. Other monoclonal antibodies and oral tyrosine kinase inhibitors, specifically erlotinib and gefitinib, have shown modest activity without a survival advantage^[13].

Prior to CRT being established as standard of care, Bonner *et al*^[14] completed a phase III study looking at the addition of cetuximab to radiation therapy in patients with locally advanced HNSCC. In this trial, 213 patients were randomized to receive either radiation therapy alone and 211 were randomized to radiation therapy given concurrently with cetuximab. Cetuximab was administered as a onetime dose of 400 mg/m² prior to starting radiation therapy, followed by 250 mg/m² weekly for the duration of radiotherapy therapy for six or seven weeks. As compared to the radiation therapy alone arm, patients who received concurrent cetuximab and radiation had a statistically significant increase in median locoregional control (LRC) of 9.5 mo (14.9 mo *vs* 24.4 mo, $P = 0.005$), progression free survival (PFS) of 17.1 mo *vs* 12.4 mo ($P = 0.006$) and overall survival (OS) of 49 mo *vs* 29.3 mo ($P = 0.03$). Toxicity was minimally increased in the cetuximab arm, with adverse events related to infusion reactions, fevers, chills, pruritus, acneiform rash, nausea, weight loss, and anemia^[14]. This trial was designed prior to adoption of CRT as standard of care for locally advanced HNSCC, and thus, the standard arm of the study was the standard treatment at that time (radiation alone).

Panitumumab, a fully monoclonal antibody, targeting EGFR was also evaluated in the treatment of HNSCC in an attempt to decrease toxicity. Concert-1, a phase II trial, randomized patients with previously untreated HNSCC in a 2:3 fashion to receive concurrent therapy using cisplatin for three doses with or without panitumumab. The primary endpoint of the study was LRC rate at 2 years. There was no statistically significant difference in LRC between the cisplatin plus radiotherapy arm (CisRT 68%) and the panitumumab plus CisRT (PCisRT 61%). Progression free survival was 35% in the CisRT arm *vs* 40% in the PCisRT group ($P = 0.61$). There was increased grade 3+ toxicity noted in the group treated with panitumumab, and this included mucositis, skin injury in the radiation field, and dysphagia^[15].

Both cisplatin and cetuximab have demonstrated survival advantages when used as single agents in combination with radiation therapy in the management of locally advanced HNSCC. RTOG 1055 was designed to evaluate whether multi-agent therapy in combination with radiation would provide added benefit. This is a phase III clinical trial in which 940 patients were randomized to receive either chemoradiation therapy with cisplatin on day 1 and 22 or the same regimen with the addition of weekly cetuximab. After a median follow-up of 2.4 years, there was no difference in progression free survival between the two arms. However, there was an increase in acute toxicity, including mucositis and skin reactions within the radiation field. Long term toxicity was similar in the two groups^[16]. While the addition of single agent cisplatin or cetuximab to radiation therapy can improve outcomes in locally advanced HNSCC, combination therapy with cisplatin and cetuximab does not improve outcomes.

While the abundance of data in HNSCC support chemotherapy using cisplatin administered at a bolus of 100 mg/m² every 3 wk during radiation, data supporting the use of cetuximab is confined to one phase III study. Additional studies are needed to directly compare concurrent chemoradiation with cetuximab *vs* cisplatin. RTOG 1016 is such a trial in progress which is comparing radiation with cetuximab to radiation with cisplatin in patients with human papillomavirus (HPV) positive HNSCC of the oropharynx. It will be several years before survival data becomes available^[17].

CHEMOTHERAPY AND RADIATION SCHEDULES

In addition to advancements in chemotherapy, there have been significant improvements in radiation treatment and delivery. RTOG 9003 showed that accelerated radiation, given over six weeks rather than seven weeks, was associated with better locoregional disease control at five years than standard radiation, although more toxic^[18]. Despite improvements in disease control, several studies have shown that accelerated radiation schedules are not a substitute for chemotherapy^[3,4]. When patients receiving concurrent chemotherapy were randomized to standard *vs* accelerated radiation, there was no benefit in the accelerated radiation arm^[5]. Therefore, conventional radiation is preferable to accelerated radiation when administered concurrently with chemotherapy.

SEQUENTIAL THERAPY: THE ROLE OF INDUCTION THERAPY

In an attempt to improve distant disease control and overall survival in unresectable locally advanced HNSCC, induction chemotherapy has emerged over the last decade as an alternative treatment modality. In the meta-analysis by Pignon *et al*^[9], 31 induction chemotherapy trials that included 5311 patients showed that induction chemotherapy

did not have a statistically significant improvement in survival with a [HR of 0.96 (0.9-1.02), *P* = 0.18]. On the other hand, induction chemotherapy did show a greater benefit in regard to distant disease control at 3.5% [HR = 0.73 (0.61-0.88), *P* = 0.001] *vs* 2.9% for concurrent platinum and 5FU studies [HR = 0.88 (0.77-1.00), *P* = 0.04]. The comparison of the two hazard ratios was insignificant (*P* = 0.12 for all trials, *P* = 0.56 for 5-FU-platin trials)^[9].

Two large subsequent clinical trials evaluated the addition of Taxotere[®] (docetaxel) to an induction regimen using cisplatin and fluorouracil in locoregionally advanced HNSCC. The TAX 324 study compared induction therapy with docetaxel, cisplatin, and fluorouracil (TPF) to cisplatin and fluorouracil (PF), followed by chemoradiotherapy. In this trial, 501 patients were randomly assigned to receive induction chemotherapy with either TPF or PF administered every 3 wk for 3 cycles. Both groups were subsequently treated with concurrent chemoradiotherapy using weekly carboplatin at an area under the curve (AUC) of 1.5. Radiation was administered to a total of 70 to 74 Gy. After a minimum follow up of 2 years, the survival benefit was significant in the TPF group with a hazard ratio for death of 0.7 (*P* = 0.006). The median overall survival was 71 mo for the TPF group *vs* 30 mo in the PF group (*P* = 0.006). There was also better LRC for the TPF group (*P* = 0.04)^[19].

Additionally, the TAX 323 study compared induction therapy with TPF to PF followed by radiotherapy alone. In this European trial, 358 patients were randomized to receive induction chemotherapy with TPF *vs* PF every 3 wk for four cycles followed by radiotherapy alone administered on different schedules (conventional, accelerated, hyperfractionated) to 66-74 Gy. After a median follow-up of 32.5 mo, there was a 2.8 mo progression free survival benefit in the TPF group. The HR for disease progression or death in the TPF group was 0.72 with a *p* value of 0.007. The main toxicity associated with the TPF regimen in both the TAX 323 and the Tax 324 was leukopenia and neutropenia^[20].

In 2010, Paccagnella *et al*^[8] reported a phase II study comparing concurrent therapy to sequential therapy using TPF as induction. One hundred and one patients were randomized to receive concurrent chemoradiotherapy *vs* induction chemotherapy (TPF) followed by concurrent treatment. The primary end point of the trial was radiologic complete response (CR) rate, evaluated 6-8 wk after the completion of concurrent therapy. This study showed superiority of sequential chemotherapy with CR of 50% compared to 21.2% (*P* = 0.004). Although the study was not powered for assessing PFS and OS, there was a 13.6 mo and 9.2 mo PFS and OS advantage respectively when induction chemotherapy was used, without an increase in toxicity^[8].

Additional data regarding the use of induction therapy is provided by two recently completed phase III studies. The PARADIGM trial randomized patients to concurrent chemoradiotherapy *vs* sequential therapy. The study was halted early due to slow accrual with only

Table 1 DeCIDE and PARADIGM Protocols

	DeCIDE	PARADIGM
Stages	IV	III, IV
Arm 1 (standard)	CRT CRT: five 14 d cycles of docetaxel (day 1), fluorouracil (day 0-4) and hydroxyurea (day 0-4) with twice daily radiation (day 1-5)	CRT cisplatin (100 mg/m ²) Q 3 wk, 12 cycles Radiation: accelerated concomitant boost over 6 wk (72 Gy)
Arm 2 (experimental, induction)	TPF (2) → CRT TPF two cycles: docetaxel 75 mg/m ² day 1, cisplatin 100 mg/m ² day 1, fluorouracil 1000 mg/m ² per day continuous for 5 d CRT: five 14 d cycles of docetaxel (day 1), fluorouracil (day 0-4) and hydroxyurea (day 0-4) with twice daily radiation (day 1-5)	TPF (3) → CRT TPF three cycles: docetaxel 75 mg/m ² , cisplatin 100 mg/m ² day ¹ , fluorouracil 1000 mg/m ² continuous for 4 d Responders to induction: CRT (carboplatin AUC 1.5, weekly) Poor responders to induction CRT (docetaxel 20 mg/m ² weekly) Radiation: accelerated concomitant boost over 6 wk (72 Gy) for poor responders. Induction chemotherapy responders 70 Gy over 7 wk

¹Statistically significant. CRT: Chemoradiation.**Table 2 DeCIDE and PARADIGM results**

Study	Patients	Randomization (induction regimens)	PFS (3 yr)	OS (3 yr)	DM
PARADIGM	145	CRT	69%	78%	11%
		TPF (3) → CRT	67%	73%	7%
DeCIDE	280	CRT	59%	73%	19% ¹
		TPF (2) → CRT	67%	75%	10% ¹

¹Statistically significant. CRT: Chemoradiation; DM: Distant metastasis.

145 out of the originally planned 330 patients accrued. Patients were randomized in a 1:1 fashion to induction therapy using TPF × 3 followed by concurrent therapy using either weekly carboplatin and conventional radiation or weekly docetaxel and accelerated boost radiotherapy (Arm A) or accelerated boost concurrent therapy using bolus cisplatin × 2 (Arm B). This study allowed for post-induction chemotherapy to be based on response to induction chemotherapy. Patients with poor response including progression of disease, not completing all cycles of TPF, gross disease left at primary site after induction, lymph nodes > 2 cm after induction, or partial response with biopsy proven residual at primary were subsequently treated with weekly docetaxel (20 mg/m²) and accelerated radiation whereas induction chemotherapy responders had weekly carboplatin (AUC 1.5) and conventional radiation as illustrated in Table 1. The primary endpoint was overall survival. After a median follow-up of 49 mo, three-year survival was excellent in both arms, 78% in the concurrent therapy arm *vs* 73% in the sequential therapy arm ($P = 0.77$) as shown in Table 2. The secondary end point of the study, progression free survival was not statistically significant at 69% in the concurrent therapy arm *vs* 67% in the induction therapy arm, $P = 0.82^{[21]}$. There was no significant difference in acute toxicity and evaluation for late toxicity is ongoing.

The DeCIDE protocol by Cohen *et al*^[22] randomized patients to concurrent CRT using 5 d of docetaxel, 5-FU, and hydroxyurea and radiation given twice daily at 1.5 Gy per fraction followed by a 9 d break *vs* two cycles of TPF followed by the same CRT regimen as demonstrated in

Table 1. Of note, radiation in this study was delivered *via* a split course, considered the standard at University of Chicago Medical Center, though this is not often used outside that institution. The study was able to recruit 280 out of 400 patients originally planned. The primary end point of the study was overall survival. After a three years of follow-up, the overall survival was 73% for the CRT arm *vs* 75% for the induction chemotherapy arm ($P = 0.70$). In terms of secondary end points, progression free survival was 59% for the CRT arm *vs* 67% for the induction therapy arm, not statistically significant with a P value of 0.18. Cumulative incidence of distant failure was 19% in the CRT *vs* 10% in the induction therapy arm, and this was statistically significant in favor of induction chemotherapy with a P value of 0.025 as noted in Table 2^[22].

DISCUSSION

Concurrent CRT is superior to radiation alone for a selected group of patients with unresectable HNSCC, including patients with stage III and IV disease, younger than 70 years of age, and who have an ECOG performance status of 0-1. The chemotherapy regimen with the most evidence is three cycles of single agent cisplatin, although other agents have also been studied and have demonstrated efficacy, such as cetuximab. In order to further improve on these results several studies have examined induction chemotherapy.

The TAX 324 and TAX 323 trials clearly demonstrated superiority of the TPF induction regimen over PF

Table 3 Tax 323 and 324 results

Study	Patients	Randomization (induction regimens)	PFS (mo)	OS (mo)	DM (%)
Tax 323	358	PF	8.2 ¹	14.5 ¹	10.3
		TPF	11 ¹	18.8 ¹	12.9
Tax 324	501	PF	13 ¹	30 ¹	9
		TPF	36 ¹	71 ¹	5

¹Statistically significant. PFS: Progression free survival; OS: Overall survival; DM: Distant metastasis.

in the management of locoregionally advanced HNSCC. However, neither study addressed or included a control arm of concurrent chemoradiation therapy, which is the current standard of care^[20] (Table 3). Though these studies resulted in FDA approval of docetaxel as part of induction chemotherapy, they were criticized for comparing two experimental regimens, rather than comparing them to the accepted standard of care^[23,24]. It remains unknown if induction chemotherapy is more effective than concurrent CRT in the treatment of local advanced, unresectable HNSCC.

The notion that induction chemotherapy reduces distant metastasis and thus improves overall survival seems compelling; however, the induction studies to date do not support this. There was no difference between the groups in rates of distant metastasis in the PARADIGM study. In the DeCIDE study, there was a decrease in distant metastasis from 19% to 10%; however, the study failed to show an improvement in OS or distant failure free survival (DFFS) for the induction arm. Both PARADIGM and DeCIDE trials were limited by several factors. Both studies had accrual difficulties, which caused each to close prior to planned accrual. The difficulty with accrual was due to competing trials in the United States at that time, patient preference, and strong physician preferences within the community. A strong pre-existing preference in regards to induction chemotherapy for more advanced disease might have created a selection bias against the risk of randomization to chemoradiotherapy alone^[21]. Additionally, the importance of HPV was not known when these studies were initiated. An increasing number of new HNSCC cases are HPV positive which has an improved prognosis. As a result of growing number of HPV related oropharyngeal cancers, the overall outcome was better than expected for both studies. This limited the study's power to detect differences in the treatment arms, and would have meant that even larger numbers would have been required in what was already a poorly accrued study. All current HNSCC studies stratify by HPV status because of the significantly improved outcome for HPV positive patients^[25].

Even with the limitations of these studies, it is clear that there is no improvement in PFS or OS to using induction chemotherapy, as opposed to CRT for all patients. It remains unclear from the current data if there is a subset of patients who may benefit from induction chemotherapy. One of the advantages of induction therapy is the theoretical ability to eliminate systemic micro-

metastatic disease and thus prevent distant failure^[26]. The TAX 324 study noted a trend towards improved distant metastasis rates with TPF *vs* PF induction therapy (5% DM with TPF *vs* 9% with PF, $P = 0.14$). TAX 323 did not confirm this trend. The PARADIGM study showed DM rates of 7% and 11% using induction and concurrent therapies, respectively, and this did not reach statistical significance. The DeCIDE study did show a reduction in DM using induction (10%) as compared to concurrent treatment (19%). There were subtle differences in study design that could have accounted for the discrepancy in reduction in rates of distant metastasis between the two studies. The PARADIGM study allowed stage III patients to enroll whereas DeCIDE was limited to stage IV. The inclusion of lower stage patients may have accounted for patients with less distant metastases in the PARADIGM study. Additionally, the CRT regimens were different in the two studies. It is possible that induction chemotherapy is more useful in split course radiation and is not beneficial in conventional RT.

Induction chemotherapy has theoretical advantages in terms of reducing distant metastasis and may be useful in patient subsets at increase risk for distant metastasis such as those with bulky, or lower cervical lymph node involvement. Induction chemotherapy may also be useful for patients who would have a delay in starting concurrent CRT. For example, it is common practice to have dental extractions of diseased teeth prior to starting radiation in order to help prevent osteoradionecrosis. After the dental extraction, it takes 2 wk for the extraction site to heal enough to begin radiation. Thus, if patients are expected to have long delays in starting CRT secondary to getting dental consult, extractions, and post-extraction healing, it may be beneficial to start induction chemotherapy while the dental issues and radiation planning is under way. At the current time, further clinical trials will be helpful in refining the role of induction chemotherapy in those subsets of patient with HNSCC most likely benefit from this treatment. Several studies have found that pre-treatment PET scans may also help guide therapy. Independent studies have shown that HNSCC lymph node SUV greater than 10 is predictive for higher rates of distant metastasis^[27]. This or other predictive tests may help determine risk for distant metastasis and potentially select patients to benefit from induction chemotherapy.

The optimal induction regimen is unclear from the limited studies performed. As seen in Tables 1 and 4, there are many differences in both induction and post-

Table 4 Tax 323 and 324 Protocols

	Tax 323 study	Tax 324 Study
Stages	III, IV	III, IV
Induction therapy regimens	TPF: docetaxel 75 mg/m ² day 1, cisplatin 75 mg/m ² day 1, fluorouracil infusion 750 mg/m ² per day continuous infusion day 1 to 5 PF: cisplatin 100 mg/m ² day 1, fluorouracil 1000 mg/m ² continuous infusion days 1 to 5	TPF: docetaxel 75 mg/m ² day 1, cisplatin 100 mg/m ² day 1, fluorouracil 1000 mg/m ² per day, continuous 24 h IV infusion for 4 d PF: cisplatin 100 mg/m ² day 1, fluorouracil 1000 mg/m ² per 24 h continuous infusion for 5 d
Concurrent therapy regimens	Start 4-7 wk after completing induction therapy: Radiation administered over 7 wk, either conventional (66 to 70 Gy), accelerated (70 Gy) or hyperfractionated (74 Gy)	Start 3-8 wk after completing induction therapy: Radiation 2 Gy per day, 5 d a week for a total of 70-74 Gy plus weekly carboplatin AUC 1.5

Gy: Gray; TPF: Docetaxel, cisplatin, and fluorouracil; PF: Cisplatin and fluorouracil.

Table 5 Concurrent regimens after induction

Study	Concurrent chemotherapy	Radiation	Notes
Tax 323	None	Conventional (66 to 70 Gy), accelerated (70 Gy) or hyperfractionated (74 Gy)	Only study not to use concurrent chemotherapy
Tax 324	Carboplatin AUC 1.5, weekly	Radiation 2 Gy per day, 5 d a week for a total of 70-74 Gy	
PARADIGM: responders	Carboplatin AUC 1.5, weekly	70 Gy over 7 wk	Regimen varied by response to induction
PARADIGM: non-responders	Docetaxel 20 mg/m ² weekly	Accelerated concomitant boost over 6 wk (72 Gy)	Regimen varied by response to induction
DeCIDE	CRT: five 14 d cycles of docetaxel (day 1), fluorouracil (day 0-4) and hydroxyurea (day 0-4)	Twice daily radiation (day 1-5)	Split course radiation

induction treatment regimens. As summarized in Table 5 there is evidence to support a wide range of post-induction chemoradiation regimens including docetaxel, carboplatin, radiation alone and the more complex split-course poly-chemotherapy University of Chicago regimen.

CONCLUSION

Chemotherapy is an important component in the treatment of local advanced HNSCC in selected patients. Most published chemotherapy data supports the use of bolus cisplatin given concurrently with radiation. Newer data supports targeted therapy with cetuximab as well. The role of alternative chemotherapy regimens is less clear. Studies looking at induction chemotherapy did not reveal a survival benefit to induction chemotherapy although it is possible that patients at increased risk for distant metastatic disease may benefit from induction chemotherapy, future studies will need to be performed to further clarify which patients are best suited to an induction chemotherapy approach.

While waiting for further data to help pick ideal patients for induction chemotherapy, at our institution we currently recommend induction chemotherapy for patients with N3 disease and patients who are expected to have a delay in starting concurrent CRT. We feel that this patient subset is most likely to benefit from induction therapy.

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Radiation-induced sarcomas of the head and neck

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Abstract

With improved outcomes associated with radiotherapy, radiation-induced sarcomas (RIS) are increasingly seen in long-term survivors of head and neck cancers, with an estimated risk of up to 0.3%. They exhibit no subsite predilection within the head and neck and can arise in any irradiated tissue of mesenchymal origin. Common histologic subtypes of RIS parallel their de novo counterparts and include osteosarcoma, chondrosarcoma, malignant fibrous histiocytoma/sarcoma nitric oxide synthase, and fibrosarcoma. While imaging features of RIS are not pathognomonic, large size, extensive local invasion with bony destruction, marked enhancement within a prior radiotherapy field, and an appropriate latency period are suggestive of a diagnosis of RIS. RIS development may be influenced by factors such as radiation dose, age at initial exposure, exposure to chemotherapeutic agents and genetic tendency. Precise pathogenetic mechanisms of RIS are poorly understood and both directly mutagenizing effects of radiotherapy as well as changes in microenvironments are thought to play a role. Management of RIS is challenging, entailing surgery in irradiated tissue and a limited scope for further radiotherapy and chemotherapy. RIS is associated with significantly poorer outcomes than stage-matched sarcomas that arise independent of irradiation

and surgical resection with clear margins seems to offer the best chance for cure.

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Key words: Post-irradiation; Nasopharyngeal carcinoma; In-field; Radiotherapy; Head and neck cancer

Core tip: Radiotherapy is an important modality in the curative management of head and neck carcinoma. However, it is also associated with significant morbidity. Radiation-induced second malignancies, particularly radiation-induced sarcomas (RIS), are arguably the most devastating sequelae associated with radiotherapy. This review examines the common trends, pathophysiology, clinical presentation, diagnosis and management of RIS in head and neck cancers.

Thiagarajan A, Iyer NG. Radiation-induced sarcomas of the head and neck. *World J Clin Oncol* 2014; 5(5): 973-981 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i5/973.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i5.973>

INTRODUCTION

Radiotherapy is a commonly used in a curative setting to treat head and neck cancers, being utilized in both definitive as well as adjuvant settings. With prolongation of survival amongst head and neck cancer patients stemming from advances in therapeutic regimens and improvements in general oncologic care, attention to treatment-related morbidity becomes increasingly important. Radiation-induced second malignancies, in particular radiation-induced sarcomas, are arguably the most devastating of the late complications of radiotherapy (Table 1). With improved oncologic outcomes, post-irradiation sarcomas are increasingly seen in long-term survivors of head and neck cancers with an estimated risk of up to 0.3%^[1,2].

Table 1 Summary of key findings

With improved oncologic outcomes, RIS are increasingly seen in long-term survivors of head and neck cancers
There is no subsite predilection; They can arise in any irradiated tissue of mesenchymal origin
Common histologic subtypes parallel their de novo counterparts
Imaging features of RIS are not pathognomonic but large size, extensive local invasion with bony destruction, and marked enhancement within a prior radiotherapy field are suggestive of a diagnosis of RIS
RIS development may be influenced by factors such as radiation dose, age at initial exposure, exposure to chemotherapeutic agents, and genetic tendency
Precise pathogenetic mechanisms of RIS are poorly understood
Management is challenging, entailing surgery in irradiated tissue and limited scope for further radiotherapy and chemotherapy
RIS is associated with significantly poorer outcomes than stage-matched de novo sarcomas
Surgical resection with clear margins appears to offer the best chance for cure

RIS: Radiation-induced sarcomas.

In order to established causality between radiation and sarcomagenesis requires the the following conditions: (1) the sarcoma should arise within the irradiated field (in the area encompassed by the 5% isodose line); (2) the sarcoma must be histologically distinct from the index lesion; and (3) there must be a latency of several years between radiation exposure and subsequent diagnosis of the sarcoma^[3,4]. This time interval is necessary to differentiate post-irradiation sarcomas from sporadic sarcomas that may have predated radiation therapy. However, the best interval to establish this distinction continues to be a subject of debate: The original stipulation for this latent period was 5 years or longer. Subsequent modifications have seen a reduction in this time interval ranging from 6 mo to 4 years^[5-7]. For post-irradiation head and neck sarcomas, arbitrary time frames of 3-4 years have been used as cutoffs based on a loose consensus that this was a sufficient gap for radiation carcinogenesis to occur^[8,9]. Finally, patients with inherited syndromes that predispose to sarcomas even in the absence of radiation such as Li-Fraumeni or Rothmund-Thomson are generally excluded from the Radiation-induced sarcomas (RIS) subgroup of patients as defined above.

Squamous cell cancers comprise the commonest histologic sub-type of radiation-induced malignancy occurring in the head and neck region. RIS is the second most common, accounting for approximately 12% of radiation-induced malignancies; lifetime risk has been estimated to be 0.03%-0.3% in patients who have been previously radiated. Radiation-induced sarcomas exhibit no predilection for any single subsite within the head and neck. They can arise within any irradiated tissue of mesenchymal origin and as connective tissue is ubiquitous, any site within the head and neck can be a primary site for RIS. In one of the larger series of post-irradiation sarcomas of the head and neck recently published by our institution, the most common subsite was found to be the nose and paranasal sinus region, consistent with the fact that the vast majority of our cases (greater than 80%) were seen in nasopharyngeal carcinoma survivors^[10]. This finding has been replicated in a few other studies from China^[11]. That said, these data represent the spectrum of RIS observed in regions where nasopharyngeal carcino-

ma is endemic and should not be generalized to all post-irradiation sarcomas of the head and neck.

RIS include osseous and soft tissue sarcomas, and the vast majority are high-grade^[12,13]. The most common histologic subtypes of RIS parallel their de novo counterparts and include osteosarcoma, chondrosarcoma, malignant fibrous histiocytoma/sarcoma nitricoxide synthase, and fibrosarcoma. Other histologies encountered include rhabdomyosarcoma (particularly in children), angiosarcoma, synovial sarcoma, and malignant peripheral nerve sheath tumors^[1,2,9]. In our series, the commonest RIS subtype was sarcoma NOS and this is in keeping with much of the published literature on post-irradiation sarcomas of the head and neck.

In general, the imaging features of RIS are not pathognomonic and are often indistinguishable from those of sporadic sarcomas or recurrent primary tumors. However, the large size, extensive local invasion with bony destruction, marked enhancement within a prior radiation therapy field, and an appropriate latency period, suggests a diagnosis of RIS^[14,15].

The development of radiation-induced sarcomas may be influenced by factors such as dose, age at initial exposure, exposure to chemotherapeutic agents, and genetic tendency. As radiation carcinogenesis is a stochastic late effect, there is no "safe" or threshold dose below which RIS are not seen; In fact, RIS have occurred at doses less than 15Gy^[16,17]. However, the risk of RIS does appear to increase with increasing radiation dose^[2,18,19]. That said, there is some uncertainty about the shape of the dose-response curve at high radiation doses. RIS is generally thought to occur at doses that induce sublethal damage in normal tissues resulting in mutagenic responses and disorganized reparative proliferation and ultimately, tumor induction. Hence, some have postulated a downturn in RIS risk at ultra-high radiation doses where lethal damage predominates but a recent systematic review of the epidemiologic studies evaluating patterns of secondary malignancy risks after high-dose fractionated radiation therapy showed no clear evidence of nonlinearity in the dose-response in the direction of a reduction in risk even at very high doses, *i.e.*, 60Gy or higher^[20].

Greater risks for secondary sarcomas have been asso-

ciated with younger age at initial diagnosis. In the Childhood Cancer Survivor Study, the risk of RIS was more than nine-fold higher amongst childhood cancer survivors when compared with the general population, with highest risk observed in patients younger than four years of age at the time of primary cancer diagnosis^[21]. The reasons for these observed variations in susceptibility to RIS with age are not well understood and may be related to biology and not just longer follow-up times after treatment. Plausible explanations for this phenomenon include higher numbers of stem cells in irradiated tissues at a young age or their high proliferative rates, rendering them more sensitive to the tumorigenic effects of radiation. In addition, the microenvironmental constraints which inhibit proliferation of initiated cells may be less effective in some organs during youth and promotion by growth hormones is likely to be greater during youth. Finally, many cases of childhood cancer involve a germline mutation, and the distinct possibility exists that this mutation may include an increased sensitivity to radiation-induced cancer.

Radiotherapy with adjuvant chemotherapy is associated with higher relative risk of RIS in children. Alkylating agents and anthracyclines have been particularly implicated in this regard. They appear to increase RIS risk by a factor of 4 or more in some studies, after adjusting for radiation therapy, with risk increasing with cumulative drug exposure^[22,23]. Whether chemotherapy also potentiates the tumorigenic effects of RT in adults is less clear.

In addition, it has been postulated that the use of newer radiation techniques such as intensity-modulated radiation therapy (IMRT) may result in an increase in radiation-induced second malignancies. The reasons for this are twofold: First, IMRT involves the use of more fields compared to three-dimensional conformal radiation therapy, and as a consequence, the integral dose to the patient is higher, *i.e.*, a larger volume of normal tissue is exposed to lower doses of radiation. Second, delivery of a specified dose to the isocenter from a modulated field, delivered by IMRT, will require the linear accelerator to be energized for longer (*i.e.*, more monitor units are needed) compared with delivering the same dose from an unmodulated field. It therefore follows that the total body dose due to leakage radiation will be increased^[24,25]. That said, radiation-induced sarcomas are thought to be primarily a complication of high-dose radiation, rarely occurring at doses below 40Gy.

Previous reports suggest that RIS develop after a median latency period of approximately 17 years, although shorter latency has been reported among pediatric patients^[26-28]. Some of these reports suggest an indirect relationship between latency and dose of radiation dose especially for doses higher than 40Gy. However this remains unproven.

PATHOPHYSIOLOGY

The precise pathogenetic mechanisms underlying suscep-

tibility to and development of radiation-induced tumors are poorly understood. The prevailing paradigm focuses on radiation-induced DNA damage leading to mutations in susceptible cells. In this regard, *p53* point mutations and genetic aberrations in the *Rb* gene have been implicated^[29-34]. However, more recent literature suggests that radiation carcinogenesis is in fact much more complex. In addition to the directly mutagenizing effects of radiotherapy, changes in microenvironments are thought to play a critical role in tumorigenesis. Several studies have demonstrated that irradiated microenvironments can independently promote genomic injury in stem cells and enhance the expression of a neoplastic phenotype^[35].

In addition, there is mounting evidence that radiotherapy can influence cell function in non-targeted tissues in diverse ways. The bystander effect, which has been observed after radiation and chemical exposures, refers to a setting in which untreated cells demonstrate abnormalities mimicking exposure, such as chromosomal instability after irradiation. Radiation-induced signals transmitted between irradiated (in-field) cells and neighboring unirradiated cells can promote the development of persistent reactive oxygen species in unirradiated cells and hence, tumorigenesis. The mechanisms underlying the bystander effect are not well-defined, but have been postulated to involve secretable factors such as cytokines and intercellular gap junctions^[36,37]. The radiation-induced sarcomas referred to in this review are, by definition, tumors arising within the irradiated region and as such, a discussion of the bystander effect is outside the scope of this review.

CLINICAL PRESENTATION

In general, radiation-induced sarcomas present in a similar manner to *de novo* primary sarcomas of the head and neck. However, radiation-associated tissue changes such as induration may render them more difficult to identify by physical examination.

In the vast majority of cases, these tumors manifest as a painless palpable mass. They may also present with skin changes on the scalp or face, or subsite-specific symptoms (*e.g.*, cranial nerve palsies with skull base tumors, dysphagia with oropharyngeal tumors, or hoarseness with laryngeal tumors).

As with sarcomas occurring elsewhere in the body, lymph node involvement is uncommon in RIS of the head and neck, occurring in only about 10% of patients. The most common histologic subtypes associated with nodal metastases are RMS and angiosarcoma.

Rarely, patients may present with symptoms attributable to metastatic disease, most often involving the lungs (*e.g.*, SOB, cough/haemoptysis, chest pain *etc.*).

DIAGNOSTIC AND STAGING EVALUATION

Computed tomography of the primary tumor site offers

Table 2 Advantages and disadvantages of computed tomography and magnetic resonance imaging in head and neck oncologic imaging

CT	MRI
Advantages	
Fast	Superior soft tissue resolution including better assessment of perineural invasion, intracranial extension of disease, marrow infiltration
Well tolerated	Multi-planar imaging capability, better definition of craniocaudal extent
Relatively inexpensive	Less image degradation caused by artifacts arising from dental amalgam
Provides assessment of tissue composition (vascularity, lipid content <i>etc.</i>)	Does not involve ionizing radiation
Ideal at demonstrating cortical bone erosion	Contrast material is less likely to produce allergic reaction
Disadvantages	
Involves exposure to small amounts of radiation	May take more time to perform
Inferior soft tissue resolution compared with MRI	More expensive
Higher risk of allergic reactions and nephrotoxicity associated with the use of iodinated contrast agents	Lower patient tolerance; Claustrophobic patients may need sedation
	Contraindicated in patients with pacemakers and other implanted metallic devices which may malfunction following exposure to strong magnetic fields
	More susceptible to motion artefact

CT: Computed tomography; MRI: Magnetic resonance imaging.

three-dimensional information about locoregional tumor extent, provides assessment of tissue composition (vascularity, lipid content *etc.*), and assists in directing biopsies for histopathologic confirmation, planning surgical extirpation, and guiding target delineation during adjuvant radiotherapy planning^[14,15]. However, in the head and neck region, magnetic resonance imaging (MRI)s offer several well-recognized advantages over computed tomography (CT)s (Table 2). Firstly, they provide superior soft tissue resolution compared with CTs. Secondly, their multiplanar imaging capability permits better definition of the craniocaudal tumor extent. Thirdly, while CTs are ideal at demonstrating cortical bone erosion, marrow infiltration is better appreciated on MRIs. Finally, MRIs are far less susceptible to image degradation caused by artifacts arising from dental amalgam^[38]. For these reasons, MRIs should be an integral part of the workup of RIS of the head and neck and combined CT and MRI use is ideal.

In addition to radiologic evaluation of the primary tumor site, CT of the chest should be routinely undertaken as a component of staging in light of the fact that the lungs are the predominant site of metastases for both soft tissue and bone sarcomas. Guidelines from the National Comprehensive Cancer Network also suggest either an FDG-PET scan and/or bone scan in the staging workup of bone sarcomas to evaluate the entire skeleton for the presence of skip lesions.

Head and neck sarcomas including RIS are staged using the same staging schema applied to sarcomas arising at other body sites. The staging system used for soft tissue sarcomas, rhabdomyosarcomas, and for primary bone sarcomas (both osteosarcomas and chondrosarcomas) are presented in Tables 3-5 respectively.

PATHOLOGIC FINDINGS

As previously mentioned, imaging features of RIS are

not pathognomonic and it is difficult to exclude primary tumor recurrence and occasionally even post-operative or post-radiotherapy changes when relying on imaging alone. Hence, examination of tissue is mandatory in establishing the diagnosis of a soft tissue or bone sarcoma. The diagnostic biopsy must be carefully planned to ensure that adequate tissue is obtained in a manner that does not compromise definitive therapy. Core needle biopsy is considered the preferred method to achieve an initial biopsy in most cases.

The vast majority of RIS are high-grade and display a significant degree of tumor necrosis^[12,13]. The histopathologic spectrum of RIS is broad and is considerably dependent on the nature of the reporting institutions and/or the clinical practice of the reporting physicians. For instance, many studies in this field exclude bone sarcomas, paediatric sarcomas as well as benign tumors and tumors of low malignant potential, *e.g.*, desmoids and dermatofibromasarcoma protuberans. In most reported series, the commonest histologic subtype of RIS encountered is sarcoma NOS (formerly referred to as malignant fibrous histiocytoma). Other encountered histologies include but are not limited to osteosarcoma, chondrosarcoma, fibrosarcoma, rhabdomyosarcoma (particularly in children), and Angiosarcoma^[1,2,9,10].

There are as yet no specific histopathologic criteria to guide distinction between radiation-induced sarcomas and sporadic sarcomas arising within the radiation field, although the morphology of tissues in the immediate vicinity may be suggestive if it shows radiation-related changes (*e.g.*, dense cellular fibrosis, atypical fibroblasts, alteration of the vascular architecture, and abundant fibrous stroma in the dermis adjacent to the sarcoma)^[39].

Likewise, there has been considerable interest in identifying molecular markers or genetic signatures that can differentiate between RIS and spontaneously occurring sarcomas. Radiation-induced angiosarcomas consistently

Table 3 TNM staging for soft tissue sarcoma

Primary tumor (T)				
TX	Primary tumor cannot be assessed			
T0	No evidence of primary tumor			
T1	Tumor 5 cm or less in greatest dimension			
T1a	Superficial tumor			
T1b	Deep tumor			
T2	Tumor more than 5 cm in greatest dimension			
T2a	Superficial tumor			
T2b	Deep tumor			
Regional lymph nodes (N)				
NX	Regional lymph nodes cannot be assessed			
N0	No regional lymph node metastasis			
N1	Regional lymph node metastasis			
Distant metastasis (M)				
M0	No distant metastasis			
M1	Distant metastasis			
Histologic grade (G)Δ				
GX	Grade cannot be assessed			
G1	Grade 1			
G2	Grade 2			
G3	Grade 3			
Anatomic stage/prognostic groups				
Stage I A	T1a	N0	M0	G1, GX
	T1b	N0	M0	G1, GX
Stage I B	T2a	N0	M0	G1, GX
	T2b	N0	M0	G1, GX
Stage II A	T1a	N0	M0	G2, G3
	T1b	N0	M0	G2, G3
Stage II B	T2a	N0	M0	G2
	T2b	N0	M0	G2
Stage III	T2a, T2b	N0	M0	G3
	Any T	N1	M0	Any G
Stage IV	Any T	Any N	M1	Any G

show MYC amplification, a finding not seen in primary angiosarcomas^[40]. Studies using microarray analysis have implicated mitochondrial genes and genes involved in antioxidant pathways in radiation-induced tumors, suggesting that mitochondrial dysfunction or chronic oxidative stress could play key roles in their pathogenesis^[39,41].

While promising, none of these markers are in clinical use. Most studies have used some modification of the Cahan criteria for classifying sarcomas as radiation-induced^[3]. While satisfying these criteria is likely to result in a high probability that the sarcoma is radiation related, there remains no gold standard for defining a radiation-associated sarcoma.

MANAGEMENT

Head and neck sarcomas are relatively rare clinical entities and radiation-induced head and neck sarcomas even more so. Their rarity coupled with their diversity of histologic subtypes makes rigorous clinical study difficult. As such, treatment algorithms for RIS of the head and neck are derived from retrospective case series and principles of management are drawn from those utilized to treat sarcomas at other body sites, rather than from large randomized clinical trials.

Management of these patients is complex. Surgical resection with clear margins seems to offer the best out-

comes for this group of patients. However, the confining and complex functional anatomy of the head and neck region and proximity to critical neurovascular structures makes adherence to traditional margin-driven therapy challenging even in de novo sarcomas^[5]. Treatment of RIS presents added challenges-entailing surgery in irradiated tissue and a limited scope for further radiotherapy and chemotherapy in selected sarcoma subtypes.

Not unexpectedly, RIS results in worse outcome compared to stage-matched de novo soft tissue and osteogenic sarcomas. Five-year disease-free survival rates for the former are 10%-30% compared to 54% for de novo tumors^[42]. The poorer outcomes could be due to: (1) difficulties and hence delayed diagnosis in previously radiated tissue; (2) compromised resection margins, due to proximity of the tumor to critical structures; (3) limited of treatment options in a maximally radiated field *i.e.*, technical difficulties of operating within an irradiated area, difficulties with reirradiation when surrounding normal tissues have been treated to near tolerance; (4) poor tumor sensitivity to chemotherapy; (5) the high-grade nature of the vast majority of RIS; and (6) host immunosuppression resulting from a combination of tumor related factors and previous treatment^[5,13,42-44].

That said, a noteworthy study of radiation-induced head and neck sarcomas conducted at our institution found that patients treated with curative intent had similar

Table 4 TNM staging for bone tumors other than lymphoma and myeloma

Primary tumor (T)				
TX	Primary tumor cannot be assessed			
T0	No evidence of primary tumor			
T1	Tumor 8 cm or less in greatest dimension			
T2	Tumor more than 8 cm in greatest dimension			
T3	Discontinuous tumors in the primary bone site			
Regional lymph nodes (N)				
NX	Regional lymph nodes cannot be assessed			
N0	No regional lymph node metastasis			
N1	Regional lymph node metastasis			
Distant metastasis (M)				
M0	No distant metastasis			
M1	Distant metastasis			
M1a	Lung			
M1b	Other distant sites			
Histologic grade (G)				
Grade is reported in registry systems by the grade value. A two-grade, three-grade, or four-grade system may be used. If a grading system is not specified, generally the following system is used:				
GX	Grade cannot be assessed			
G1	Well differentiated-low grade			
G2	Moderately differentiated-low grade			
G3	Poorly differentiated-high grade			
G4	Undifferentiated-high grade			
Anatomic stage/prognostic groups				
Stage I A	T1	N0	M0	G1, 2 Low grade, GX
Stage I B	T2	N0	M0	G1, 2 Low grade, GX
	T3	N0	M0	G1, 2 Low grade, GX
Stage II A	T1	N0	M0	G3, 4 High grade
Stage II B	T2	N0	M0	G3, 4 High grade
Stage III	T3	N0	M0	G3, 4 High grade
Stage IV A	Any T	N0	M1a	Any G
Stage IV B	Any T	N1	Any M	Any G
	Any T	Any N	M1b	Any G

outcomes regardless of whether they were radiation-induced or de novo sarcomas^[10]. This finding has a number of important implications. Firstly, heightened awareness of this entity and early recognition through careful surveillance of previously irradiated patients to detect tumors at an earlier stage would theoretically increase the likelihood of curative treatment. Secondly, optimal management not only demands multidisciplinary involvement of head and neck, neuro-, and reconstructive surgeons to maximize resectability, but also radiation oncologists and medical oncologists to consider the role of re-irradiation and/or adjuvant systemic therapy respectively, preferably in the context of a clinical trial.

Adjuvant radiotherapy may have a role in treatment of RIS of the head and neck, but its major limitation is the amount of prior radiation delivered in the same field. Factors that need to be considered include the previously treated volume and dose fractionation schedule, critical tissues and organs at risk, and time elapsed since the first treatment course. Reirradiation should only be considered if there are no other practical alternatives to treatment, since there is an increased risk of serious complications. General principles in patients undergoing reirradiation include the use of hyperfractionated radiotherapy regimens, use of highly conformal radiotherapy techniques such as brachytherapy, IMRT or increasingly, intensity-modulated proton therapy, use of previously unirradiated

normal tissue flaps for surgical resections, and the use of chemotherapy in association with lower-dose RT^[45]. In this regard, tertiary centers with high-volumes of head and neck sarcoma patients and extensive experience in re-irradiation are best suited to plan therapy in patients with RIS^[46].

The benefit of chemotherapy for head and neck soft tissue sarcomas after optimal local therapy is uncertain^[47]. Even for large, high-grade extremity sarcomas, the role of adjuvant chemotherapy is controversial, and existing data suggests that a survival benefit, if one exists, is small. However, there is some evidence suggesting improved local control with adjuvant chemotherapy^[48], which may be of particular relevance to head and neck sarcomas where treatment failure is usually consequent to local.

Likewise, there is little data addressing the benefit of chemotherapy specifically in RIS. Some investigators believe that chemotherapy will prove to be less effective in RIS compared with de novo sarcomas due to fibrotic changes in the previously irradiated field, thus preventing chemotherapeutic agents from reaching adequate concentrations in target organs. The contribution of chemotherapy to outcomes was addressed in a retrospective study of 80 cases of RIS treated between 1975 and 1995; the majority of analyzed cases were soft tissue sarcomas. Treatment included surgery alone, surgery plus chemotherapy, surgery plus radiotherapy with or without

Table 5 TNM staging system for rhabdomyosarcoma

Stage	Sites	Tumor stage invasiveness	T stage size	N	M
1	Orbit Head and neck Genitourinary Biliary tract	T1 or T2	a or b	Any N	M0
2	Bladder/prostate Extremity Cranial paraneural Other Δ	T1 or T2	a	N0 or NX	M0
3	Bladder/prostate Extremity Cranial paraneural Other Δ	T1 or T2	a b	N1 Any N	M0
4	All	T1 or T2	a or b	N0 or N1	M1

T: Tumor stage; T1: Confined to anatomic site of origin; T2: Extension; a: ≤ 5 cm in diameter; b: > 5 cm in diameter; N: Regional nodes; N0: Not clinically involved; N1: Clinically involved; NX: Clinical status unknown; M: Metastases; M0: No distant metastases; M1: Distant metastases present.

chemotherapy, chemotherapy alone, radiotherapy alone, and best supportive care. Overall survival was shortest in patients undergoing chemotherapy alone (median: 6 mo), and longest in those who underwent surgery alone (median: 42 mo). It was intermediate in patients who underwent surgery plus chemotherapy (median 28 mo). Interpretation of this data is limited by the retrospective nature of this study with small sample sizes and inherent selection biases, the heterogeneity of systemic agents used, as well as suboptimal chemotherapy administration often limited by performance status^[49].

While the majority of trials have evaluated the role of adjuvant chemotherapy in the management of soft tissue sarcomas, neoadjuvant chemotherapy has also been used in this setting and has several theoretical advantages: (1) tumor cytoreduction in bulky disease both to facilitate curative surgical resection and to permit smaller, less morbid surgery; (2) early treatment of micrometastases; and (3) avoidance of delay in commencement of systemic therapy due to postoperative complications. Potential disadvantages include impaired wound healing and delayed time to definitive local treatment particularly in the event that chemotherapy is ineffective. The discussion and decisions regarding neoadjuvant and adjuvant chemotherapy should be individualized and take into account factors such as patient age, comorbidities, performance status, histopathologic subtype of the sarcoma, as well as wishes of the patient. Needless to say, any systemic therapy should preferably be undertaken in the context of a clinical trial where tumor outcomes and toxicities are closely monitored.

On the other hand, there are certain clinical scenarios where the use of chemotherapy is less controversial. For instance, radiation-associated bone sarcomas are generally treated with chemotherapy in addition to surgery^[50]. Systemic therapy is also a routine component of treatment for several soft tissue sarcomas that occur predominantly

in children (*i.e.*, rhabdomyosarcoma, Ewing sarcoma)^[27]. Although these soft tissue sarcoma subtypes are particularly rare as radiation-associated sarcomas, most modern treatment plans utilize initial induction chemotherapy followed by local treatment, then additional adjuvant chemotherapy.

CONCLUSION

Since a significant proportion of head and neck cancer patients treated curatively receive high-dose radiotherapy as a component of their oncologic care, it is critical that clinicians are aware of radiation-induced sarcomas as a potential toxicity. RIS typically occurs after prolonged latent periods, occasionally spanning decades following initial radiotherapy and a high index of clinical suspicion assumes great importance in the outcome of these patients. Any suspicious masses should be biopsied, and if RIS is detected, the treatment of choice, where possible, is surgical resection with negative margins as this appears to offer the best chance for long-term survival. Adjuvant chemotherapy and re-irradiation may have a role in carefully selected cases and should preferably be undertaken in the context of a clinical trial. Future studies analyzing the genetics of RIS are also warranted to identify mechanisms responsible for sarcomagenesis and to attempt to target them in efforts to improve outcome.

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Role of FDG PET-CT in evaluation of locoregional nodal disease for initial staging of breast cancer

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Abstract

Fluorodeoxyglucose positron emission tomography/computed tomography (FDG PET/CT) is not indicated or recommended in the initial staging of early breast cancer. Although it is valuable for detecting distant metastasis, providing prognostic information, identifying recurrence and evaluating response to chemotherapy, the role of FDG PET/CT in evaluating locoregional nodal status for initial staging of breast cancer has not yet been well-defined in clinical practice. FDG PET/CT has high specificity but compromised sensitivity for identifying axillary nodal disease in breast cancer. Positive axillary FDG PET/CT is a good predictor of axillary disease and correlates well with sentinel lymph node biopsy (SLNB). FDG PET/CT may help to identify patients with high axillary lymph node burden who could then move directly to axillary lymph node dissection (ALND) and would not require the additional step of SLNB. However, FDG PET/CT cannot replace SLNB or ALND due to unsatisfactory sensitivity. The spatial resolution of PET instruments precludes the detection of small nodal metastases. Although there is still disagreement regarding the management of internal mammary node (IMN) disease in breast cancer, it is known that IMN involvement is of prognostic significance, and IMN metastasis has been associated with higher rates of distant metastasis and lower overall survival rates. Limited clinical observations

suggested that FDG PET/CT has advantages over conventional modalities in detecting and uncovering occult extra-axillary especially IMN lesions with upstaging the disease and an impact on the adjuvant management.

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Key words: Breast cancer; Fluorodeoxyglucose positron emission tomography/computed tomography; Locoregional nodal disease; Axillary lymph node; Internal mammary lymph node; Axillary lymph node dissection; Sentinel lymph node biopsy

Core tip: The presence and extent of locoregional nodal metastasis at diagnosis is the single most important prognostic factor in breast cancer. The predominant lymphatic drainage pathway from the breast cancer is toward the axilla. Fluorodeoxyglucose positron emission tomography/computed tomography (FDG PET/CT) is a good predictor of axillary disease and correlates well with sentinel lymph node biopsy (SLNB). FDG PET/CT may help to identify patients with high axillary lymph node burden who could then move directly to axillary lymph node dissection (ALND) and would not require the additional step of SLNB. However, FDG PET/CT cannot replace SLNB or ALND due to unsatisfactory sensitivity secondary to the limitation of its spatial resolution. The internal mammary node (IMN) involvement is of prognostic significance in breast cancer, and IMN metastasis has been associated with higher rate of distant metastasis and lower overall survival rates. Limited preliminary data indicated that FDG PET/CT plays a role in identification of positive IMN, and it is superior to conventional imaging modalities.

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INTRODUCTION

Breast cancer is the most common solid malignancy in women in the Western world, and a woman's lifetime risk of developing breast cancer exceeds 10%^[1]. The treatment and prognosis of breast cancer depend on the size of the lesion, pathologic grade, estrogen receptor status, locoregional nodal involvement and metastatic disease. Of these, the presence and extent of locoregional nodal metastasis at diagnosis is the single most important prognostic factor in breast cancer, which is reflected by 5-year survival rates of 99% in the absence of nodal metastasis, and 84% and 23% respectively for locoregional and distant metastatic disease^[2,3]. An increase in the number of tumor-positive axillary nodes is related to a worsened prognosis irrespective of primary tumor size. Approximately 30% breast cancer with local or locoregional-confined disease eventually relapse^[4,5]. Therefore, accurate evaluation of locoregional nodal status in breast cancer remains essential because of its implications for treatment and prognosis.

Imaging plays a critical role in the initial evaluation of breast cancer and serves as an important adjunct to surgical, pathologic and clinical staging. Several imaging modalities are used in the diagnosis and staging of breast cancer, including mammography, ultrasonography, computed tomography (CT), magnetic resonance imaging and positron emission tomography/computed tomography (PET/CT). For initial assessment of primary breast cancer, mammography is still the most used modality usually complemented with ultrasonography for the evaluation of axillary nodal involvement. Today metabolic imaging PET/CT with F18-fluoro-2-deoxy-D-glucose (FDG) has gained widespread clinical applications in oncology, and is accepted as a standard care in many malignancies. FDG is an analog of glucose and is used as a tracer of glycolysis. Malignant tissue and cells often demonstrate increased rate of glycolysis for rapid proliferation, due to increased numbers of glucose transporter protein and increased intracellular hexokinase and phosphofructokinase levels^[6]. The intensity of FDG is proportional to the rate of glycolysis, therefore is potentially semi-quantitative with standardized uptake value (SUV). It is well known that FDG PET/CT is valuable for detecting distant metastasis, providing prognostic information, identifying recurrence, and evaluating response to chemotherapy^[7]. However, knowledge of the value of FDG PET-CT for locoregional nodal staging is limited and somehow controversial. This review aims to present the role of FDG PET/CT in evaluating locoregional nodal status of breast cancer.

FDG PET/CT FOR AXILLARY LYMPH NODES

The most important prognostic factor in breast cancer is the axillary lymph node status. However, management of the axilla in patients with operable breast cancer is

still one of most controversial areas in clinical oncology. The best procedure to examine the axillary lymph nodes is still axillary lymph node dissection (ALND) or sentinel lymph node biopsy (SLNB). SLNB remains the gold standard for axillary nodal evaluation. Lymphoscintigraphy is a radionuclide imaging method to localize sentinel node in the axilla for biopsy. However, SLNB and ALNB carry significant surgical complications^[8].

The accuracy of FDG PET-CT for detecting axillary nodal lesion has been studied primarily in early-stage disease. Veronesi *et al*^[9] compared SLNB and FDG PET/CT in detecting occult axillary metastases in 236 consecutive patients. All patients with pathological confirmed cancer and negative axillae on clinical examinations underwent FDG PET/CT and SLNB. PET/CT identified positive axillary nodes in 43 patients (18.2%), 38 of these were confirmed true-positive on ALND (34) and SLNB (4). The results indicated a high positive predictive value of FDG PET/CT, and those with FDG PET positive axillae preoperatively should proceed directly to ALND without the need for SLNB. But SLNB identified 65 axillary positive cases, which was greater than the number of positive cases identified by FDG PET/CT. FDG PET/CT was unable to detect occult axillary metastases in one third of cases.

In a prospective multicenter trial, FDG PET-CT was performed in 360 women with newly diagnosed invasive breast cancer, and the PET results were compared to pathologic findings^[10]. Overall, FDG PET was 61% sensitivity and 80% specificity for axillary metastases, with a positive predictive value of 62% and a negative predictive value of 79%. Patients with false negative PET had significantly smaller and fewer tumor-positive lymph nodes than true-positive cases. The results indicated the limitation of FDG PET in detection of micrometastases and small, tumor-infiltrated axillary lymph nodes.

Another prospective study was obtained by Ueda *et al*^[11], which evaluated the utility of FDG PET/CT in combination with ultrasound for axillary staging in breast cancer. 183 patients underwent both FDG PET/CT and ultrasound within 5 wk of surgery. All patients had SLNB and/or ALND. FDG PET/CT was positive in the axillary lymph nodes in 40 (22%) patients, 34 (85%) of those were true-positive on correlation with surgical pathology. Of 143 negative PET/CTs, 25 (17%) were false-negative. The sensitivity and specificity of FDG PET/CT were 58% and 95%, respectively. The authors concluded that FDG PET/CT added incremental diagnostic confidence to axillary ultrasonography.

Kim *et al*^[12] tried to determine whether pre-surgical FDG PET/CT could be used as a guide for ALND or SLNB. 137 patients with biopsy-proven breast cancer underwent preoperative FDG PET/CTs, those who were positive in the axillae went to ALND directly, and those who were negative in the axillae proceeded to SLNB. There were 8 false negative and no false positive scans. The overall sensitivity and specificity of FDG PET/CT in predicting axillary metastases were 77% and 100%. 27

patients were spared unnecessary SLNB by using FDG PET/CT.

A study by Choi *et al*^[13] suggested that FDG PET/CT has similar sensitivity and specificity to breast ultrasonography in detecting axillary nodal lesions. One hundred and fifty-four consecutive biopsy-proven invasive breast cancer patients were enrolled in this study. All patients had preoperative FDG PET/CT and conventional image studies. Postoperative histopathologic results were used as the final standard. The sensitivity and specificity of FDG PET/CT to detect metastatic axilla were 37.3% and 95.8%, respectively; whereas the corresponding estimates of breast ultrasonography were 41.2% and 93.7%, respectively.

A meta-analysis by Cooper *et al*^[14] examined 7 studies with a combined 862 patients who underwent PET-CT for breast cancer staging. The mean sensitivity for detecting nodal metastasis was 56%. However, although the overall sensitivity of nodal evaluation in early-stage breast cancer is low, PET-CT still outperformed traditional imaging modalities.

The sensitivity of FDG PET-CT for axillary nodal metastasis depends on both axillary tumor burden and FDG avidity of primary tumor^[15,16]. A significantly higher proportion of metastases are detected in patients with aggressive histologic features.

In a study of 61 consecutive breast cancer patients by Heusner *et al*^[17], FDG PET/CT was obtained and compared to either SLNB or ALND, or both. Of 24 histologically positive axillae, FDG PET/CT predicted lymph node involvement in 17. In 14 true positive PET/CT cases, the mean diameter of the nodal lesions was 9 mm (range 4–22 mm). In 10 false negative PET/CT cases, the mean diameter of the axillary nodes was 3 mm (range 0.8–6 mm). The overall sensitivity was 58%, and specificity was 92%.

In a more recently published Ontario Clinical Oncology Group Study with 325 patient enrollments and ALND or SLNB as the gold standard following FDG PET/CT, the sensitivity, specificity, positive predictive value and negative predictive value were 23.7%, 99.6%, 95.8% and 75.4%^[18]. Using logistic regression, primary tumor size was predictive for prevalence of the nodal lesion and for PET sensitivity.

Table 1 outlines the major studies about the role of FDG PET/CT in evaluation of axillary nodal disease in breast cancer.

In conclusion, the data above indicate that positive axillary FDG PET/CT is a good predictor of axillary disease and correlates well with SLNB. FDG PET/CT may help to identify patients with high axillary lymph node burden who could then move directly to ALND and would not require the additional step of SLNB^[1]. However, FDG PET/CT cannot replace SLNB or ALND due to unsatisfactory sensitivity. False-negative FDG PET/CT in the evaluation of the axillary disease is mainly secondary to small size of the lymph nodes. A negative scan cannot exclude micrometastasis in the axilla. The spatial resolution (approximately 5–6 mm) of PET instruments

precludes the detection of small nodal metastases.

FDG PET/CT FOR INTERNAL MAMMARY LYMPH NODES

Although the predominant lymphatic drainage pathway from the breast cancer is toward the axilla, nodal metastases outside the axilla may be present in up to 56% of breast cancer^[1]. The sites of extra-axillary nodes include the internal mammary chain, interpectoral space, supraclavicular/infraclavicular region^[19,20]. In which, breast cancer drainage to internal mammary node (IMN) happens in as many as 30% of patients^[21,22]. Most of IMN metastases have concomitant axillary metastases, but 8%–10% patients with breast cancer may have IMN metastases only^[23].

The presence of IMN metastasis is classified as stage N3. IMN involvement is of prognostic significance in breast cancer, and IMN metastasis has been associated with higher rate of distant metastasis and lower overall survival rates^[24–28]. Yao *et al*^[24] examined the relationship between lymphoscintigraphic evidence of IMN drainage and survival in early stage breast cancer patients. The results show a near 3-fold increase mortality in IMN positive patients. Prognosis of patients with both axillary and IMN metastases is poor when compared with axillary nodal metastasis only^[29]. IMN involvement might predict treatment failure, early recurrence or distant metastasis in breast cancer^[30]. Multiple studies have consistently found that medial breast cancers carry a worse prognosis compared with lateral cancer, even after adjusting for other known prognostic factors. Because there is no plausible evidence that medial tumors are more biologically aggressive, it is likely that the worse outcome is a result of the un-treatment of IMN metastases, which is more common in medial cancer^[31,32].

The assessment of IMN remains a challenge. IMN metastasis is often clinically occult. Visualization of IMN drainage by lymphoscintigraphy depends on the use of peritumoral injection^[24], and drainage to IMN varies by locations of tumors^[24]. The peritumoral, or intratumoral injection of radiotracer was a useful method for identifying a significant portion of tumors that have primary IMN drainage because anatomic studies have shown that IMN is supplied primarily by retro-mammary lymphatic^[33,34]. With peritumoral or intratumoral injection technique, up to 25% IMN drainage could be identified by lymphoscintigraphy^[21,34]. However, subareolar injection, which improves axillary lymph node detection, rarely shows IMN uptake due to the superficial lymphatic channels. Although the gold standard for establishing the status of IMN metastasis is surgical, most surgeons do not routinely perform IMN sampling or dissection due to the relative inaccessibility and lack of convincing data for established survival benefit^[26,33]. In studies where biopsy was performed for hot internal mammary sentinel node on lymphoscintigraphy, tumor was detected patho-

Table 1 The studies evaluating the role of fluorodeoxyglucose positron emission tomography/computed tomography in axillary lymph nodes in breast cancer

Author	Year	Patient No.	Ref.	PET/CT Sensitivity (%)	PET/CT Specificity (%)	PET/CT PPV (%)	PET/CT NPV (%)	Conclusions
Wahl <i>et al</i> ^[10]	2004	360	ALND	61	80	62	79	FDG PET was limited in detection of micrometastasis
Veronesi <i>et al</i> ^[9]	2007	236	SLNB	37	96	88	66	High specificity of FDG PET/CT indicated that patients with positive PET should have ALND directly
Ueda <i>et al</i> ^[11]	2008	183	SLNB and/or ALND	58	95	85	83	Diagnostic accuracy of PET/CT was nearly equal to ultrasound
Kim <i>et al</i> ^[12]	2009	137	ALND or SLNB	77	100	100	94	FDG PET/CT could help to select patients for either ALND or SLNB
Heusner <i>et al</i> ^[17]	2009	61	SLNB	58	92	82	77	FDG PET/CT could not replace invasive approaches for axillary staging
Choi <i>et al</i> ^[13]	2012	154	Biopsy or additional imaging and follow-ups	37	96	83	74	FDG PET/CT could not be recommended as a primary diagnostic procedure
Groheux <i>et al</i> ^[16]	2011	70	SLNB or US-FNA	63	91	63	91	FDG PET/CT might impact cancer management in small portions of patients
Koolen <i>et al</i> ^[3]	2012	290	SLNB or US-FNA	82	92	98	53	FDG PET/CT could be recommended as a standard staging procedure
Pritchard <i>et al</i> ^[18]	2012	325	SLNB or ALND	24	100	96	75	FDG PET/CT was not sufficiently sensitive to detect positive axillary nodes

FDG PET/CT: Fluorodeoxyglucose positron emission tomography/computed tomography; PPV: Positive predictive value; NPV: Negative predictive value; ALND: Axillary lymph node dissection; SLNB: Sentinel lymph node biopsy.

logically in 8% to 27% of patients^[33]. Usually, if there is drainage to IMN, there is concomitant drainage to axillary node. In such case, general practice is surgical excision of only axillary sentinel node^[22]. Systemic treatment strategy is rarely influenced by IMN metastasis, due to concurrent axillary nodal metastasis and unfavorable primary tumor characteristics. Most of these patients would need adjuvant therapy such as chemotherapy and/or radiation and/or hormonal therapy. However, two recent European studies showed that IMN dissection improved accuracy of breast cancer staging and survival^[35,36]. The patients with IMN metastases had a better 5-year survival after IMN radiotherapy and chemotherapy^[34]. Heuts *et al*^[36] also suggested that tailored adjuvant systemic therapy and additional parasternal radiotherapy have a beneficial effect on the prognosis of these patients. However, the value of IMN radiation is uncertain and subject of debate partially due to a concern about the risk of cardiac toxicity associated with IMN radiotherapy^[36].

Although data are limited regarding the role of FDG PET/CT for IMN metastasis in breast cancer, some studies had demonstrated that FDG PET is superior to conventional diagnostic techniques in the detection of extra-axillary nodal metastases, particular to the IMN. Eubank *et al*^[37] compared the detection rates of CT and FDG PET/CT in 73 patients with recurrent or metastatic breast cancer. All patients had CT and PET/CT within 30 d of each other. The prevalence of abnormal FDG uptake in the IMN or mediastinum doubles that of abnormal CT findings in the extra-axillary nodal regions. FDG PET/CT could uncover disease in these nodal regions not detected by conventional staging methods. In

another prospective study with 154 preoperative patients with breast cancer by Choi *et al*^[13], 7 extra-axillary nodal lesions were detected by FDG PET/CT only although all patients had additional conventional imaging studies. Aukema *et al*^[38] found that in 60 breast cancer patients with tumor size greater than 3 cm and/or proven axillary nodal metastasis, pre-chemotherapy FDG PET/CT detected abnormal IMN in 8 patients. In 4 of the 8 patients, treatment planning was changed and radiotherapy was added. Koolen *et al*^[3] reported that in 310 patients with breast cancer and scheduled for neoadjuvant chemotherapy, FDG PET-CT detected 26 (8%) abnormal IMN. PET/CT findings helped selecting patients for postoperative internal mammary chain radiotherapy. Bernsdorf *et al*^[39] analyzed FDG PET/CT data of 103 consecutive patients with newly diagnosed operable breast cancer and tumors greater than 2 cm. Extra-axillary lymph node involvement was detected in the internal mammary chain by PET/CT in 10 patients, 5 of them had adjuvant treatment modifications. Recently, Wang *et al*^[40] reported the role of FDG PET/CT in the detection of IMN metastases with pathologic correlation in a large series of patients. One hundred and ten of 1259 patient had FDG avid IMNs on PET/CT. Twenty-five patients underwent ultrasound-guided fine needle aspiration of suspicious IMN based on PET/CT, and 20 IMNs (80%) were cytologically proven metastases from the primary breast malignancies. The results indicated a very high likelihood of malignant involvement of FDG avid IMNs. In another recently reported study by Koolen *et al*^[41], pre-chemotherapy FDG PET/CT scans identified IMN nodal lesions in 17 of 278 patients with breast cancer. The results show

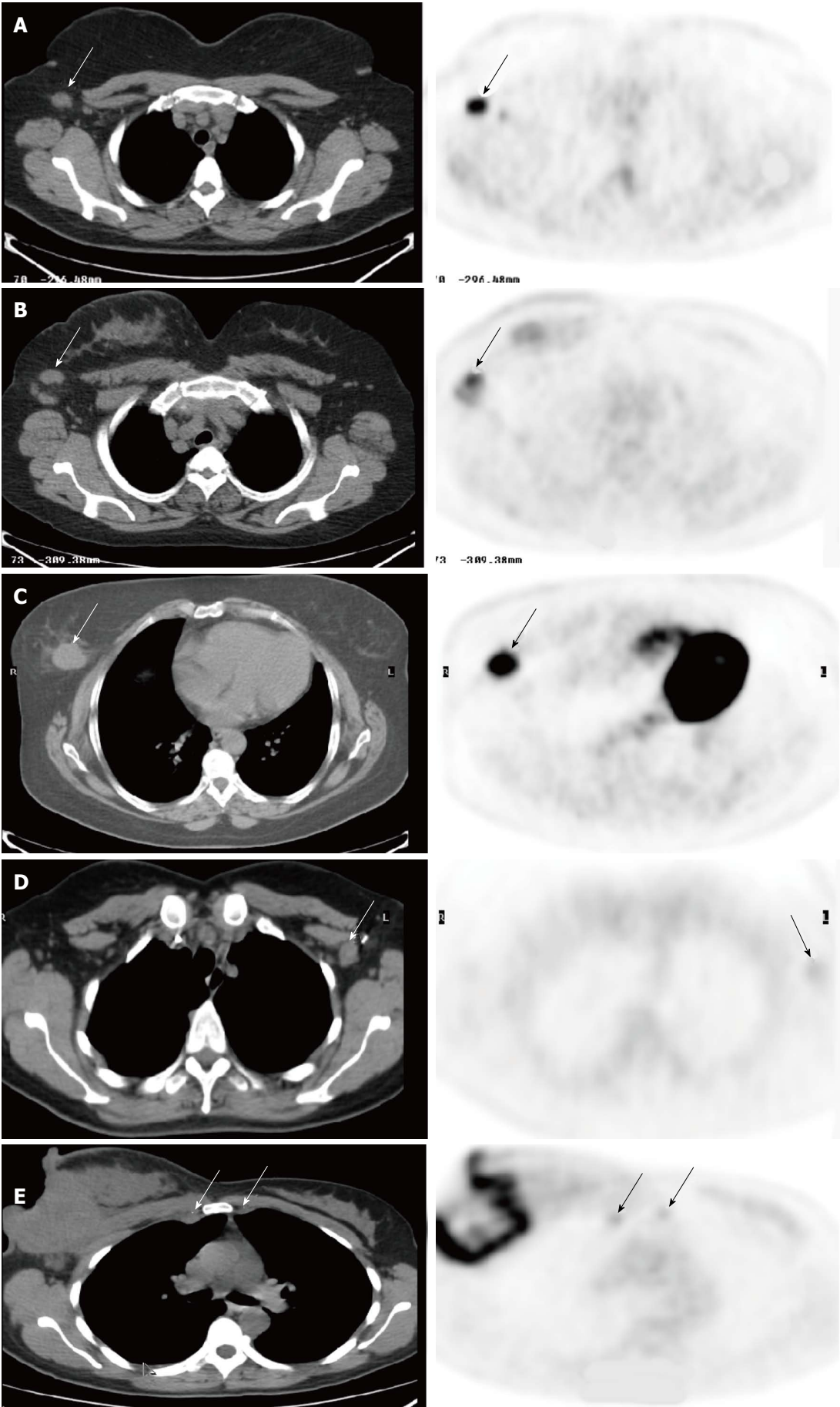


Figure 1 Fluorodeoxyglucose positron emission tomography/computed tomography. A: In a 61-year-old woman with newly diagnosed breast cancer. There was

a single palpable axillary lymph node on physical examination. The images demonstrated a 1.5 cm axillary node with intense uptake (SUV 10, arrows), consistent with metastasis. There were also additional smaller axillary nodes with abnormal uptake. The patient underwent axillary lymph node dissection (ALND), and surgical pathology revealed that two axillary nodes were positive; B: In a 51-year-old woman with newly diagnosed right breast cancer. There were palpable right axillary lymph nodes on physical examination. The images demonstrated a few enlarged right axillary nodes with intense uptake (SUV 6.5, arrows). The patient had ALND, which showed three metastatic lymph nodes; C: In a 54-year-old woman with newly diagnosed right breast cancer. The images showed a 3.0 cm fluorodeoxyglucose (FDG) avid tumor in the right breast (arrows), but were negative in the right axilla. The patient had lymphoscintigraphy for sentinel lymph node biopsy, which was negative for axillary metastasis; D: In a 40-year-old woman with history of left breast cancer and post lumpectomy. On physical examination, there was a palpable lymph node in the left axilla. Positron emission tomography/computed tomography (PET/CT) showed a 1.5 cm left axillary lymph node with very mild uptake (SUV 1.4, arrows), consistent with a benign etiology. Subsequent SLNB confirmed lymphadenitis; E: FDG PET/CT in a 41-year-old woman with newly diagnosed inflammatory carcinoma of the right breast. In addition to large right breast necrotic mass and axillary nodal lesions, there are a 1.2 cm right internal mammary node (IMN) and a 1.0 cm left IMN, and both are mildly FDG avid (SUV 3.2) and suspicious for IMN metastases (arrows). The patient was excluded as a surgical candidate based on FDG PET/CT findings. She received chemotherapy and radiation. SUV: Standardized uptake value.

that FDG PET/CT contributes to pretreatment staging and therapy planning, upstaging substantial proportion of patients to the high risk group, thereby potentially changing prognosis and possibly implicating postoperative irradiation.

In conclusion, there is a significant disagreement in the oncologic community regarding the management of IMNs in breast cancer, most likely due to the difficulties in assessment of IMN and conflicting evidence of significance of IMN treatment. Lymphoscintigraphy may help to identify the IMN drainage, but the IMN sampling or dissection is much more difficult than for axillary nodes, and only a small portion of hot sentinel IMNs are positive for metastases on pathological studies. As a non-invasive imaging modality, FDG PET/CT plays a role in identification of positive IMN, which may renew the oncologists' interest in IMN management. But to date, the data regarding FDG PET/CT for IMN in breast cancer are very limited, and more clinical studies are warranted.

CASE EXAMPLES

Figure 1 illustrate the role of FDG PET/CT in locoregional nodal staging of breast cancer. In the case 1 and 2 (Figure 1A and B), FDG PET/CT was positive in the axillae. The patients avoided SLNB and directly had ALND. Pathology confirmed true-positive PET/CT findings.

FDG PET/CT was negative in the axilla in the case 3 (Figure 1C), and suggested a benign axillary lymph node in the case 4 (Figure 1D). In spite of negative PET/CT results, both patients had SLNB due to large primary tumor (the case 3) or palpable axillary lymph node (the case 4). SLNB demonstrated negative axillary disease consistent with PET/CT findings.

In the Case 5 (Figure 1E), FDG PET/CT identified positive IMNs, which updated staging, and subsequently changed the patients' managements.

CONCLUSION

Although FDG PET/CT is valuable for detecting distant metastasis, identifying recurrence and evaluating response to chemotherapy, the role of FDG PET/CT in evaluating locoregional nodal status for initial staging of breast cancer has not yet been well-defined in clinical practice. FDG PET/CT is not recommended as a routine imaging modality for initial staging of early breast cancer^[42],

although the direct scientific evidence to support this recommendation is limited. FDG PET/CT has high specificity but compromised sensitivity for identifying axillary nodal disease in breast cancer. Positive axillary FDG PET/CT is a good predictor of axillary disease and correlates well with SLNB. FDG PET/CT may help to identify patients with high axillary lymph node burden who could then move directly to ALND and would not require the additional step of SLNB. However, FDG PET/CT cannot replace SLNB or ALND due to unsatisfactory sensitivity. The spatial resolution of PET instruments precludes the detection of small nodal metastases.

There are substantial disparities in regard to the significance of IMN in breast cancer, and evaluation of IMN status is difficult in practice. Limited Data suggested that FDG PET/CT has advantages over conventional modalities in detecting and uncovering occult extra-axillary especially IMN lesions with upstaging the disease and an impact on the adjuvant management.

Positive FDG PET/CT is highly predictive for locoregional nodal disease in locally advanced breast cancer. Although the indications and role in initial staging of breast cancer remain to be validated, FDG PET/CT can be used in concert with other imaging modalities especially for patients with high risk.

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Overcoming endocrine resistance in metastatic breast cancer: Current evidence and future directions

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Abstract

About 75% of all breast cancers are estrogen receptor (ER)-positive. They generally have a more favorable clinical behavior, prognosis, and pattern of recurrence, and endocrine therapy forms the backbone of treatment. Anti-estrogens (such as tamoxifen and fulvestrant) and aromatase inhibitors (such as anastrozole, letrozole, and exemestane) can effectively control the disease and induce tumor responses in a large proportion of patients. However, the majority of patients progress during endocrine therapy (acquired resistance) and a proportion of patients may fail to respond to initial therapy (de novo resistance). Endocrine resistance is therefore of clinical concern and there is great interest in strategies that delay or circumvent it. A deeper knowledge of the molecular mechanisms that drive endocrine resistance has recently led to development of new strategies that have the promise to effectively

overcome it. Many resistance mechanisms have been described, and the crosstalk between ER and growth factor receptor signaling pathways seems to represent one of the most relevant. Compounds that are able to inhibit key elements of these pathways and restore endocrine sensitivity have been studied and more are currently under development. The aim of this review is to summarize the molecular pathophysiology of endocrine resistance in breast cancer and its impact on current clinical management.

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Key words: Everolimus; Mammalian target of rapamycin; PI3K inhibitors; Estrogen receptor; Endocrine resistance

Core tip: Endocrine therapy forms the backbone of treatment for hormone receptor (HR)-positive metastatic breast cancer (MBC) patients. Unfortunately, resistance to endocrine agents develops in the majority of patients. A deeper knowledge of the molecular mechanisms that drive endocrine resistance has boosted the development of strategies designed to overcome resistance to endocrine therapies. In particular, co-targeting of receptor tyrosine kinase and intracellular signaling pathways (such as the PI3K-Akt-mTOR pathway) has emerged as a particularly promising strategy. We predict that the development of new drugs with a strong underlying biological rationale will quickly result in more personalized treatment of patients with HR-positive MBC and further improve outcomes.

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INTRODUCTION

Breast cancer is a leading cause of female death worldwide^[1]. There has been a continuous decline in mortality over recent years as a direct result of improvements in early diagnosis and increased availability of more effective treatments^[2,3]. However, despite these improvements, metastatic breast cancer (MBC) remains a largely incurable disease and new treatments need to prolong survival, relieve symptoms, and delay progression.

Approximately 75% of breast cancers express either or both the estrogen receptor (ER) and progesterone receptor (PgR)^[4]. Hormone receptor (HR)-positive and negative disease differ in terms of clinical behavior, prognosis, patterns of recurrence, and aggressiveness. Patients with HR-positive disease are likely to have more indolent disease, bone metastases, and late recurrences^[5]. For most HR-positive MBC patients, endocrine therapy is the preferential initial treatment and has a positive impact on survival.

Recently, a number of compounds with different mechanisms of action, low toxicity, and superior efficacy have become available for patients with HR-positive disease. Three classes of endocrine therapies are commonly used to treat HR-positive MBC: selective estrogen receptor modifiers (SERMs), such as tamoxifen, which directly bind to the ER and block its transcriptional activity; selective estrogen receptor downregulators (SERDs), such as fulvestrant, which bind to ER and induce its degradation; and aromatase inhibitors (AIs), such as letrozole, anastrozole, and exemestane, which reduce the production of estrogen *via* inhibition of the aromatase enzyme in peripheral tissues and within the tumor itself^[6].

Unfortunately, although long-term remission is possible^[7], the majority of patients develop resistance to endocrine therapy^[8]. Moreover, a proportion of patients may have primary resistance to endocrine therapy^[9]. There is therefore a lot of interest in developing strategies that delay the onset of endocrine resistance or circumvent acquired resistance to specific drugs.

It has recently been suggested that dysregulation of growth factor signaling networks and crosstalk between overexpressed growth factor receptors and ER play an important role in the endocrine-resistant phenotype^[10]. Manipulating these networks is an attractive and potentially effective strategy that aims to delay the onset, or eventually overcome, resistance to endocrine therapies.

The aims of this review are to provide an overview of the known mechanisms of resistance to endocrine therapies and to focus on emerging strategies aimed at circumventing its development.

THE BIOLOGY OF THE ER

The ER is mainly a nuclear protein that modulates gene expression *via* several different pathways. A schematic of the biology of ER signaling is presented in Figure 1.

The “classical” pathway

Estrogen is a steroidal hormone that passively diffuses

through cell membranes to enter the cell. The “classical” ER pathway is initiated by estrogen-induced dimerization of ER and subsequent binding to specific DNA promoter regions, known as estrogen response elements (EREs), which activates transcription of genes involved in promoting cellular proliferation and survival^[11]. ER can also inhibit gene expression, particularly those involved in downregulation of the cell cycle or pro-apoptotic actions. The transcriptional activity of ER is regulated by a number of co-activators (for example, members of the p160 family of nuclear receptor co-activators such as SRC1 and SRC2) that bind to ER to form large complexes^[12,13]. In breast cancer cells, SERMs such as tamoxifen lead to the formation of ER-co-repressor complexes that inhibit ER-dependent transcriptional activity to induce anti-proliferative and pro-apoptotic effects.

The “non-classical” pathway

In addition to the “classical” regulation of gene expression, ER also regulates genes that do not harbor EREs in their promoter regions in a “non-classical” manner. ER can, in fact, interact with other proteins that are known to be involved in promoting gene expression, such as Fos and Jun^[14].

Non-nuclear activities of the ER

Although the majority of cellular ER localizes in the nucleus, the ER can also localize in the cytoplasm and cell membrane, where it can interact with receptor tyrosine kinase (RTK) growth factor receptors, such as the epidermal growth factor receptor (EGFR), human epidermal growth factor receptor-2 (HER2), or insulin-like growth factor-1 receptor (IGF-1R)^[15]. In fact, the ER plays a key role in this complex intracellular signaling network and is strictly linked to other signaling networks^[16]. A complex network of bi-directional crosstalk exists at multiple levels in breast cancer cells, whereby the ER pathway and growth factor receptor signaling pathways interact and potentiate one another, resulting in dysregulated proliferation and growth^[12].

Therefore, through direct DNA binding, co-activation, or molecular crosstalk, ER can influence tumor cell proliferation, survival, and malignant progression by amplifying the intracellular proliferative signals from RTKs and their downstream effectors.

Putative mechanisms of endocrine resistance

There is strong evidence that crosstalk between growth factor receptor and ER pathways can mediate resistance to endocrine therapy. The ER exists as part of a highly complex and adaptive signaling network that enables cancer cells to escape simple perturbations, such as those presented by the currently available endocrine therapies.

For example, overexpression of members of the EGFR family of RTKs, particularly HER2, has been described as a molecular alteration that is able to confer *de novo* resistance to anti-estrogens^[12]. HER2 directly phosphorylates ER and its co-regulators, leading to enhanced

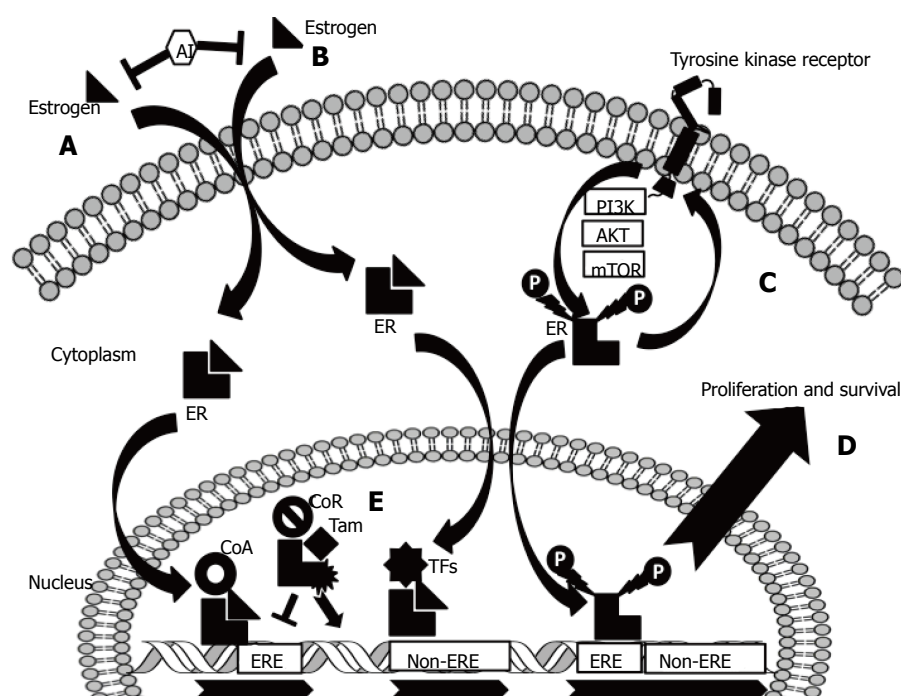


Figure 1 The biology of the estrogen receptor and a schematic representation of the key mechanisms of endocrine resistance. A: Estrogen induces gene regulation via the “classical” pathway. Estrogen passively diffuses through cell membranes and binds to the estrogen receptor (ER), inducing receptor dimerization. This complex recruits co-activators (CoA) and binds regions of DNA known as estrogen response elements (EREs), promoting transcription. Aromatase inhibitors (AIs) negatively regulate ER activity by reducing circulating estrogen levels; B: The ER can also cooperate with other transcription factors (TFs) and regulate the transcription of genes not harbouring EREs via the “non-classical” pathway; C: ER strictly interacts with receptor tyrosine kinases (RTKs) via their downstream effectors. ER can, in fact, be directly phosphorylated and activated, the final result being gene expression and a cascade of second intracellular effectors (the non-nuclear activity of ER); D: This strict and bi-directional crosstalk between ER and RTKs and downstream effectors is responsible for endocrine resistance; E: In breast cancer cells, SERMs [such as tamoxifen (Tam)] bind ER and induce the recruitment of co-repressors (CoR) that negatively regulate the activity of ER. Mutated forms of ER are able to enhance gene expression in spite of the presence of Tam.

ligand-independent gene expression, even in the presence of negative regulators such as SERMs.

There are data to suggest that patients with early breast cancers that overexpress HER2 obtain less benefit from adjuvant tamoxifen than those with HER2-negative tumors; furthermore, HER2 overexpression seems to be predictive of a poor clinical response to tamoxifen^[17,18]. EGFR overexpression is also predictive of decreased benefit from tamoxifen^[19,20] and increased risk of disease progression during anti-estrogen treatment^[21].

There is emerging evidence to suggest that long-term estrogen deprivation can directly induce the transcription of growth factor receptors such as EGFR, HER2, and IGF-1R, resulting in increased activity of their downstream mediators and increased cellular proliferation, the final result being escape from estrogen deprivation and ligand-autonomous growth^[22-24].

Another interaction that seems to be crucial in mediating resistance to endocrine therapies involves the phosphatidylinositol 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) pathway, an ubiquitous signal transduction pathway that is also interconnected with other RTKs, including, but not limited to, the EGFR family (Figure 1)^[25-27]. This pathway regulates many cellular functions, not least growth and proliferation, differentiation, metabolism, migration, and survival^[28], and it is abnormally activated in many different cancer types,

including breast cancer, in which it has an important role in the development of anti-cancer drug resistance.

Dysregulation of this pathway is crucial in the development of acquired endocrine resistance. The pathway can become activated *via* increased upstream signaling due to activation of RTKs, PI3K-activating mutations, or decreased expression of negative regulators of the pathway, such as through loss of the tumor suppressor PTEN (phosphatase and tensin homolog). For example, several studies have established a link between upregulated Akt protein expression and/or phosphorylation and resistance to endocrine therapy^[29,30], and it is known that an mTOR subunit phosphorylates and activates the functional domain 1 of the ER^[31,32].

In a preclinical study, deGraffenried *et al.*^[33] reported that breast cancer cells with high Akt activity are resistant to hormonal therapy but that sensitivity could be restored with the use of mTOR inhibitors. Furthermore, in another study of ER-positive breast cancer cells, a combination of mTOR inhibitor and letrozole acted synergistically to inhibit proliferation and trigger apoptosis^[34].

However, several other mechanisms have been described that contribute to endocrine resistance. For example, loss of ER expression in the evolution from primary to metastatic disease may contribute to the emergence of estrogen resistance; data from clinical studies suggest that 17% of ER-positive patients treated with adjuvant

tamoxifen may convert to an ER-negative phenotype at the time of relapse^[35].

Mutations in *ESR1*, the gene encoding ER, also seem to negatively affect responses to hormonal therapy^[36,37]. Recently Toy *et al.*^[36] reported frequent mutations in *ESR1* that affect the ligand-binding domain (LBD) of ER in metastatic hormone-resistant breast cancers after prolonged exposure to hormonal therapy. These highly recurrent mutations mainly affected p.Tyr537Ser, p.Tyr537Asn, and p.Asp538Gly, and as a consequence caused an agonist conformation of the receptor. In addition, they noted that LBD-mutant receptors have a hormone-independent active state that is likely to promote resistance to estrogen-depriving therapies. Interestingly, mutant ER retains some sensitivity to drugs that directly target the receptor, suggesting that more potent ER antagonists may be of substantial therapeutic benefit in this subgroup of individuals.

There may also be individual biological variability in drug metabolism that might influence responses to therapy. For example, about 8% of Caucasian women fail to convert tamoxifen to its active metabolite, endoxifen, which has been suggested to be a mechanism of *de novo* resistance^[38].

In summary, multiple complex and adaptive mechanisms contribute to the development of endocrine resistance (Figure 1). As our understanding of the mechanisms that underpin resistance improves, the goal of future studies is to prolong responses to endocrine manipulation and potentially restore endocrine sensitivity in those tumors that have become resistant, with or without drugs that target interconnected pathways. Based on this theory, we describe three different approaches to overcome endocrine resistance that have recently been explored clinically in randomized trials.

OVERCOMING ENDOCRINE RESISTANCE

Combined inhibition of the ER and RTKs

Combined inhibition with ER- and HER2-targeting agents: HER2 is amplified and/or overexpressed (positive) in around 15% to 20% of human breast cancers. Although overexpression of HER2 is a marker of aggressiveness and poor prognosis, HER2-positive cells are sensitive to anti-HER2 targeted therapy, such as trastuzumab^[39,40]. About half of HER2-positive breast cancers co-express hormone receptors and this is associated with resistance to both tamoxifen and AIs, as shown in a number of pre-clinical and clinical studies^[25]. As a result of this pre-clinical evidence, several trials have explored using a combination of endocrine and HER2-targeting agents to overcome endocrine resistance.

Specifically, three trials have been published to date. The “Trastuzumab and Anastrozole Directed Against ER-Positive HER2-Positive Mammary Carcinoma” (Tandem) phase 3 study compared anastrozole alone with the combination of anastrozole and trastuzumab as first-line treatment for patients with HER2/HR-positive ad-

vanced breast cancer^[41]. The results showed that the combination of trastuzumab and anastrozole doubled median progression free survival (PFS) (2.4 mo *vs* 4.8 mo) and significantly increased the overall response rate (ORR) (6.8% *vs* 20.3%), compared to anastrozole alone. Side effects were modest and manageable (maximum grade 2) and consisted mainly of fatigue, vomiting, diarrhea, pyrexia, and arthralgia. There was no statistically significant treatment difference in overall survival; however, this may have been due to 70% of patients in the anastrozole arm crossing over to receive trastuzumab after progression on anastrozole alone.

The “Efficacy and Safety of Letrozole Combined With Trastuzumab in Patients With Metastatic Breast” (eLEcTRA) study prematurely closed due to slow recruitment. The design was the same as Tandem but a different AI (letrozole) was prescribed^[42]. Similar to Tandem, eLEcTRA showed that the addition of trastuzumab to letrozole was associated with improved PFS and clinical benefit rate (CBR) at the cost of a modest increase in overall toxicity.

The third study was “EGF30008”, a large, phase 3, double-blind, randomized-controlled trial conducted in 1286 women with HR-positive breast cancer; they were not selected on the basis of HER2 status (of the 1286 patients enrolled, 219 had HER2-positive tumors)^[43,44]. These patients were randomized to daily oral treatment with letrozole (2.5 mg) plus the dual HER1-HER2 tyrosine kinase inhibitor lapatinib (1500 mg) *vs* letrozole (2.5 mg) plus placebo. In the ER-positive/HER2-positive population (*n* = 219), the addition of lapatinib to letrozole resulted in a significantly lower risk of disease progression than with letrozole alone. The PFS was 8.2 mo in the combined arm *vs* 3.0 mo in the placebo arm. The ORR (28% *vs* 15%) and CBR (48% *vs* 29%) were also significantly greater in lapatinib treated women. In contrast to the other two studies, the addition of lapatinib was accompanied by a significant increase in the grade 1 and 2 side effects commonly associated with dual tyrosine kinase inhibition, namely diarrhea (68%) and cutaneous rash (46%). The impact of lapatinib plus letrozole on OS has not been reported. However, based upon a clinically meaningful increase in PFS, the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have approved lapatinib in combination with an aromatase inhibitor in this setting.

As expected, the HER2-negative patients enrolled in EGF30008 derived no benefit in PFS from the addition of lapatinib to letrozole. Interestingly, however, in the sub-group of “tamoxifen-resistant” patients (*i.e.*, those relapsing during or within six months from the completion of adjuvant tamoxifen treatment), the improvement in PFS was similar to HER2-positive patients, suggesting that the disruption of crosstalk between the ER and RTK signaling pathways might restore sensitivity to anti-estrogens.

Recently, Finn *et al.*^[45] showed that weak ER expression is associated with worse outcomes for postmeno-

pausal women with advanced HR-positive disease when treated with letrozole alone compared to a combination with lapatinib. Their data suggest that the population of patients with low quantitative expression of ER within the HER2-negative population may be most likely to benefit from the addition of lapatinib to letrozole, at least in terms of PFS improvement. They hypothesize that this benefit could be related to the anti-EGFR effect of lapatinib.

In conclusion, these three trials suggest that the combination of an anti-HER2 agent and an AI has significant clinical benefit and improves PFS compared to endocrine therapy alone. No significant differences in overall survival (OS) were observed in any of the trials, possibly due to the influence of crossover and/or the number of lines of treatment received after progression. Interestingly, these three trials also confirm that HER2-positive patients have relative endocrine resistance; in fact, women receiving endocrine therapy alone had response rates ranging only from 7% to 15% and median time-to-progression (TTP) ranging from 2.4 to 3.3 mo.

These three trials provide proof-of-concept that HER2-associated endocrine resistance may be reverted by targeting HER2 and that combination therapy represents a therapeutic opportunity for patients with these particular clinicopathological features.

Combined inhibition with ER- and EGFR-targeting agents

As previously discussed, the crosstalk between the ER and EGFR has been reported to mediate endocrine resistance. Therefore, combination strategies have been evaluated in the clinic^[12,46].

Although the clinical and prognostic role of EGFR in breast cancer has yet to be fully characterized and is mainly restricted to “basal-like” tumors, a few randomized trials have explored the effect of combined ER and EGFR targeting in women with MBCs not selected on the basis of EGFR status^[47].

NCT00229697 was a randomized phase II trial that evaluated the addition of the pure EGFR inhibitor gefitinib to tamoxifen in patients with HR-positive advanced breast cancer^[48]. Patients with newly metastatic disease, or who had recurred after adjuvant tamoxifen, during/after adjuvant AI, or after first-line AI, were randomized to receive tamoxifen plus placebo or tamoxifen plus gefitinib. A trend towards benefit from the combination therapy was seen in patients with tamoxifen-sensitive disease, with an increase in median PFS from 8.8 to 10.9 mo. In the AI-resistant population, no improvement in outcome was observed.

Another randomized phase II trial (NCT00077025), presented by Cristofanilli *et al.*^[49], evaluated the efficacy and tolerability of anastrozole combined with gefitinib or anastrozole with placebo in tamoxifen-resistant women with HR-positive MBC^[49]. Unfortunately, this study was closed prematurely due to slow accrual, but the data that were gathered showed that PFS was longer in patients

receiving the combination therapy than for those patients receiving anastrozole plus placebo (14.7 mo *vs* 8.4 mo).

Both of these studies suggest that the observed benefit of EGFR inhibition can be explained by EGFR activation as a mechanism of adaptation to tamoxifen inhibition. It would be therefore interesting to explore this association in an EGFR overexpressing population, like in the neoadjuvant study published by Polychronis *et al.*^[50]. In this study, both the combination of anastrozole and gefitinib and, interestingly, gefitinib alone showed clinical activity. Although the ORR was similar in both arms, patients assigned to gefitinib and anastrozole had a greater decrease in tumor proliferation (as measured by Ki67 labeling), than those assigned gefitinib and placebo.

Combined inhibition of the ER and PI3K-Akt-mTOR signaling

Crosstalk between the ER signaling pathway and the PI3K-Akt-mTOR signaling pathway is thought to play a crucial role in the development of resistance to endocrine therapy (Figure 2). Specifically, PI3K-Akt-mTOR pathway upregulation is associated with ligand-independent activation of ER and an associated increase in expression of genes regulated by ER, albeit in the presence of anti-estrogens^[30]. Moreover, several studies have shown that this effect can be reverted using mTOR inhibitors, such as everolimus or temsirolimus^[33,34].

These data provide a strong rationale for combining agents that target this pathway and anti-estrogens in an attempt to restore endocrine sensitivity. Based on this, we present a series of clinical studies below that explore the efficacy of this approach.

Everolimus

Everolimus, the 40-O-(2-hydroxyethyl) derivative of sirolimus (a rapamycin analogue), is an oral mTOR inhibitor that binds with high affinity to its intracellular receptor FKBP12, a protein belonging to the immunophilin family. The everolimus-FKBP12 complex interacts with mTOR to inhibit downstream signaling^[51,52].

In the phase II trial “TAMRAD”, Bachelot *et al.*^[53] evaluated the efficacy and safety of everolimus in combination with tamoxifen in 111 patients with MBC who had relapsed after first line treatment with AIs. Fifty-four patients were randomized to receive everolimus 10 mg/d and tamoxifen 20 mg/d, and the remainder received tamoxifen alone. Patients were stratified in two sub-groups: those who progressed during or within six months after the end of treatment with adjuvant AIs or progressed during the first six months of AIs with metastatic disease were defined having *ex novo* or primary resistance, whereas those who relapsed six or more months after completion of adjuvant AIs or after the first six months of therapy with AIs with metastatic disease were defined having acquired resistance.

The CBR was higher in patients treated with everolimus (61% *vs* 42%; $P = 0.045$) and TTP was longer in the combination arm (8.6 mo *vs* 4.5 mo; HR 0.54; 95%CI:

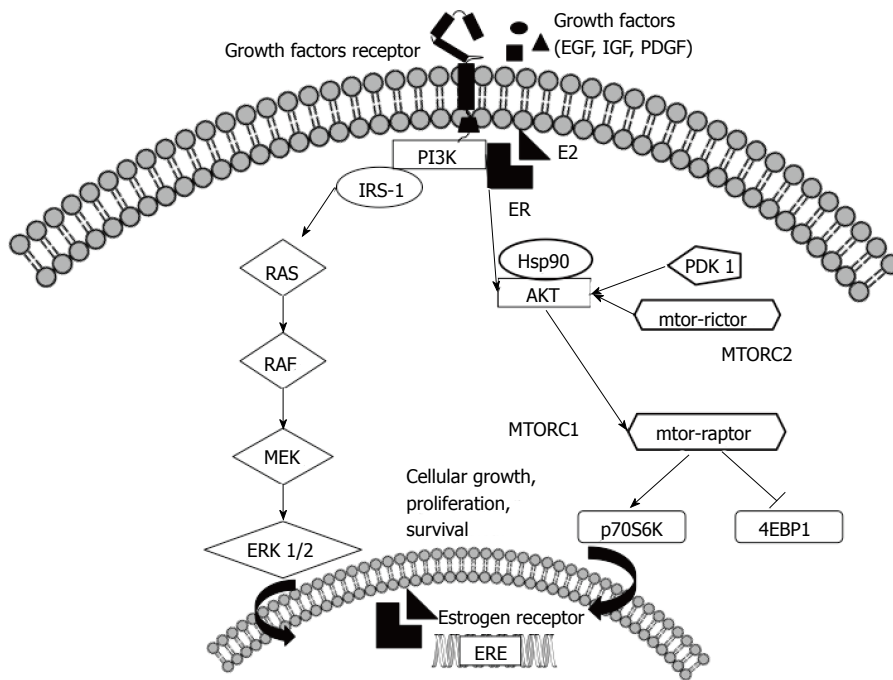


Figure 2 A representation of the molecular crosstalk between estrogen receptor and the receptor tyrosine kinases and PI3K-Akt-mTOR axes. In breast cancer, the PI3K-Akt-mTOR pathway modulates responses to signals communicated through growth factor receptors and the estrogen receptor (ER), and this crosstalk is important for sensitivity to anti-endocrine therapy. In particular, Akt and ERK1/2 phosphorylate ER on key residues involved in the induction of ligand-independent activation of DNA transcription. Furthermore, the converse occurs: estradiol, bound to membrane ER, interacts with and activates a regulatory subunit of PI3K. The mammalian target of rapamycin (mTOR) signaling cascade is another key regulatory pathway that controls proliferation and survival in cancer cells and plays an important role in the molecular crosstalk with the ER pathway. Two mTOR-interacting proteins, raptor and rictor, define distinct branches of the mTOR pathway: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Both active mTORC1 (via the phosphorylation of downstream targets, such as 4E-BP1 and p70S6 Kinase) and active mTORC2 contribute to promoting cellular survival and proliferation. EGF: Epidermal growth factor; IGF: Insulin-like growth factor; PDGF: Platelet derived growth factor; PI3K: Phosphatidylinositol-3-phosphate kinase; E2: Estradiol; IRS-1: Insulin receptor substrate-1; RAS-RAF-MEK-ERK: Mitogen activated protein kinase pathway; HSP90: Heat shock protein 90; PDK-1: Pyruvate dehydrogenase lipoamide kinase isozyme 1; p70S6K: Protein 70S6 kinase; 4EBP1: Eukaryotic translation initiation factor 4E-binding protein 1; ERE: Estrogen response element.

0.36-0.81). The subgroup analysis showed that the benefit of the combination therapy was greater in patients with acquired resistance (74% in the secondary resistance subgroup *vs* 46% in the primary resistance subgroup).

Baselga *et al.*^[54] explored the activity of this combination in the neoadjuvant setting. In a phase II trial, 270 postmenopausal patients with ER-positive breast cancer were randomized to receive letrozole 2.5 mg/d plus everolimus 10 mg/d or letrozole 2.5 mg/d plus placebo for 16 wk prior to surgery. The primary endpoint was clinical response. The clinical response rates were 68.1% *vs* 59.1% in the combination and placebo arms, respectively ($P = 0.062$). Moreover, everolimus showed greater anti-proliferative activity (57% *vs* 30% in the everolimus and placebo arm, respectively; $P < 0.01$), defined as the reduction in cell proliferation assessed in pre- and post-surgical biopsy specimens.

Following these phase II results, a larger randomized, double-blind, phase III study was conducted by the same group^[55]. The 'BOLERO-2' study enrolled 724 patients with HR-positive advanced breast cancer who had recurred or progressed after previous therapy with a non-steroidal AI (letrozole or anastrozole). Patients were randomized to receive exemestane 25 mg/d plus everolimus 10 mg/d or exemestane 25 mg/d plus placebo. The

primary endpoint was PFS and the secondary endpoints were OS, ORR, CBR, safety, and quality of life.

The trial was stopped early because the pre-planned interim analysis showed a better PFS in the combination therapy arm (6.9 mo *vs* 2.8 mo in the combination and exemestane alone arms, respectively; $P < 0.001$) and a 57% reduction of risk of progression (HR = 0.43; 95%CI: 0.35-0.54; $P < 0.001$). These data were confirmed in the final PFS analysis conducted at a median follow-up of 18 mo^[56]. PFS was 7.8 mo *vs* 3.2 mo (HR = 0.45; 95%CI: 0.38-0.54; $P < 0.001$) in the combination and placebo arms, respectively, and the magnitude of benefit was irrespective of clinicopathological characteristics, including previous treatment. The ORRs were 12.6% *vs* 1.7% ($P < 0.001$) in the combination and placebo arms, respectively; the CBR was better in the combination arm (51.3% *vs* 26.4% in the everolimus and placebo arms, respectively; $P < 0.001$). The final OS results are still not available and are awaited with interest.

Although generally well tolerated, all the clinical studies have reported toxicity related to everolimus. Data from BOLERO-2 showed that a greater proportion of patients discontinued treatment in the everolimus arm than in the placebo arm (19% *vs* 4%, respectively) due to adverse events. However, no significant difference

in overall quality of life was reported between the two arms^[57]. The most commonly reported toxicities related to everolimus were stomatitis, fatigue, rash, anorexia, and diarrhea; a less common but life-threatening adverse event was non-infectious pneumonia (presenting as an acute deterioration in respiratory function with ground glass or patchy opacities on computed tomography scans), which was reported in about 3% of patients. This non-infectious pneumonia seemed to be immunologically mediated and the clinical management often required immediate drug interruption and high doses of corticosteroids. Other concerning toxicities reported were hyperglycemia, hypercholesterolemia, and hypertriglyceridemia^[58,59].

Temsirolimus

Temsirolimus is a compound that, similar to everolimus, inhibits the kinase activity of mTOR by complexing with FKBP12. However, it differs from everolimus in its pharmacokinetics and toxicity profile^[60].

In a randomized phase II study, Carpenter *et al.*^[61] explored the activity and safety of oral temsirolimus with letrozole in heavily pre-treated ER-positive MBC patients. This trial had a three-arm design: one arm received letrozole alone, whereas the other two arms received letrozole plus temsirolimus daily (10 mg) or intermittently (30 mg), respectively. One-year PFS was higher in both combination arms with letrozole alone (69%, 62%, and 48%, respectively).

However, these results were not confirmed in a subsequent larger randomized phase III trial conducted by Chow *et al.*^[62] in heavily pre-treated MBC patients; no improvement in PFS was seen in the investigational arm and the study was stopped early.

Temsirolimus has also been evaluated in AI-naïve patients. In a randomized phase III study, 1112 postmenopausal women with ER-positive locally advanced or metastatic BC with no prior exposure to AIs were randomly assigned to receive letrozole plus oral temsirolimus 30 mg/d for five days every two weeks or placebo with the same schedule^[63]. The independent data monitoring committee also stopped this trial early at the second pre-defined interim analysis because the study was deemed unlikely to reach its primary endpoint. The published data showed no difference in PFS (8.9 and 9.0 mo, respectively; $P = 0.25$) between the groups at a median follow-up of 9.5 mo.

PI3K inhibitors

Alterations in the *PIK3CA* gene are the most common somatic mutations in breast cancer, and both crosstalk between the ER and PI3K pathways and PI3K activation are thought to play a role in endocrine resistance^[64,65].

Specifically, PI3K pathway alterations occur in about 70% of breast cancers and include mutations and/or amplifications of the genes encoding the PI3K catalytic subunits, p110 α (*PIK3CA*) and p110 β (*PIK3CB*), the PI3K regulatory subunit p85 α (*PIK3R1*), and the PI3K effectors *AKT1*, *AKT2*, and *PDK1*. The loss of lipid

phosphatases, such as PTEN and INPP4B (inositol polyphosphate-4-phosphatase type II), can also activate the pathway^[66-69].

In 2012, the Cancer Genome Atlas Network described that luminal ER+ tumors commonly harbor PI3K mutations, 49% in luminal A and 32% in luminal B^[70]. Fu *et al.*^[71] have recently shown that activation of RTK signaling induces transcription of growth-related genes and causes decreases in ER levels and activity, leading to an inferior response to endocrine therapy. Co-targeting this pathway with ER and PI3K inhibitors therefore appears to be a promising therapeutic opportunity for patients with ER+ breast cancer. In support of this, Fu *et al.*^[71] found that the combination of tamoxifen with a dual PI3K/mTOR inhibitor (BEZ-235) additively reduces cell growth in different ER-positive HER2-negative breast cancer cell line models^[71,72]. Furthermore, Sanchez *et al.*^[73] suggested in pre-clinical testing that fulvestrant may sensitize long-term estrogen deprived ER+ breast cancer cells to the therapeutic effects of PI3K inhibitors, with an associated synergistic increase in apoptosis.

At the most recent San Antonio Breast Cancer Conference, Juric *et al.*^[74] presented results from a phase 1b study of the PI3K α inhibitor GDC-0032 in combination with fulvestrant in patients with ER+ advanced breast cancer. GDC-0032 was administered to 17 patients at a range of doses (six to nine mg/d) in combination with fulvestrant 500 mg every four weeks (with loading dose of 500 mg at day one, 14, and 28). The combination appeared to be well tolerated and had promising preliminary efficacy, with a final recommended dose of six mg per day. No dose limiting toxicities (DLTs) were observed and the main adverse events were gastrointestinal toxicities (anorexia, nausea, and diarrhea), metabolic toxicity (hyperglycemia), and rash. Metabolic partial responses were observed in eight out of 11 patients (73%), including those previously treated with fulvestrant^[75].

At the same conference, another phase 1 trial reported on BKM120, a novel oral pan-PI3K inhibitor, in combination with fulvestrant in postmenopausal women with ER-positive MBC. Fulvestrant 500 mg IM was administered monthly on day one of each 28-d cycle (following the loading dose) and BKM120 was administered daily on day one to 28 of each cycle. 18 patients have been treated at three doses of BKM (80 and 100 mg/d continuously and 100 mg/d, five days on and two days off). Both BKM120 100 mg schedules (continuous or intermittent) with fulvestrant were tolerable without DLTs. Liver toxicity (assessed by ALT) has been reported with BKM120, especially with continuous dosing, and often requires dose reduction but not interruption. The results of this trial were promising, with over 50% clinical benefit, one partial response, and five prolonged disease stabilizations.

Phase III studies of this combination have also been started in the same setting and preliminary information was reported at the 2012 American Society of Clinical Oncology (ASCO) annual meeting. For example, the BELLE (buparlisib breast cancer clinical evaluation) tri-

Table 1 Ongoing clinical trials of PI3K inhibitors in combination with endocrine therapy in hormone receptor-positive metastatic breast cancer

Treatment	Disease conditions	Trial status	Trial number
Phase I			
BYL719 + letrozole	Postmenopausal women hormone receptor-positive stage IV breast cancer	Ongoing	NCT01791478
BKM120 + fulvestrant	Postmenopausal women estrogen receptor-positive stage IV breast cancer	Ongoing	NCT01339442
BKM120 or BEZ235 + letrozole	Postmenopausal women hormone receptor-positive stage IV breast cancer	Ongoing, not recruiting	NCT01248494
XL147 or XL765 + letrozole	Postmenopausal women hormone receptor-positive stage IV breast cancer	Completed	NCT01082068
Phase II			
PF-04691502 + exemestane <i>vs</i> exemestane alone	Estrogen receptor-positive stage IV breast cancer	Withdrawn prior to enrolment	NCT01658176
PF-04691502 + letrozole <i>vs</i> letrozole alone	Postmenopausal women estrogen receptor-positive early (phase II) and advanced (phase I b) breast cancer	Terminated	NCT01430585
GDC-0941 or GDC-0980/placebo + fulvestrant	Postmenopausal women estrogen receptor-positive, AI treated, stage III B-IV breast cancer	Ongoing	NCT01437566
Phase III			
BKM120/placebo + fulvestrant	Postmenopausal women hormone receptor-positive, AI treated, stage III B-IV breast cancer progressed on or after mTOR inhibitor-based treatment	Ongoing	NCT01633060
BKM120/placebo + fulvestrant	Postmenopausal women hormone receptor-positive, stage III B-IV breast cancer refractory to AIs	Ongoing	NCT01610284

als are investigating the safety and efficacy of buparlisib (BKM120) with fulvestrant.

BELLE2 is a phase III of BKM120 plus fulvestrant in HR-positive HER2-negative advanced breast cancer that has progressed on or after AI therapy, while BELLE3 is a similar phase III trial in patients with advanced breast cancer previously treated with AIs and refractory to endocrine and mTOR inhibitor combination therapy. The results from these trials will not be available for a few years (NCT01610284 and NCT01633060). BELLE4 is a phase II, randomized, double-blind and placebo-controlled study of BKM120 in combination with paclitaxel in patients with HER2-negative, locally advanced or metastatic breast cancer, with or without PI3K pathway activation. Other combination trials using different PI3K inhibitors are currently recruiting, for example BYL719 with letrozole or fulvestrant, and ongoing trials of PI3K inhibitors combined with endocrine agents are summarized in Table 1.

Multiple targeting of ER

Although the functional crosstalk between different molecular pathways and ER are thought to be the largest contributor to the development of endocrine resistance, many other mechanisms have also been described. For example, cells that express mutated ER circumvent inhibition by tamoxifen or long-term estrogen deprivation, as described above, and due to its peculiar mechanism of action, fulvestrant appears to be more active in these situations. Fulvestrant mediates the down-modulation and accelerated degradation of ER, thereby reducing its activity and its availability to other interacting molecules. Moreover, preclinical data suggest that fulvestrant retains and enhances its antitumor activity in the low estrogen

environment, such as in the presence of AIs^[76]. These data support a strong rationale to explore the activity of combining fulvestrant with AIs.

To this end, three large randomized trials have assessed this approach in postmenopausal women with ER-positive MBC^[77-79]. Mehta *et al*^[79] explored the activity of fulvestrant (500 mg loading dose, followed by 250 mg on days 14 and 28 and monthly thereafter) in combination with anastrozole compared to anastrozole alone (1 mg/d in both arms) in the first-line setting in women with MBC previously exposed to AIs and tamoxifen in the adjuvant setting. Overall, the study was positive in terms of its primary endpoint, with a small but statistically significant 1.5-mo increase in median PFS. However, the combination was only beneficial in the tamoxifen-naïve population. No differences in ORR and CBR were observed in the two arms of the trial.

In the second study, conducted by Bergh *et al*^[77], women with HR-positive MBC were randomized to receive the same two treatments as above in the first-line setting. Sensitivity to AIs was defined as either no prior exposure or administration of these drugs in the adjuvant setting and relapse occurring after one year from completion of adjuvant endocrine therapy. This trial failed to show differences between the study arms in the primary endpoint of TTP, or in ORR, CBR, and OS.

In the third study, recently published by Johnston *et al*^[78], patients with MBC resistant to AIs were randomized to fulvestrant (dose and schedule as above) plus anastrozole (1 mg/d), fulvestrant plus placebo or anastrozole, or to exemestane 25 mg/d. Patients were eligible if they progressed while on AIs after a period of at least 12 mo for adjuvant therapy or six months for metastatic disease.

This study also reported no differences in terms of PFS, OS, ORR, and CBR between the treatment arms.

CONCLUSIONS AND FUTURE PERSPECTIVES

Endocrine therapy was traditionally thought to be less effective than chemotherapy for the treatment of women with MBC and was consequently demoted to a secondary role. Recently, our understanding of ER biology has improved and, in parallel, our therapeutic armamentarium has expanded with the development of several classes of compounds with different mechanisms of action. As a result, endocrine therapy is the confirmed leader in the treatment of HR-positive MBC due to greater efficacy and negligible toxicity.

However, most women treated with endocrine therapies develop resistance, and several mechanisms of resistance have been described. In particular, ER appears to be a key player in a complex network of signaling pathways that leads to proliferation and survival of cancer cells. Due to the adaptability of this network, cells can easily escape simple perturbations, such as those presented by the currently available endocrine therapies.

Moreover, these observations have provided the rationale for developing drugs that target other interconnected pathways. Combinations of endocrine agents with or without these drugs have recently been tested in randomized trials, with exciting results.

In this paper, we have described three possible strategies to overcome endocrine resistance, some of which are already becoming part of clinical practice.

Of these, co-targeting the RTK signaling pathways and intracellular signaling networks is the most effective. Lapatinib has recently been approved in patients with HER2- and ER-positive breast cancer, and everolimus has been approved in combination with exemestane for women refractory to AIs. Many other drugs that target intracellular signaling networks, especially the PI3K-mTOR-Akt axis, are currently under development and some of these have shown promising results.

However, recent advances in the understanding of the biology of ER signaling and of the molecular markers of resistance have highlighted that ER and its pathway remain central to endocrine resistance. These findings are likely to translate into new strategies to overcome endocrine resistance in the near future. For example, targeting tumors with specific ER mutations with more potent and specific anti-estrogens seems to be a fascinating approach.

All these advances have positively impacted on survival of women with HR-positive MBC. They chart a course towards the biology-based selection of treatments and a more rational use of chemotherapy to improve efficacy and limit toxicity in women with breast cancer.

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Immune therapy for human papillomaviruses-related cancers

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by the immune system, leading to the development of cancer lesions. When this occurs, radiotherapy and chemotherapy are then used. Unfortunately, about 50% of the HPV-cancer patients still die. In the past decade, a better knowledge of the natural history of the virus-host interaction and of the immune response against this viral infection has brought new therapeutic strategies geared to modulate the immune system to generate an efficient virus-specific cytotoxic response. Novel HPV protein-expressing vaccines have shown some significant clinical efficacy and systemic HPV-specific cytotoxic T cell responses. This review will describe the current status of the several therapeutic strategies used to treat HPV-induced lesions, and discuss the various new therapies now being tested.

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Key words: Human papillomavirus; T cell; Immunoglobulin; Antibody; Vaccinia virus

Abstract

Human papillomaviruses (HPVs) are a large family of double strand DNA viruses comprising more than 180 types. Infection with HPV is very common and it is associated with benign and malignant proliferation of skin and squamous mucosae. Many HPVs, considered low-risk such as HPV 6 and 11, produce warts; while high-risk viruses, such as HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, and 58, induce tumors. About 5% of all cancers in men and women are associated with HPV infection. Because there are not antiviral drugs for HPV infection, current therapies for low-risk HPV infections involve physical removal of the lesion by cryotherapy, trichloroacetic acid, laser, or surgical removal. Surgical procedures are effective in the treatment of pre-cancerous lesions, however after these procedures, many recurrences appear due to new re-infections, or to failure of the procedure to eliminate the HPV. In addition, HPV can inhibit recognition of malignant cells

Core tip: Infection with human papillomavirus (HPV) is very common and it is associated with benign and malignant proliferation of skin and mucosae. Low-risk HPV produce warts; while high-risk HPV induce tumors. Because there are not antiviral drugs for HPV infection, current therapies involve surgical removal of the lesion. Unfortunately, after surgery many recurrences still appear and about 50% of the HPV-cancer patients die. In the past decade, new therapeutic strategies geared to generate an efficient virus-specific cytotoxic response have been developed. This review describes the current status of the several therapeutic strategies used to treat HPV-induced lesions.

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INTRODUCTION

In the mid 70s human papilloma virus (HPV) was described as a mild viral infection that caused benign warts, that in most cases would regress spontaneously^[1]. In few years however, it became evident that there are many HPV with selective tropism for various cutaneous mucosae, and that HPV infection could cause severe diseases. Especially, some HPVs infecting the ano-genital area are real oncogenic viruses that lead to cervical carcinoma^[2], the seventh most common cancer in the world^[3] and the second most common cancer among women^[4].

More than 180 types of HPV have been identified from different lesions, classified by DNA sequences, and named sequentially^[5]. HPV have selective tropism for cutaneous or mucosal epithelia and are divided into two groups: low-risk that cause benign lesions, named papillomas (small wart-like neoplasias) that usually regress on their own; and high-risk that cause malignant transformation and develop into large tumors. The two most common low-risk HPV are HPV 6 and HPV 11. They are responsible for 90% of genital warts and recurrent respiratory papillomas^[6]. There are about 15 high-risk HPV causing around 95% of all cervical carcinomas^[7]. The types HPV 16 and HPV 18 account for about 50% and 14% of all cases respectively^[4]. High-risk HPV are also involved in other types of cancer. Around 80% of tumors of the anus, 60% of the vagina, and 40% of vulva and penis are induced by HPV, mainly the type HPV 16^[8]. Also, around 60% of tongue, tongue^[9], and tonsil tumors are also caused by HPV^[10].

Natural history studies of HPV show that 60% of sexually active people will be infected with at least one high-risk HPV during their lifetime^[11]. Most of these infections are eliminated by the immune system in about 1 or 2 years from exposure^[12]. However, in the remaining cases, the virus persists for a long time causing lesions that can progress into cancer^[13]. Early detection of HPV-induced lesions becomes important in order to prevent the development of cancer. The best way to diagnose an HPV infection is to confirm the presence of HPV DNA in the lesion by hybridization or by PCR^[14,15]. This type of testing is expensive and difficult to implement in poor parts of the world^[16]. Therefore, regular screening of cytological (Pap smear) or colposcopic abnormalities, which are signs of HPV infection, continues to be an effective preventive strategy for cervical cancer^[17] particularly in developing countries. Unfortunately, this strategy is not without problems. There is great variability due to the lack of trained personnel resulting in high rate of false negative results^[18]. Clearly a combination of screening strategies is needed to detect as early as possible HPV-induced lesions.

Once HPV lesions are detected, the main therapeutic approach involves physical elimination of the lesion by cryotherapy, trichloroacetic acid, laser or surgical removal^[19]. In pre-cancerous lesions however, surgical procedures alone are not very effective, since recurrences

occur at rates of 20%-30% or more with lesions both at previously treated sites due to failure of the procedure to eliminate the HPV, and at new sites due to new infections^[20]. In addition, HPV can inhibit recognition of malignant cells by the immune system^[21], leading to the development of cancer lesions. When this occurs, radiotherapy and chemotherapy are then used with relative success, since about 50% of the HPV-cancer patients still die^[22]. Clearly, new therapeutic strategies are in urgent need to control the burden of HPV-related cancer^[23].

The fact that most HPV infections are cleared spontaneously shows that the immune system can effectively eliminate virus-infected cells. But, in the case of persistent infections, the immune system clearly has failed. HPV has evolved several mechanisms to evade the immune system. First, HPV infects keratinocytes, which are distant from immune cells, presents a minimal expression of viral proteins in the keratinocyte, and thus does not induce lysis of keratinocytes. In addition, HPV down-regulates the expression of major histocompatibility complex (MHC) class I molecules, Toll-like receptor (TLR) 9, and cytokines such as, interferon and interleukin (IL)-8^[21]. New therapeutic approaches take advantage of our knowledge on how the immune system deals with viral infections and eliminates virus-infected cells mainly through cytotoxic activity of various leukocytes, mainly T cells^[21]. These new therapies involve the use of anticancer vaccines and intralesion immunotherapy with the idea to activate specific cytotoxicity towards HPV-infected cells^[24,25]. In this review, we describe the current status of the several therapeutic strategies used to treat HPV-induced lesions, and discuss the various new therapies now being tested.

PAPILLOMAVIRUS

Papillomavirus belong to the Papovaviridae family of DNA viruses. These viruses contain a double strand DNA with 8000 pb approximately arranged in an 8 well-defined genes (Figure 1). Six early genes are involved in virus replication and two late genes are involved in virus assembly. HPV infect the epithelium of the cervix, and their replication is closely linked to the differentiation of the epithelium (Figure 2)^[26,27].

IMMUNE RESPONSE TO HPV

Protection against viral infections is provided by both arms of the immune system. First, HPV can infect cells through damaged skin tissue. When the damage reaches the basal layer of the epidermis, the virions can infect dividing keratinocytes. The initial inflammatory response induced by tissue damage leads to infiltration of immune cells mainly neutrophils, followed by macrophages and later lymphocytes. These nonspecific innate immune cells detect "danger" by recognizing viral molecules, such as the double-stranded DNA HPV genome or the L1 and L2 capsid proteins. These molecules are detected by pat-

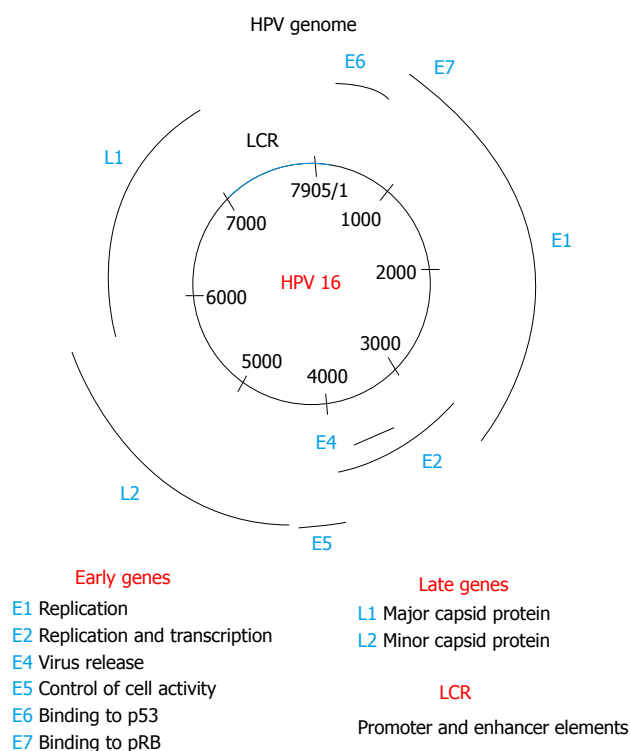


Figure 1 Human papilloma virus genome. The genomic organization of the human papilloma virus 16 (HPV 16) is shown. The double strand DNA is close to 8000 base pairs. The early genes code for proteins involved in viral replication and transcription: E1, E2, E6, and E7 genes. The E4 and E5 genes are expressed a little later and have functions in immune evasion and virus release. The late genes code for the virus structural proteins: the major capsid protein L1, and the minor capsid protein L2. The E6 and E7 proteins of the high-risk HPV types have oncogenic properties. The long consensus repeat (LCR) sequence contains the promoter and enhancer elements of the virus.

tern recognition receptors, such as Toll-like receptors^[28], which signal to activate defense mechanisms *via* the production of inflammatory cytokines such as IL-1 β , IL-6, IL-8, IL-12, and α -, β - and λ -interferon (IFN) to activate natural killer (NK) cells^[29] and other immune cells. Later, antigen presenting cells (APCs), such as Langerhans cells or dendritic cells (DCs) in the skin and mucosa^[30], can uptake viral antigens and process them into small peptides that are presented together with MHC (HLA in humans) molecules on the cell membrane, to lymphocytes for initiation of an adaptive immune response (Figure 3). For this process, DCs migrate to lymph nodes where they undergo maturation through the expression of co-stimulatory molecules. The processed viral antigen/MHC complex on DCs binds to antigen-specific T cell receptors on naïve CD4⁺ and CD8⁺ T cells. This binding induces T cell proliferation, IL-2 production, and activation of T cells. Activated CD4⁺ helper T cells can differentiate into Th1, Th2, or Treg/Th3 phenotypes depending on the cytokines they produce. Th1 produce IL-2, IL-12, IL-15, tumor necrosis factor (TNF)- α , and λ -IFN; Th2 produce IL-4, IL-5, IL-6, IL-10, and IL-13; and Treg/Th3 produce IL-10, transforming growth factor (TGF)- β , and λ -IFN. On the other hand, activated CD8⁺ T cells differentiate into cytotoxic T lymphocytes (CTL) which secrete the

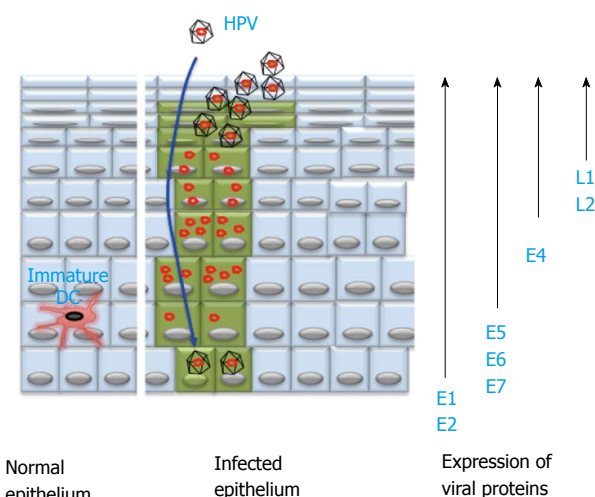


Figure 2 Papilloma virus replication is tissue specific. The human papilloma virus (HPV) infects a keratinocyte in the basal layer of the epithelium after a micro trauma (a small cut of the epithelium that uncovers the basal membrane). The virus DNA is maintained in the proliferating cells at a low-copy number (E1 and E2 viral proteins are expressed). When the infected cells begin to differentiate into mature keratinocytes, the virus activates other genes (E4, E6, E7 viral proteins are expressed) and replicates its DNA to a high-copy number. In the top layers of the epithelium all viral proteins (including E4 and the capsid proteins L1 and L2) are expressed. Thousands of new virions are formed and released from the cells without causing cell death.

proteolytic enzymes, granzyme and perforin^[31]. CTLs migrate back to the infected sites and destroy HPV-infected cells (Figure 3).

The majority of HPV infections are cleared spontaneously within two years from infection and without any clinical manifestation by immune-competent individuals^[12,26]. In spontaneously regressing HPV-related lesions, infiltration of CD4⁺ and CD8⁺ T cells has been detected, while in persistent lesions these immune cells are not seen^[32]. In addition, in immunosuppressed individuals such as organ transplant recipients^[33] or human immunodeficiency virus (HIV)-infected people a higher incidence of HPV-related lesions are observed^[34]. These observations indicate that the adaptive immune response against the virus is important and for the most cases effective. This adaptive response comprises elements of humoral and cellular immunity^[21]. However, HPV has also evolved mechanisms both to avoid initial detection and to interfere with adaptive response that allow the virus to persist and lesions to progress into cancer.

Humoral response

The majority of patients with HPV infections present a humoral immune response that is detected by the presence of antibodies against different papillomavirus proteins such as L1, E2, and E4 proteins in the first stage of infection. At later times when viral DNA gets integrated into cellular genome, antibodies against E6 and E7 proteins can be detected in low- and high-grade lesions as well. However, this antibody response is usually weak and variable and it does not seem to protect from future re-

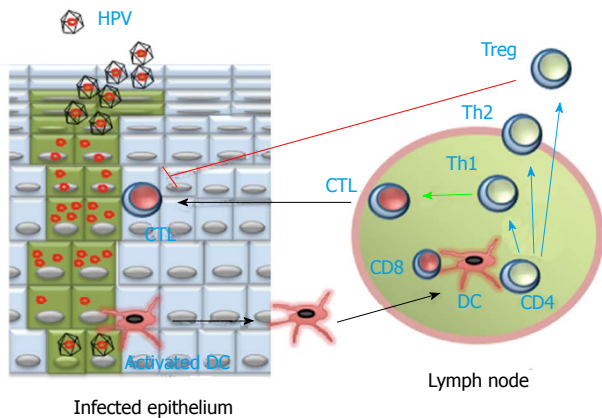


Figure 3 Cellular immune response against human papilloma virus. Dendritic cells (DC) can take human papilloma virus (HPV) antigens from the infected epithelium, and then migrate (black arrows) to lymph nodes. There, DC present the processed antigen together with MHC class I and class II molecules, to CD8+ T cells and CD4+ T cells, respectively. CD4+ T cells proliferate and differentiate (blue arrows) into T helper cells, either Th1 or Th2, depending on the type of cytokines they produce. CD8+ T cells differentiate (blue arrow), with help (green arrow) from Th1 cells, into cytotoxic T cells (CTL). Then, CTL migrate (black arrow) back to the infected epithelium to destroy virus-infected cells. CD4+ T cells can also differentiate (blue arrow) into regulatory T cells (Treg), which inhibit (red line) the cytotoxic activity of CTL.

infections^[35]. For high-risk HPVs, the seroconversion rate is about 50% within 18 mo of detecting the corresponding HPV DNA^[36], and important differences have been found in the proportion of seropositivity among HPV 16 DNA-positive individuals, depending on the site of the cancer around the ano-genital area, indicating that cancer development may lead to changes in antibody responses in a site-specific fashion^[37]. Thus, humoral responses are not efficient at eliminating established HPV lesions. In addition, antibody titers can persist for many years even after the virus is cleared, so seropositivity is a useful marker for past infection rather than current infection.

Cellular response

Cell-mediated immune responses are more important in clearing HPV-related lesions. HPV-specific CD4⁺ (helper) and CD8⁺ (cytotoxic) T cells are generated to fight and eliminate infected cells (Figure 3). Patients who successfully eliminated previous HPV 16 infections present memory T cell responses to viral proteins^[38], and in patients with spontaneous regression of grade 3 vulvar intraepithelial neoplasia strong CD4⁺ and CD8⁺ T cell responses are found^[32]. A high ratio of CD4⁺ to CD8⁺ T cells^[39,40] with a predominance of Th1 cytokines^[41,42] is also observed during regressing lesions. Thus both CD4⁺ and CD8⁺ T cells are important for elimination of HPV infection. In contrast, patients with cervical intraepithelial neoplasia or cervical cancer present deficient T cell responses^[39]. A low ratio of CD4⁺ to CD8⁺ T cells^[42,43] and a strong shift to Th2 cytokine profile^[44-46] are observed in persistent lesions. Also, responses that include HPV-specific Treg that inhibit cytotoxic activity^[38]. Thus, an efficient cytotoxic cell-mediated immune response is critical for elimination of HPV-related lesions. Unfortunately,

the virus has also evolved mechanisms to interfere with the immune response.

HPV mechanisms to evade the immune system

The best mechanism against the immune system is to avoid detection. HPV replication takes place in areas where immune surveillance is poor. In the stratified squamous epithelium of the uterine cervix, surveillance by DCs greatly declines towards the keratinized layers (Figures 2 and 3). In addition, the virus links its own replication with the differentiation state of the keratinocyte. At the beginning of the infection, in the keratinocytes of the basal layer, expression of viral genes is minimal. Expression of viral gene products up-regulates progressively with differentiation and upward migration of keratinocytes (Figure 2). In this way, HPV late proteins, which are the most immunogenic, are expressed at areas of poor immune surveillance (Figure 3). In addition, new virions are released through the normal rupture of surface epithelium, reducing any possible inflammatory response and avoiding uptake by DCs. Therefore, HPV replication is a local phenomenon with minimal activation of the immune system.

In addition of keeping a low profile during replication, HPV has other mechanisms that help the virus interfere with the immune response^[21]. The E6 and E7 proteins block IFN production by the infected cell. E6 inhibits the transcription factor IRF-3 that binds to the -IFN promoter, downregulating expression of interferon-responsive genes^[47]. E7 also inhibits the expression of -IFN responsive genes^[48,49]. In addition both E6 and E7 reduce the expression of TLR 9^[50], and cytokines, such as IL-8^[51], and IL-18^[52], which are pro-inflammatory molecules. The proteins E5, E6, and E7 downregulate the expression of MHC class I molecules, reducing recognition of the HPV-infected cell by NK cells and by specific CTLs^[53].

Moreover, a reduced inflammation state is found in persistent lesions and in tumors. This condition correlates with a change in the cytokine profile produced at the site of infection. A shift to Th2 cytokines is also common in persistent lesions^[45,54]. This leads to an inhibitory state for helper CD4⁺ T cells. In addition, Tregs have been found infiltrating tumors, especially in the early stage of tumor progression^[55]. Tregs are able to strongly inhibit activation of CD4⁺ T cells, cytokine production, and activation of CTLs^[56]. In patients with cervical cancer, larger numbers of Tregs and increased suppressive activity have been observed, both in the tumor and in draining lymph nodes^[57,58]. Presence of these Tregs associates with an increased risk for progression of premalignant lesions to cancer^[59]. Thus, when trying to activate the immune system special attention should be paid to the possible development of HPV-specific Tregs that may block the cytotoxic response needed to eliminate virus-infected cells^[60]. Indeed, it has been proposed that immunogenic tumors (those with abundant tumor-infiltrating lymphocytes) may display potent immunosuppressive potential^[61]

and should be treated with Tregs-depleting agents or with inhibitors of co-stimulatory molecules such as cytotoxic T-lymphocyte antigen-4 (CTLA-4) with the therapeutic monoclonal antibody ipilimumab^[62].

There is much information in the literature regarding cellular immune responses in patients with HPV infection, with cervical lesions, and with cervical cancer. These reports are beyond the limits of this review, but a general description of the cell responses from HPV infection to development of cancer is as follows. During the initial infection an inflammatory state favors activation of innate immune cells^[63,64] and secretion of cytokines that induce CD4⁺ T cell activation^[39]. As mentioned before in regressing lesions, both CD4⁺ and CD8⁺ T cells are found with a high ratio of CD4⁺ to CD8⁺ T cells, and also cytokines of the Th1 type are produced^[42,43]. However, when the CD4⁺ to CD8⁺ T cell ratio is low and a shift to Th2 type cytokines is observed a persistent infection is found in lesions^[45,46] and a progression to cervical cancer is more likely^[44,54,65]. Finally the presence of Tregs in lesions seems to increase the risk for progression of premalignant lesions to cancer^[59,66]. Thus, any therapeutic approach must be able to induce a strong HPV-specific immune cell response that involves CD4⁺, CD8⁺ cells, and Th1 type cytokines.

PREVENTION

In principle, if HPV infection could be prevented, HPV-associated cancers would disappear. Following this idea, HPV vaccines that could prevent infection were developed in the last decade. Taking advantage of the fact that capsid L1 proteins spontaneously assemble in virus-like particles (VLP) without viral DNA, it was possible to have the immunogenic L1 proteins in a non-infective form. Two pharmaceutical companies, Merck in the United States and Glaxo Smith Kline (GSK) in Europe, used VLP produced by overexpressing HPV L1 protein in yeast or insect cells, to create prophylactic vaccines. Gardasil® (Merck) is a quadrivalent vaccine against HPV types 6, 11, 16, and 18^[67], and Cervarix® (GSK) is a bivalent vaccine against types 16 and 18^[68]. Both vaccines have been approved and are commercially available. They induce an antibody response that prevents new infections with high efficacy against HPV 16 and 18 (Cervarix®)^[69,70] and HPV-6, 11, 16, and 18 (Gardasil®)^[71]. They promise, in the long-term (30-50 years) to reduce the incidence of disease associated with the vaccine HPV types^[72]. However, this type of protection could only be achieved with a large vaccination coverage (larger than 50%) of uninfected people, which are boys and girls 10 years old, to predate the debut of sexual activity. Unfortunately, full vaccination coverage of large populations will not be easy in many parts of the world^[73], and a large unvaccinated population will remain at a high risk of HPV-related disease, and in need of treatment.

In addition, other factors indicate that current vaccines will not be useful to prevent all types of HPV-

related cancers. These vaccines target only HPV 16 and 18 and in the case of Gardasil® HPV 6 and 11. Despite some cross-reactivity^[69], these vaccines show a small prophylactic effect on many HPV subtypes not included in the vaccine^[74,75]. Also, the HPV subtype distribution in cervical cancer varies throughout the world^[76,77]. For example, in Japan less than 60% of cervical cancer cases are due to HPV 16 and 18^[24]. Thus, current vaccines cannot cover all oncogenic types even in a single population, and their general use in other parts of the world is questionable^[75]. To be completely effective, future vaccines would need to be multivalent for all described oncogenic HPV types. But these formulations will certainly be much more expensive than current vaccines. Despite government subsidies trying to achieve full vaccination coverage, weaknesses in current prophylactic vaccines dictate that even vaccinated females must continue cervical cancer screening^[78]. Clearly, large numbers of people remain at risk of developing HPV-related disease, and thus virus-specific therapies remain a priority.

CURRENT THERAPIES

HPV infections that are not cleared by the immune system usually persist in latent form as cells of the basal layer maintain low viral copy numbers indefinitely. Tissue damage or immunosuppression can then induce an active infection that depending on the HPV type, may progress to neoplasias. HPV infection of the ano-genital area produces two types of lesions: warts (condyloma acuminata) and squamous intraepithelial lesions. Condylomata are mostly related to low-risk HPV infections, and represent a low risk of malignant transformation. In contrast, squamous intraepithelial lesions are related to high-risk HPV infections. These malignant lesions display various degrees of histological abnormalities. For the cervix these lesions are classified as cervical intraepithelial neoplasia (CIN) 1, mild, CIN 2, moderate, and CIN 3, severe. A similar classification is used for vaginal intraepithelial neoplasia, vulvar (VIN), penis (PIN) and anus (AIN) intraepithelial neoplasias. All of these lesions can progress to invasive cancer. Treatment for CIN and similar lesions has predominantly involved ablative therapies which purpose is to eliminate the damaged HPV-infected tissue, leaving the healthy tissue of the cervix intact^[79]. Hysterectomy is not acceptable as primary therapy for high-grade CIN because most patients are women of reproductive age^[80]. Ablative therapies include cryotherapy, excision procedures (conization), laser therapy, and electrosurgery^[81].

Ablative therapies

Cryotherapy: Also known as cryosurgery, it is a surgical treatment to freeze and destroy abnormal tissue in the cervix. It is performed with a cryoprobe, which is a metal rod cold enough to freeze and eliminate the tissue. The procedure is more effective when the tissue is frozen for 3 min, allowed to thaw for about 5 min, and frozen again



Figure 4 Conization of human papilloma virus cervical intraepithelial neoplasia 3 lesions. Colposcopy of papillomavirus cervical intraepithelial neoplasia 3 lesions from a patient treated with conization. Photographs of cervix before (A) and immediately after conization (B).

for another 3 min. Cryotherapy is widely used because it is easy of use and can be performed at the doctor's office. In fact, in many countries it is the only option available outside surgical settings. So, it is important that diagnosis and visualization of lesions be accurate before the cryotherapy is performed, in order to avoid missing small or deep lesions. During colposcopy, vinegar (acetic acid) or iodine (Lugol's solution) are applied to vagina and cervix with a swab or cotton balls to see areas of abnormal tissue more clearly, prior to cryotherapy. In summary cryotherapy is used mainly when ectropion (cervical eversion), cervicitis, or an HPV infection is present. If low- and high-grade lesions are detected after histology, then conization is recommended.

Excision procedures: For larger lesions excision procedures are used. The most commonly procedures are the loop electrosurgical excision procedure (LEEP) and the cold knife cone biopsy (conization). These methods are relatively inexpensive (between United States \$ 500 and United States \$ 1000^[82]) and can be performed at the doctor's office (Dysplasia clinic) in an outpatient setting. LEEP uses a thin, low-voltage electrified wire loop to cut out abnormal tissue, which is either visible during colposcopy, or hidden in the cervical canal and not visible during colposcopy. Conization is an extensive form of cervical biopsy used to remove abnormal tissue. It is called a cone biopsy because a cone-shaped piece of

tissue is removed. Conization removes abnormal tissue, usually high in the cervical canal, and also some normal tissue around the damaged tissue, so that a perimeter of normal cells is left in the cervix. Usually conization is the standard treatment if invasive disease (CIN 1, CIN 2, and CIN 3 lesions) is suspected, because it can remove tissue deep in the endocervical canal and can avoid diathermy artifacts (Figure 4).

Laser therapy: In this type of surgery a highly focused beam of light is used to obliterate the damaged tissue. Commonly a carbon dioxide laser is used to create the light beam. The laser light destroys the tissue and cauterizes the wound at the same time, reducing bleeding and pain, and shortening the healing time.

Electrosurgery: Electrofulguration is a type of electro-surgery used to burn the cancerous tissue^[83]. It consists of applying a moderately damped alternating electrical current through an electrode that is held close to the tissue so that sparks hit and destroy the lesion.

Hysterectomy: In this surgery, complete removal of the uterus, and other reproductive organs, such as ovaries, fallopian tubes and surrounding structures, is performed. This radical procedure is done when initiation of invasive cancer in female patients is diagnosed.

The choice to use one treatment over another depends mainly on infrastructure and is usually made by the provider. All these ablative therapies are very effective in initial treatments where elimination of lesions can be as high as 80%. However, removal of damaged tissue, does not always guarantee elimination of viral DNA, and high rates of recurrence (up to 40%) are found^[20] even after several treatments^[84].

Non-surgical therapies

Non-surgical therapies that kill cells on contact are also used to eliminate HPV-infected lesions^[85]. Some of these alternative treatments can be self applied by the patient, and generally are the first line of treatment for genital warts^[86].

Trichloroacetic acid: Trichloroacetic acid is applied topically by the physician directly on warts. The acid action produces a chemical burn of the wart. This treatment is effective in clearing up to the 50% of lesions; it does not present systemic toxicity and can be used during pregnancy. However, it is associated with adverse effects such as ulceration, dermal scarring and secondary infections.

Podophyllotoxin: Podophyllotoxin is a non-alkaloid lignan extracted from the roots of *Podophyllum* species (e.g., *P. peltatum*-Mayapple)^[87]. This toxin is used as an initial treatment for genital warts. It is found as a gel (Condylox) or as a solution or cream (Wartec), that can be self-applied by the patient. The mechanism of action is not clear, but it may be related to the binding of lignans to micro-

tubule proteins, inducing cell cycle arrest at metaphase. Podophyllotoxin treatment can achieve 50% clearance, but recurrence rates are 25%-30%^[84]. Podophyllotoxin is contraindicated in pregnancy.

Imiquimod: Imiquimod is a low-molecular-weight imidazoquinoline compound that modulates the innate immune system. In the form of a patient-applied cream (Al-dara) it is used to treat genital warts. Reduction in genital warts was observed in 50% of patients that used the 5% cream for 12 wk, with only 19% recurrence^[88]. Imiquimod was also reported to have certain therapeutic effects on basal cell carcinoma^[89], vaginal^[90], vulvar^[91], and anal intraepithelial neoplasia^[92], but the drug is not licensed for these treatments. Imiquimod has important inflammatory side effects, including itching, erythema, burning, irritation, tenderness, ulceration, and pain, thus its use on mucosal tissue is limited^[93].

The mechanism of action of Imiquimod is not completely clear, but it is known that Imiquimod is an agonist for TLR 7, commonly involved in pathogen recognition^[94]. Imiquimod binding to TLR 7 activates immune cells such as dendritic cells, macrophages, and keratinocytes to produce γ -IFN^[95] and other pro-inflammatory cytokines, such as IL-6 and TNF- α ^[96,97]. There is also evidence that Imiquimod, when applied to skin, can activate antigen presenting cells, which then migrate to local lymph nodes to induce an adaptive immune response^[95]. Because Imiquimod induces IFN production, it should be used with caution on intra-epithelial lesions infected with high-risk HPV types. Both α -IFN and β -IFN inhibit cell growth of keratinocytes with episomal HPV DNA, leading to virus elimination. However, IFNs do not affect cell growth of cells containing integrated HPV DNA. The result is that it selects for keratinocytes with integrated HPV DNA^[98,99]. The continued use of Imiquimod may then select for transformed cells with integrated HPV, and induce progression to high-grade intra-epithelial neoplasia.

Polyphenon: Polyphenon is a high-grade extract of green tea leaves from *Camellia sinensis*, manufactured by the Mitsui Norin Co., Ltd. of Japan. In the form of a topical ointment it can be self applied by the patient for treatment of genital warts. It has shown efficacy (54%) to reduce warts in treated patients with fewer recurrences^[100]. The main component in polyphenol is the catechin (a plant natural phenol belonging to the flavonoids family) (-) Epigallocatechin gallate (EGCG). EGCG is a potent antioxidant that reduces nitric oxide production by inhibiting inducible nitric oxide synthase *via* inactivation of the transcription factor nuclear factor-B (NF-B)^[101,102]. EGCG has also been shown to induce inhibition of growth and apoptosis^[103].

Cidofovir: Cidofovir is an acyclic nucleoside phosphonate analog to cytidine. It is used for the treatment of cytomegalovirus infections^[104], and it is also used off-license

as a topical treatment against benign low-risk HPV infections^[105]. Cidofovir is phosphorylated in the cell and then blocks incorporation of cytidine into viral DNA thus arresting virus replication. Small trials with CIN patients have shown promising efficacy^[106], but safety issues are a concern^[107].

NOVEL IMMUNOTHERAPIES

Prophylactic vaccination as discussed above, concentrates on preventing virus infection of epithelial cells, by generating a humoral immune response with antibodies that recognize viral capsid proteins and neutralize the virus. However, this approach is not effective for treating existing infections or established HPV-related diseases. Thus, a high prevalence and mortality of cervical cancer continues around the world, especially in developing countries. An effective therapy for established disease should stimulate a cellular immune response, with activation of both CD4⁺ (helper) and CD8⁺ (cytotoxic) T cells, which can recognize and eliminate virus infected cells^[108]. Cytotoxic cells need to recognize a viral antigen expressed in the infected cells. Several of the HPV proteins are potential targets to induce a good immune cytotoxic response. Capsid proteins L1 and L2 are poor antigens for a therapeutic vaccine, since these proteins are expressed mainly in terminally differentiated keratinocytes and in advanced-stage infections of cervical tissue low levels of these protein are detected (Figure 2). In contrast, HPV early proteins, such as E1, E2, E6, and E7, are expressed in multiple stages of the virus infection, and in HPV-associated cancers the E6 and E7 proteins are constitutively expressed (Figure 2). Thus, these proteins are all good antigenic targets for a therapeutic vaccine.

E2 is a DNA-binding protein involved in activation or repression of different HPV promoters^[109], and it also has a relevant role in controlling migration of viral DNA to daughter cells during mitosis of infected cells^[110]. Due to its importance in the whole virus replication cycle, E2 protein expression is maintained throughout most stages of infection (Figure 2) and thus, it becomes an excellent target for stimulating the immune system to eliminate infected cells independently of the stage of pathogenesis. Based on this, earlier studies using dogs immunized against papilloma E1 and E2 proteins, found that dogs were protected from papilloma growth after viral challenge, or had complete regression of papilloma after post challenge immunization^[111,112]. These results in animal models led to the design of new vaccines intended to induce a cellular immune response to the E2 protein. In clinical trials very encouraging results have been obtained with this approach (see next section)^[113,114].

The oncogenic HPV E6 and E7 proteins are essential for progression to, and maintenance of, the malignant phenotype. Therefore, these two proteins are also possible antigenic targets for therapeutic vaccines. The high-risk HPV 16 and 18 E6 and E7 proteins have been selected predominantly for induction of a cellular response.

Table 1 Immune therapies for human papilloma virus-related lesions in clinical trials

Name	Target protein	Platform	Immune response/clinical efficacy	Ref.
Peptide-based				
HPV16-SLP	E7 E6, E7	Synthetic peptides restricted to HLA-A2 Synthetic overlapping long peptides	T helper cell response, but no CTL response HPV-specific T cell response. Neither tumor regression nor prevention of progressive disease	[118,152] [120-122]
Protein-based L1VLPE7	E7	Chimeric L1-E7 VLP	Histological reduction to CIN 1 in 39% of treated patients compared to 35% of placebo recipients. This trend was not significant	[123]
SGN-00101 HPV16 E6/E7	E7 E6, E7	Fusion protein of M. bovis Hsp and HPV 16 E7 Fusion protein of HPV 16 E6 and E7 proteins administered with the adjuvant ISCOMATRIX	Complete regression in 35% of patients with CIN 3 Demonstrated important CD8+ T cell responses	[125,126] [128]
TA-CIN	E6, E7, L2	Recombinant fusion protein consisting of E6, E7, and L2 from HPV	Imiquimod, for 8 wk, followed by 3 doses of TA-CIN induced complete regression in 32% of patients with VIN	[131]
DNA vaccine pConE6E7	E6, E7	DNA vaccine that encodes a HPV 16 or 18 consensus E6/E7 fusion gene	Cellular immune response against HPV E6 and E7 antigens and protection against HPV E6 and E7-expressing tumors	[133,134]
ZYC101a	E7	Microencapsulated DNA vaccine encoding E7-specific CTL epitopes	In younger patients a 70% reduction of lesions was detected	[135]
Amolimogene	E6, E7	A poly (lactide co-glycolide) encapsulated plasmid DNA encoding T cell epitopes from HPV 16 and 18	Increase in HPV-specific T cells to epitopes from HPV 16, 18, 6 and 11. No correlation between cellular immunity and clinical response	[136]
Sig-E7(detox)-HSP70	E7	Fusion protein between HPV 16 E7 and heat shock protein 70	Low frequency and weak HPV E7-specific T-cell responses with no correlation to clinical results	[137]
Recombinant virus TA-HPV	E6, E7	Vaccinia virus encoding modified HPV 16/18 E6 and E7 proteins	60% patients with high-grade VIN had partial reduction in lesion diameter, and an increase in lesion-infiltrating CD4+ and CD8+ T cells	[142,143]
TG4001	E6, E7	Vaccinia virus encoding modified HPV 16 E6 and E7 proteins, and the human IL-2	Ten patients (48%) with CIN 3 showed promising clinical responses at 6 mo, but no data on related immune response	[144]
MVA E2	E2	Vaccinia virus (MVA) encoding bovine papilloma virus (BPV) E2	19 out of 34 patients with high-grade CIN 3 lesions had complete regression. Specific cytotoxic activity against cancer cells correlated with clinical outcome	[113,114]
MVA-E1	E1	Vaccinia virus encoding the E1 sequence of HPV 16	MVA-E1 into C57BL/6 mice resulted in sustained HPV 16 E1-specific T cells with cytotoxic activity	[147]

HPV: Human papillomavirus; CTL: Cytotoxic T lymphocytes; VLP: Virus-like particles; VIN: Vulvar; CIN: Cervical intraepithelial neoplasia.

Immunization has been tried with different delivery systems in transplantable rodent tumor models^[115] and in some clinical trials. The types of therapeutic vaccines tested so far in clinical trials can be grouped into five categories: peptide-based, protein-based, DNA vaccination, viral vectors, and DC-based immunization (Table 1). These therapeutic approaches are described next.

Peptide-based vaccines

Peptides are easy to use, inexpensive, and safe, but they are in general weakly immunogenic and they need to be mixed with adjuvants to improve their recognition by the immune system. The design of peptides involves identification of what parts of the HPV antigen are immunogenic. Short peptides may miss the relevant epitope needed for stimulation of an efficient immune response. Thus, current preparations contain mixtures of peptides. In addition, the polymorphic nature of the MHC may result in some peptides not being presented because the

corresponding HLA molecule is missing in particular patients. Therefore, the peptide vaccine approach has also used restricted HLA-binding peptides. A potential problem with this strategy is that exogenously added peptides may load onto MHC class I molecules on cells other than antigen presenting cells, leading to tolerance instead of stimulation because non-APC lack co-stimulatory molecules^[116]. The best approach seems to be the use of long overlapping peptides, which appear to be processed and presented better than whole proteins by DC^[117].

A vaccine consisting of two synthetic HPV 16 E7 peptides (representing HLA-A*0201-restricted cytotoxic T lymphocyte epitopes) and one helper peptide (a pan-HLA-DR-binding T-helper epitope) emulsified in adjuvant was tried in a phase I - II clinical trial with cervical carcinoma patients. The peptide preparation was safe, and induced proliferative immune responses in 4 out of 15 patients. Unfortunately, no cytotoxic T lymphocyte responses against the HPV 16 E7 peptides were detect-

ed^[118]. In a different study, 20 patients with HPV 16-positive, high-grade vulvar intraepithelial neoplasia were immunized with a mix of long peptides from the HPV 16 viral oncoproteins E6 and E7 in incomplete Freund's adjuvant. Five patients had complete regression, and HPV 16 was not longer detected in four of them. These patients also presented an increased T cell response^[119].

Another peptide vaccine (HPV 16-SLP) consists of a mix of 9 HPV16 E6 and 4 HPV 16 E7 overlapping long peptides. This vaccine was used to immunize patients with resected HPV 16-positive cervical cancer. HPV 16-specific T-cell immune responses were found with a preference of CD4⁺ -IFN- γ -producing T cells. Interestingly, there was also proliferation of T cells with a CD4⁺, CD25⁺, Foxp3⁺ phenotype that is associated with Treg, suggesting that the response against HPV was not completely effective^[120]. In another study, this vaccine was given to women with high-grade cervical squamous intraepithelial lesions. Immunization induced increased HPV 16-specific T-cell immunity, but not infiltration of HPV 16-specific T cells into the lesions, nor HPV clearance at the time of LEEP excision^[121]. More recently, in a small trial including 20 patients with HPV 16-positive advanced or recurrent gynecological carcinoma, HPV 16-SLP was subcutaneously administered with Montanide ISA-51 adjuvant. A good HPV-specific T cell response was detected. However, no tumor regression nor prevention of progressive disease were found^[122].

Recombinant proteins

Another approach for HPV immunization is the use of complete or recombinant proteins. Recombinant proteins have the advantage of providing all potential epitopes of an antigen, after processing by APC; but they are also more difficult to produce than peptides. Despite their longer size, proteins present low immunogenicity and they need to be mixed with adjuvants. For this reason, the E6 and E7 protein vaccines are made of viral proteins fused to proteins with more immunogenicity such as capsid proteins or bacterial heat shock proteins (Hsp).

L1VLPE7: L1VLPE7 is made of chimeric VLPs consisting of a carboxyl-terminally truncated HPV 16 L1 protein fused to the amino-terminal part of the HPV 16 E7 protein. The recombinant fusion proteins self-assembles into VLPs. In a small placebo-controlled clinical trial with 39 HPV 16-infected CIN 2/3 patients, these chimeric VLPs were able to induce antibodies with high titers against HPV 16 L1 and low titers against HPV 16 E7. Also cellular immune responses against both proteins were detected. Unfortunately, only a small no significant trend for histological improvement to CIN 1 or normal was observed in 39% of the patients^[123].

Another similar vaccine made with a recombinant HPV 16 L1(Δ N26)-E7(Δ C38) protein, expressed in *E. coli* has been tested in a murine of cervical cancer. This chimeric protein also assembles into chimeric VLPs. Immunization with these chimeric VLPs induced neutralizing antibodies and triggered cell-mediated immune

responses^[124].

SGN-00101: SGN-00101 is a fusion protein consisting of a Hsp from *Mycobacterium bovis* and HPV 16 E7 protein. This protein was given to patients with CIN 3, *via* three monthly subcutaneous immunizations. In 22% of patients regression to CIN 1 was observed. However, it was unclear whether this response was due to natural regression rather than the treatment^[125]. In another study, SGN-00101 was given in four injections 3 wk apart to patients with high-grade CIN. With this protocol seven of 20 patients (35%) had a complete regression that correlated with immune response^[126]. This vaccine also showed a partial response in 60% HIV-infected men with anal intraepithelial neoplasia^[127].

HPV16 E6/E7: A fusion protein formed by HPV 16 E6 and E7 proteins was mixed with the adjuvant ISCOMATRIX and given to patients with CIN. A specific HPV 16 E6 and E7 immune response was detected with an important contribution of CD8⁺ CTL response in immunized individuals than in placebo recipients. Elimination of HPV 16 DNA from lesions was detected in some treated patients and placebo recipients, but it did not correlate to immune status of patients^[128].

TA-CIN: Tissue antigen-cervical intraepithelial neoplasia (TA-CIN) is a recombinant fusion protein consisting of E6, E7, and L2 from HPV 16 and HPV 18. This fusion protein was tested in 29 patients with ano-genital intraepithelial neoplasia. They received three intramuscular doses of TA-CIN at four weekly intervals. Several immune parameters indicated an HPV-specific response. However, there was not a correlation between induction of systemic immunity and clinical outcome^[129]. Another study examined immunization of patients with vulval intraepithelial neoplasia with TA-CIN followed by one dose of a recombinant vaccinia virus encoding HPV 16 and 18 E6/E7 (TA-HPV). Only six out of 29 patients presented partial regression, and CTL activity was found in only four patients. Due to the poor outcome, this protocol has been abandoned^[130]. More recently a different protocol with TA-CIN was examined in patients with vulval intraepithelial neoplasia. In a phase II clinical trial 19 patients were given a topical application of the immunomodulator, Imiquimod, for 8 wk, followed by 3 doses of TA-CIN at four-week intervals. Complete regression was observed in 32% (6 out of 19) patients at week 10, increasing to 58% (11 out of 19) at week 20. At this time, there was also a significant local infiltration of CD8⁺ and CD4⁺ T cells in lesions of responding patients. Interestingly, non-responders had an increase of Treg cells. It seems that the inflammatory state induced by Imiquimod enhances the immune response, but the therapeutic effect still depends on the individual immune response of patients^[131].

DNA-based vaccines

Another interesting approach for immunization against HPV early proteins has been the use of plasmid DNA that

codes for the corresponding viral proteins. It is known that plasmid DNA, when injected into the skin or muscle can induce immune responses to encoded antigens. The process is relatively inefficient, but new technological advancements such as improved physical methods of delivery can induce more-potent cellular and humoral responses^[132].

pConE6E7: A DNA vaccine that encodes a HPV 16 or 18 consensus E6/E7 fusion gene (pConE6E7) has been tested in mice and rhesus monkeys. Immunization induced a potent cellular immune response against HPV 18 E6 and E7 antigens^[133]. Moreover, prophylactic immunization with this vaccine also induced complete protection against HPV E6 and E7-expressing tumors. In the case of established HPV-tumors, the vaccine was able to delay the growth of tumors^[134].

ZYC101a: ZYC101a is a microencapsulated DNA vaccine. It consists of plasmid DNA encoding E7-specific CTL epitopes from HPV 16 and 18 embedded in biodegradable micro particles. In a placebo-controlled trial, patients with histologically confirmed CIN 2/3 neoplasia were treated with three intramuscular doses of ZYC101a. Forty three percent of patients presented regression, compared to 27% of patients receiving placebo, but the difference was not statistically significant. However, in a subset of younger patients (less than 25 years of age) a significant reduction of lesions was detected in treated patients (70%) *vs* patients receiving placebo (23%). Unfortunately, no correlation between cytotoxic activity and clinical outcome was detected^[135].

Amolimogene: Amolimogene is another encapsulated plasmid DNA proteins of HPV types 16 and 18. In a phase II trial of patients with HPV-associated high-grade CIN, 11 out of 21 patients had elevated CD8⁺ T cell responses to HPV 16 and/or 18 peptides, and seven of these patients also had increases to corresponding HPV 6 and/or 11 peptides. No correlation between cellular immunity and clinical response was reported^[136].

Sig-E7 (detox)-HSP70: Sig-E7(detox)-HSP70 is a DNA vaccine encoding a fusion protein between HPV 16 E7 protein and heat shock protein 70. This vaccine was evaluated in patients with HPV 16 positive CIN 2/3. Patients received three intramuscular immunizations with increasing doses of plasmid DNA. Low frequency and weak HPV E7-specific T-cell responses were detected after treatment. Regression of lesions was seen in only 3 out of 9 patients, but the difference was not significant. Thus, no correlation was found between immune response and clinical outcome^[137].

Recombinant virus

The therapeutic approaches described above have given interesting results at the level of *in vitro* or animal models. Clinical results are at best slightly better than those expected for spontaneous regression, and in many cases

no clear correlation between the immune response and the clinical outcome were found. Another approach that has shown better results for treatment of HPV-induced lesions is the use of recombinant viruses. With this method, the HPV early gene products can be delivered directly into the cells. All the immune system responses against viruses get activated and a much better presentation of antigens takes place leading to a stronger cellular immune response. The highly attenuated poxvirus strain modified vaccinia virus Ankara (MVA) has become the vector of choice for novel HPV therapeutic vaccines^[115]. MVA is a non-replicating derivative of the uniquely successful smallpox vaccine, thus its use in humans is completely safe. In addition MVA is genetically stable, easy to manufacture, and very immunogenic^[138,139], in part due to cross-presentation of dying vaccinia virus-infected cells by DC to CD8⁺ T cells^[140]. Several MVA vectors against various diseases are now being evaluated in phase I/II clinical trials^[141].

TA-HPV: TA-HPV is a vaccinia virus encoding modified versions of the E6 and E7 genes from HPV 16 and HPV 18. In a phase II trial of HPV 16-positive high-grade vulval intraepithelial neoplasia, patients were immunized intramuscularly with TA-HPV. Forty two percent of patients showed partial reduction in total lesion diameter at 24 wk. Although, there was no increase in cytotoxic activity against selected individual HLA class I-restricted HPV 16 E6/7 peptides^[142]. In another study, eight out of 13 patients with high-grade VIN presented a partial reduction in lesion diameter, and an increase in lesion-infiltrating CD4⁺ and CD8⁺ T cells, however no increase in cytotoxic activity was detected^[143].

TG4001: TG4001 is a recombinant vaccinia virus (MVA) encoding a modified sequence of HPV 16 E6 and E7 proteins, and the human IL-2 gene. In a clinical study, 21 patients with HPV 16-related CIN 2/3 received three weekly subcutaneous injections of TG4001. Ten patients (48%) showed promising clinical responses at six months after treatment, but the related immune response was not reported^[144].

MVA E2: MVA E2 is also a recombinant vaccinia virus (MVA) encoding bovine papilloma virus E2 protein^[145]. In a series of studies, MVA E2 was evaluated in patients who had established HPV-induced CIN lesions. In a phase I / II clinical trial, for CIN 1 to CIN 3 lesions, 36 women received the recombinant virus vaccine at a total of 10⁷ MVA E2 virus particles injected directly into the uterus once every week over a 6-wk period. Ninety four percent (34) of patients showed complete elimination of precancerous lesions after treatment. In the other two patients, precancerous lesions were reduced from grade CIN 3 to CIN 1. In addition, 50% of patients eliminated completely the HPV, and in the remaining 50% of patients, HPV DNA was only 10% of the original viral load^[113]. Later, in a phase II clinical trial for high-grade



Figure 5 Effects of vaccinia virus Ankara E2 on cervix and vulva. Colposcopy of papillomavirus-induced intraepithelial lesions from patients treated with the therapeutic recombinant vaccinia virus Ankara E2. Photographs of (A) cervix with a cervical intraepithelial neoplasia 3 lesion, and (B) vulva with a condyloma lesion, before and after treatment. MVA: Modified vaccinia virus Ankara.

lesions (CIN 2 and CIN 3), 19 out of 34 (56%) patients had a complete regression, while in 11 (32%) more patients the lesions were reduced by 90%-60%. Specific cytotoxic activity against cancer cells correlated with clinical outcome^[114]. More recently, in a phase III clinical trial, 1176 female patients with ano-genital intraepithelial lesions were injected directly into the uterus, vulva, or anus lesions with 10^7 MEL-1 (MVA E2) virus particles. One thousand and forty-five out of 1176 (89%) treated patients showed complete elimination of lesions, and generated a specific cytotoxic response against papilloma-transformed cells (Figure 5) (Rosales, R. Manuscript in preparation). Thus, local application of MEL-1 vaccine is an excellent therapy for stimulating the immune system and creating regression of HPV-induced intraepithelial lesions. In support to this conclusion, a recent report, using the murine model of cervical cancer with HPV 16 E6- and E7-expressing TC-1 tumor cells, indicated that another recombinant HPV vaccine (TA-HPV) increased its efficacy when it was administered directly into the tumor. An augment in E7-specific CD8⁺ T cells was found in the blood, together with a significant decrease in tumor size^[146].

MVA-E1: As described above, both HPV E1 and E2 proteins are expressed early in the HPV life cycle, and are necessary to maintain coordinated viral replication and gene expression during differentiation of keratinocytes

along the epithelium. Thus, E1 is also a good candidate target for immunotherapy. Recently a new recombinant vaccinia virus has been reported, MVA-E1. This new vaccine consists of the MVA vector encoding the E1 sequence of HPV 16. Multiple injections of MVA-E1 into C57BL/6 mice resulted in sustained HPV 16 E1-specific cellular immune response involving T cells with cytotoxic activity^[147].

DC-based vaccines

Another approach to develop a cellular therapeutic vaccine is the use of dendritic cells pulsed with HPV antigens. Autologous monocytes are differentiated into DCs *in vitro* and then loaded with recombinant HPV proteins. The cells are then administered back to the patient in order to stimulate a better cellular immune response. Previous studies used autologous DCs loaded with HPV 16 or HPV 18 E7 protein to induce *in vitro* a specific T cell response. In 18/20 T cell lines from healthy blood donors E7-specific T cells were detected^[148]. This approach was also evaluated in patients with cervical cancer. Autologous monocyte-derived DCs were pulsed with recombinant HPV 16 E7 or HPV 18 E7 oncoprotein and administered to 15 stage IV cervical cancer patients^[149]. Four out of 11 patients had T cell proliferative responses, and three out of 11 patients showed γ -IFN production by ELISpot assay. Treatment was well-tolerated with no side effects. Unfortunately, an objective clinical response

was not observed^[149]. In a different study, a pilot trial gave autologous DCs pulsed with HPV E7 to four cervical cancer patients. E7-specific γ -IFN secreting CD8⁺ T cells were detected in all patients after vaccination^[150]. Later, in a phase I trial, autologous DCs pulsed with HPV 16/18 E7 protein and keyhole limpet hemocyanin were injected five times subcutaneously at 21-d intervals into 10 cervical cancer patients. Eight out of 10 patients had elevated counts of E7-specific γ -IFN secreting CD8⁺ T cells. Contribution of this immune response to therapy could not be evaluated since all patients were treated by surgery^[151]. These results show that this approach may be useful in the future for some difficult advanced cervical cancer patients. Another autologous DC vaccine for prostate cancer treatment, Provenge® (first FDA-approved therapeutic cancer vaccine) has given good results with metastatic patients. Thus, a great interest for new DC vaccines exists. However, this kind of treatment is labor-intensive and expensive, and has to be performed individually for each patient.

CONCLUSION

HPV infections remain an important public health issue because they are associated to cervical carcinoma, the second most common cancer among women^[4]. Two approved prophylactic vaccines, promise in the long run a reduction in HPV-related cancer incidence. However, the fact that full vaccination coverage of large populations will no be easy in many parts of the world^[73], and that these vaccines target only HPV 16 and 18 (HPV types responsible for about 60% of all cervical cancers), a large population will remain at a high risk of HPV-related disease, and in need of treatment.

Treatment for CIN and similar lesions has predominantly involved ablative therapies which purpose is to eliminate the damaged HPV-infected tissue, leaving the healthy tissue of the cervix intact^[79]. Ablative therapies include cryotherapy, excision procedures, laser therapy, and electrosurgery^[81]. Unfortunately, these surgical procedures are only effective in the treatment of pre-cancerous lesions, since after surgery many recurrences appear due to new re-infections.

Novel HPV protein-expressing vaccines are being evaluated for their potential to stimulate the immune system and generate a cellular cytotoxic response against the HPV-infected tissue or tumor. These new therapeutic vaccines can be grouped into five categories: peptide-based, protein-based, DNA vaccination, viral vectors, and DC-based immunization. They have shown variable results, but the ones using recombinant virus have demonstrated significant clinical efficacy and systemic HPV-specific cytotoxic T cell responses, particularly when the recombinant vaccinia virus is administered directly to lesions. Thus, recombinant vaccinia therapies are today the best candidates for a successful treatment of HPV-induced cancers.

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Estrogen receptors as the novel therapeutic biomarker in non-small cell lung cancer

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Abstract

Although a wide range of studies have addressed the relationship between estrogen receptor (ER) expression and prognosis in non-small cell lung cancer (NSCLC), that relationship remains controversial. This is in large part because there is no consensus on the rate of ER expression in NSCLC or on the intracellular distribution of ER expression. This suggests that establishing the relationship between ER expression and prognosis will require standardization of the antibodies used as well as the definition of a positive response. For example, it is supposed from previous studies that ERs in the cytoplasm and nucleus have different relationships to prognosis than ERs in the cytoplasm. Moreover, ER signaling in NSCLC is known to be affected by aromatase, progesterone receptor and epidermal growth factor receptor mutation. However, there has been little functional analysis these mutants and subtypes. This review will focus on what is known about the role of ERs in NSCLC and whether ER can be a useful prognostic marker or therapeutic target in NSCLC.

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Key words: Estrogen receptor; Non-small cell lung cancer; Epidermal growth factor receptor; Fulvestrant; Combined therapy

Core tip: Although there were many studies regarding

the role of estrogen receptor (ER) in non-small cell lung cancer (NSCLC), the rate of ER expression or the intracellular distribution of ER remains controversial. This suggests that establishing the relationship between ER expression and prognosis will require standardization of the antibodies used as well as the definition of a positive response. Furthermore, there has been little functional analysis for ER variants. This review will focus on what is known about the role of ERs in NSCLC and whether ER can be a useful prognostic marker or therapeutic target in NSCLC.

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INTRODUCTION

The estrogen receptor (ER) is one of the main targets of breast cancer therapy, and hormone therapy is generally administered to ER-positive breast cancer patients. There are two known ER subtypes, α and β . ER- α is the conventional receptor and is a useful prognostic marker in breast cancer. On the other hand, ER- β is a recently identified ER subtype, widely expressed in various organs including mammary gland and uterus. ER has also been detected in lung cancer cells. The first report of ER expression in lung cancer appeared in 1982^[1]. At that time, ERs were detected using radioimmunoassays, and the detection rate was relatively low. The first use of immunohistochemical staining to detect the ER in lung cancer tissue was reported by Canver *et al*^[2] in 1994. Since then, several different anti-ER antibodies have been employed to detect the receptor, and it has become apparent that there are differences in the detection rates and localization of the antigen, depending upon which antibody is used or the definition of a positive result^[3-30]. Further-

Table 1 Previous studies involving immunohistochemical detection of estrogen receptor- α in non-small cell lung cancer

Ref.	Antibody clone	Location	Detection rates
Canver <i>et al</i> ^[2]	NS	Nucleus	97%
Ollayos <i>et al</i> ^[3]	NS	Nucleus	7%
Su <i>et al</i> ^[4]	NS	Nucleus	6%
Di Nunno <i>et al</i> ^[5]	1D5	None	0
Omoto <i>et al</i> ^[6]	1D5	None	0
Dabbs <i>et al</i> ^[7]	1D5/6F11	None/nucleus	0/67%
Radzikowska <i>et al</i> ^[8]	1D5/6F11	Nucleus	3%/3%
Kawai <i>et al</i> ^[9]	HC-20	Cytoplasm	73%
Schwartz <i>et al</i> ^[10]	1D5/6F11	None	0/0
Wu <i>et al</i> ^[11]	NS	Cytoplasm	3%
Schwartz <i>et al</i> ^[12]	HC-20	Cytoplasm	66%
Márquez-Garbán <i>et al</i> ^[13]	HC-20	Nucleus/cytoplasm	45%/75%
Skov <i>et al</i> ^[14]	1D5	Nucleus/cytoplasm	3%/55%
Niikawa <i>et al</i> ^[15]	6F11	Nucleus	54%
Nose <i>et al</i> ^[16]	HC-20	Cytoplasm	84%
Raso <i>et al</i> ^[17]	6F11	Nucleus	36%
	HC-20	Nucleus/cytoplasm	5%/42%
	1D5	Nucleus/cytoplasm	34%/18%
Abe <i>et al</i> ^[18]	6F11	Nucleus	1%
Gomez-Fernandez <i>et al</i> ^[19]	1D5/6F11/SP-1	Nucleus	8%/14%/27%
Mauro <i>et al</i> ^[20]	6F11+HC-20	Nucleus/cytoplasm	38%/71%
Stabile <i>et al</i> ^[21]	HC-20	Nucleus/cytoplasm	39%/54%
Sun <i>et al</i> ^[22]	HC-20	Cytoplasm	36%
Rades <i>et al</i> ^[23]	1D5	NS	19%
Shimizu <i>et al</i> ^[24]	HC-20	Cytoplasm	47%
Rouquette <i>et al</i> ^[25]	1D5	Nucleus	9%
	F10	Nucleus	8%

NS: Indicates not specified.

more, Stabile *et al*^[31] demonstrated that both NSCLC and normal lung express ERs and show biological responses to estrogen. Consequently, the role of ER in lung cancer remains controversial.

It is now known that ER affects other signals, such as that mediated *via* epidermal growth factor receptor (EGFR)^[32]. Based on this finding, a clinical trial of the target plus hormone therapy is now ongoing^[33]. However, many questions remain unanswered. This is in part because the reasons for differences in the ER detection rate and the apparently different functions of ERs at different sites remain unclear. This review will focus on previous findings to assess the potential utility of ER as a prognostic factor and as the basis for novel therapeutic strategies for NSCLC, as well as the challenges that will need to be overcome in the future.

IMMUNOHISTOCHEMICAL DETECTION OF ER IN NSCLC

In breast cancer cells, the intracellular localization of ER- α is generally performed using clone 1D5 antibody, the epitope for which is in the N-terminus of ER- α . Using this antibody, ER- α is detected in the nucleus. Nuclear ER- α has also been detected using clone 6F11 antibody, which was raised against the full-length form of the receptor molecule^[7,8,15,17-20,25]. On the other hand, the rate of ER- α detection in NSCLC using clone 1D5 is very low, from 0%-7%^[5-8,10,17,19,23,25]. Moreover, ER- α is report-

edly located not only in the nucleus, but also in the cytoplasm and in the plasma membrane^[9,11-14,16,17,20-22,24]. Cytoplasmic and plasma membrane ER- α is mainly detected using clone HC-20 antibody, the epitope for which is in the C-terminus. The detection rate with this antibody is 70%-80%, much higher than with clone 1D5^[5-14,16,17,19-25]; indeed, we confirmed that ER- α detected using clone HC-20 is nearly always missed by clone 1D5. This suggests that ER- α detected by clone HC-20 may have an N-terminal deletion mutation that prevents its translocation to the nucleus^[9,31].

The reports published to date on the immunohistochemical detection of ER- α expression in NSCLC are listed in Table 1. It is noteworthy that the positivity rates vary depending on the definition of "positive" used and on the antibody. To establish ER- α as a prognostic marker in NSCLC, it will necessary to standardize the definition of "positive" based on the use of a particular antibody.

ER- β was first identified in 1996^[34], and the first report of ER- β expression in NSCLC was from Omoto *et al*^[6] in 2001. They observed that ER- β is expressed in lung carcinomas as well as in normal lung tissue. They also showed that adenocarcinomas expressed significantly more ER- β than squamous cell carcinomas. Unlike ER- α , strong expression of ER- β is observed in the cytoplasm as well as the nucleus of NSCLC cells. The reports published to date on immunohistochemical detection of ER- β expression in NSCLC are listed in Table 2. Three

Table 2 Previous studies involving immunohistochemical detection of estrogen receptor-α in non-small cell lung cancer

Ref.	Antibody clone	Location	Detection rates
Omoto <i>et al</i> ^[6]	NS	Nucleus	67%
Kawai <i>et al</i> ^[9]	H-150	Nucleus	51%
Schwartz <i>et al</i> ^[10]	PPG5/10	Nucleus	61%
Wu <i>et al</i> ^[11]	NS	Nucleus	46%
Márquez-Garbán <i>et al</i> ^[13]	Polyclonal	Nucleus	52%
		Cytoplasm	69%
Skov <i>et al</i> ^[14]	PPG5/10	Nucleus	84%
Niikawa <i>et al</i> ^[15]	MS-ER β13-PX1	Nucleus	90%
Ali <i>et al</i> ^[26]	PPG5/10	Nucleus	75%
Nose <i>et al</i> ^[16]	H-150	Nucleus	74%
Raso <i>et al</i> ^[17]	H-150	Nucleus	56%
		Cytoplasm	98%
	14C8	Nucleus	42%
		Cytoplasm	19%
Abe <i>et al</i> ^[18]	14C8	Nucleus	71%
Mauro <i>et al</i> ^[20]	NS	Nucleus	40%
		Cytoplasm	64%
Navaratnam <i>et al</i> ^[27]	14C8	Nucleus	49%
Rouquette <i>et al</i> ^[25]	Polyclonal	Nucleus	38%
Karlsson <i>et al</i> ^[28]	14C8	Nucleus	86%
Liu <i>et al</i> ^[29]	PPG5/10	Nucleus	45%
		Cytoplasm	59%

NS: Indicates not specified.

antibody clones were mainly used in those studies. The epitopes for clones H-150 and 14C8 are in the N-terminus of ER-β, and their detection rates in the nucleus are 51%-74% and 42%-71%, respectively^[9,16-18,27,28]. The epitope for the third clone, PPG5/10, is in the C-terminus, and the detection rate in the nucleus is 61%-84%^[10,14,26,29]. In recent years, expression of ER-β in NSCLC has been the focus of study more frequently than ER-α, including immunohistochemical analysis of ER-β variants^[29]. However, further study will be needed to determine which ER-β variant has the most impact in NSCLC. In immunohistochemical study, it should be made clear which ER expression (*i.e.*, type or location) is more responsible for the NSCLC progression. In addition, it should be also evaluated which antibody is reliable for detecting ER as the biomarker for NSCLC therapy.

On the other hand, there were some new studies on RNA expression of ERs in NSCLC^[35,36]. Brueckl *et al*^[35] reported that ER-α high expression was of significant positive prognostic value and patients with ER-α high tumors did not have any benefit from adjuvant chemotherapy. Atmaca *et al*^[36] demonstrated that ER-α mRNA expression was an independent prognostic factor in metastatic NSCLC. These studies were interesting because of different approach from immunohistochemistry, however, there were small size and needed to be more studied in the future.

ER AS A PROGNOSTIC MARKER IN NSCLC

It is well known that ER-α expression is a useful prog-

Table 3 Previous studies of estrogen receptors as prognostic markers in non-small cell lung cancer

Ref.	ER subtype	Methods	Location	Prognosis
Kawai <i>et al</i> ^[9]	α	IHC	Cytoplasm	Worse
	β	IHC	Nucleus	Better
Wu <i>et al</i> ^[11]	β	IHC	Nucleus	Better
Schwartz <i>et al</i> ^[10]	β	IHC	Nucleus	Better (male)
Skov <i>et al</i> ^[14]	β	IHC	Nucleus	Better (male)
Nose <i>et al</i> ^[16]	α	IHC	Cytoplasm	Worse
Abe <i>et al</i> ^[18]	β	IHC	Nucleus	Better
Mauro <i>et al</i> ^[20]	β	IHC	Nucleus	Better
Olivo-Marston <i>et al</i> ^[30]	α	RT-PCR	NS	Worse
Stabile <i>et al</i> ^[21]	β	IHC	Cytoplasm	Worse
Rouquette <i>et al</i> ^[25]	α	IHC	Nucleus	Better
Rades <i>et al</i> ^[23]	α	IHC	NS	Worse
Karlsson <i>et al</i> ^[28]	β	IHC	Nucleus	Better (ADCA)
Liu <i>et al</i> ^[29]	β2,5	IHC	Cytoplasm	Better

ER: Estrogen receptors; NS: Indicates not specified; ADCA: Adenocarcinoma; IHC: Immunohistochemistry; RT-PCR: Reverse transcription polymerase chain reaction.

nostic marker in breast cancer^[37,38]. In 2005, we first proposed that ERs could potentially serve as prognostic factors in NSCLC^[9]. In that study, we observed that cytoplasmic ER-α was predictive of a survival rate in NSCLC. The reports published to date on the relationship between ER expression and prognosis in NSCLC are listed in Table 3. Unlike in breast cancer, expression of ER-α in NSCLC cells is mainly observed in the cytoplasm, and its detection is associated with a poorer prognosis. Among those studies, only one reported that ER-α is predictive of a better prognosis in NSCLC^[25]. In that paper, unlike the others, ER-α was detected in the nucleus. Recently, Mauro *et al*^[20] reported that nuclear immunostaining for ER-α expression declines with age. However, it is unclear whether the age-related reduction in nuclear ER-α is associated with prognosis in NSCLC. The cytoplasmic ER-α in NSCLC is a variant type and may be associated with non-genomic signaling. However, it is still unclear whether or not cytoplasmic ER-α affects wild type nuclear ER-α. Thus, many questions remain unanswered about the role of ER-α in NSCLC.

In the time since we first reported that ER-β expression was an independent factor associated with a better prognosis of NSCLC^[9], there have been several other studies on the relation between ER-β and prognosis in NSCLC (Table 3). Most found that nuclear ER-β was predictive of a better prognosis in NSCLC. When nuclear ER-β is high in NSCLC, nuclear ER-α is low; *i.e.*, wild type ER-α is low. It is therefore thought that ER-β dominates estrogen signaling in NSCLC. On the contrary, a study by Stabile *et al*^[21] found that cytoplasmic ER-β is associated with a poorer prognosis. Interestingly, both ER-α and ER-β are associated with a poor prognosis in NSCLC when they are detected in the cytoplasm. This might be indicative of the non-genomic actions of ER variants.

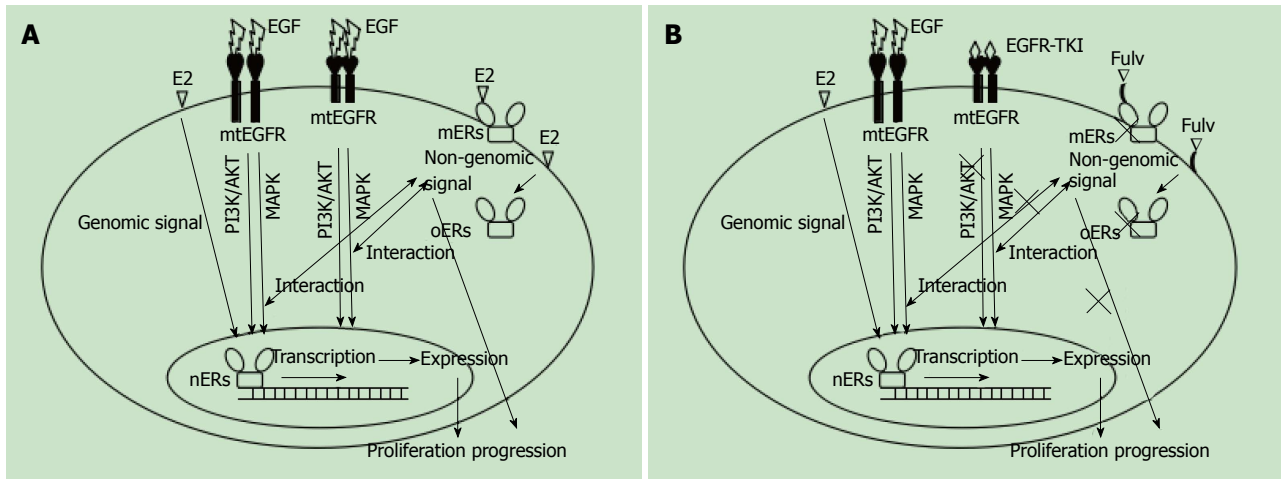


Figure 1 Intracellular estrogen receptor and epidermal growth factor receptor signaling pathway in non-small cell lung cancer. **A:** Mechanisms of ER and EGFR signaling in NSCLC. E2 (17 β -estradiol)-bound ER acts in part as a transcription factor in the nucleus. Once the ER binds to the DNA in the estrogen response element of a gene, it generally recruits co-activator complexes to modulate gene transcription (genomic signal). In addition, E2-bound ER also acts in the cytoplasm (non-genomic signal) by interacting with downstream mediators of EGFR signaling, such as MAPK and PI3K/AKT. EGFR is a cell membrane receptor tyrosine kinase that transmits a signal when it binds EGF; **B:** Mechanisms of combination therapy for NSCLC. The estrogen antagonist fulv binds to ERs and blocks estrogen signaling. EGFR-TKIs, such as gefitinib or erlotinib, bind to mtEGFR and inhibit EGFR signaling. Both treatments block the interaction between ER and EGFR signaling. EGFR: Epidermal growth factor receptor; ER: Estrogen receptor; TKI: Tyrosine kinase inhibitor.

THE ROLE OF ER VARIANTS IN CANCER CELLS

The role of ER variants in cancer cells has attracted much interest^[39-43]. It now appears that the oncogenic potential of ER variants derives from their ability to suppress the action of the normal hormone receptor, thereby acting as a dominant negative oncogene, or from their ability to activate hormone-responsive genes in a hormone-independent manner^[44]. ER has two functional domains: AF-1, which associates with a hormone-independent signaling pathway, and AF-2, which associates with a hormone-dependent pathway. Consequently, the oncogenic activity of an ER variant likely depends on specific site of its mutation.

In breast cancer, ER variants are often co-expressed with the wild type receptor, which can affect disease sensitivity to hormone therapy^[40,42]. For example, expression of the variant ER- α 36 affects estrogen signaling and is associated with tamoxifen resistance in breast cancer^[45]. In addition, the localization of ER variants in breast cancer cells reportedly differs from that of wild type ER^[42,46].

There has been very little study of ER- α variants in NSCLC, although they appear to be present, given the observed antibody-dependent variation in the ER positivity rate and intracellular localization. There have also been reports that the ER- β 1, 2 and 5 variants have distinct distributions within NSCLC cells and distinct effects on prognosis^[29]. However, there has been no analysis of the relationship between wild type ER- β and its variants, or their influence on estrogen signaling.

INTERACTION OF ASSOCIATED FACTORS WITH ER IN NSCLC

EGFR is a receptor tyrosine kinase involved in path-

ways leading to DNA synthesis and cell proliferation^[47]. Evidence suggests that ER interacts with one or more of the downstream mediators of EGFR signaling in NSCLC^[32,48-51], and that mutation of EGFR makes it susceptible to EGFR-tyrosine kinase inhibitor (EGFR-TKI), and thus a therapeutic target^[52,53]. The scheme of the interaction of between ER and EGFR signaling in NSCLC is shown in Figure 1A. It is well known that cancers usually become resistant to EGFR-TKI^[54], which raises the possibility that this drug resistance is related to the interaction between the EGFR and ER signals. For example, Stabile *et al*^[32] showed that ER signaling is activated by EGFR-TKI in lung cancer cells, while EGFR signaling is activated by anti-estrogen drugs.

Other ER-related mediators include progesterone receptor (PR), androgen receptor (AR) and aromatase. The functional importance of PR and AR in NSCLC remains unknown because expression levels are very low. Aromatase catalyzes the synthesis of estrogen in adipose tissue, and is associated with endogenous estrogen expression in NSCLC^[15]. In addition, BRCA1 is a regulator of ER signaling in breast cancer^[55-57], and was also recently detected in NSCLC^[58-60]. Rosell *et al*^[61] suggested that expression of BRCA1 and an EGFR variant carrying a T790M mutation (known as the EGFR-TKI resistant gene) is predictive of outcome and could provide the basis for alternative individualized treatment to patients with NSCLC. Although it is not yet clear whether BRCA1 is associated with ER in NSCLC, such an association could be a critical determinant of ER signaling in NSCLC.

NOVEL THERAPEUTIC STRATEGIES TARGETING ER AND EGFR IN NSCLC

Clinical trials of lung cancer treatments targeting ER and EGFR are currently ongoing^[62]. In these trials, fulvestrant,

an ER antagonist, is used in nearly all tests. Fulvestrant acts on ER, blocking its signal, irrespective of whether the receptor is localized in the nucleus, cytoplasm or cell membrane (Figure 1B)^[63]. It is therefore thought that fulvestrant would be effective, even in tamoxifen-resistant breast cancers^[64]. However, fulvestrant is a selective ER- α antagonist, and in one report fulvestrant acted to stabilize, and thus enhance, ER- β signaling^[63]. In NSCLC, extranuclear ER- α is a variant type thought to be involved in non-genomic signaling^[31], whereas ER- β is localized in the nucleus, where it exerts genomic effects and associates with a better prognosis^[9-11,14,18,20]. It is possible that the genomic signal mediated by ER- β is enhanced by fulvestrant's blocking of the non-genomic signal of the ER- α variant. Further studies will be needed to resolve the mechanism underlying the therapeutic effects by fulvestrant in NSCLC.

Erlotinib and gefitinib are two tyrosine kinase inhibitors used in the treatment of lung cancer. Erlotinib is used far more to target EGFR and, notably, the effects of both these medications may vary depending upon the race and/or gender of the patient. The intratumoral concentration of gefitinib reaches levels 40 times higher than that in blood^[65]. In other words, gefitinib is well distributed to the target tissue. By contrast, intratumoral concentrations of erlotinib are generally lower than its blood concentration^[65,66]. Nonetheless, erlotinib's IC50 for EGFR is more than 10 times lower than that of gefitinib^[67]. That is, the effective anti-EGFR dose of erlotinib is substantially lower than that of gefitinib. Clinical findings show that both drugs produce an effective response against mutant EGFR, but are less effective against wild type EGFR. To establish a combination therapy targeting both ER and EGFR signaling, development of an antagonist effective against wild type EGFR will be necessary. For example, if the intratumoral concentration of erlotinib could be increased without raising its blood concentration, it may be possible to enhance its antitumor effects without worsening its side effects. With that aim, the use of erlotinib with the angiogenesis inhibitor bevacizumab is being considered^[68]. In addition, because EGFR forms a heterodimer with HER-2, the use of the HER-2 inhibitor trastuzumab may be an effective approach to treatment. It may also be useful to consider the interaction of EGFR and/or ER with the ALK fusion protein, which is expressed exclusively with the variant receptors.

CONCLUSION

The role of ER in NSCLC is gradually becoming clearer. However, ER is involved in a complicated network through its interaction with a variety of mediators. In conventional immunohistochemical studies, wild type ER and its variants are handled similarly, but this may not be the best approach to future development of new strategies for treating NSCLC. Instead, it will be important to establish novel treatments based on the specific ER types

dominant in the particular lung cancer being treated, and to select the most effective drugs in that context.

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Incidence and management of Ziv-aflibercept related toxicities in colorectal cancer

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common side effects were diarrhea, stomatitis, fatigue, hypertension, weight loss, loss of appetite, abdominal pain, and headache. As the use of Ziv has become more widespread in oncology practices, familiarity with the toxicity profile of the drug and the use of practice guidelines for their treatment has become increasingly important. This review will address the toxicities noted in trials using Ziv for the treatment of mCRC, and will provide recommendations for toxicity management.

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Key words: Ziv-aflibercept; Colon cancer; Metastatic; Bleeding; Hypertension

Core tip: Ziv is an anti-angiogenic agent which has shown survival benefit in colorectal cancer. Side effects of this drug include hypertension, bleeding, perforation and delayed wound healing among others. In this paper, we review the side effects of Ziv and discuss how to manage those toxicities.

Abstract

Ziv-aflibercept (Zaltrap, Ziv) is a humanized fusion protein constructed by joining the vascular endothelial growth factor (VEGF) binding portions of human VEGF receptors 1 and 2 to the Fc portion of human immunoglobulin IgG1. Recently, a randomized, open-label, phase III study compared 5-fluorouracil, leucovorin, irinotecan (FOLFIRI)/Ziv with FOLFIRI/placebo in patients who had been previously treated with oxaliplatin based chemotherapy for metastatic colon cancer (mCRC). Patients who had received prior bevacizumab therapy were also eligible. This study showed that the addition of Ziv improved overall survival with median survival time of 13.5 mo *vs* 12.06 mo in ziv *vs* placebo arm. Ziv also improved progression free survival from 4.67 mo to 6.9 mo with a response rate of 19.8% in the Ziv/FOLFIRI group *vs* 11.1% in FOLFIRI alone group. This led to the approval of Ziv in combination with FOLFIRI in metastatic colon cancer patients treated with prior oxaliplatin regimens. The most

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INTRODUCTION

Angiogenesis is critical for tumor proliferation and metastasis for several malignant tumors^[1]. Vascular endothelial growth factor (VEGF) is a homodimeric protein that binds to and activates receptors VEGFR1 and VEGFR2, located on the vascular endothelium^[2]. This leads in stimulation of downstream signaling leading to inhibition of apoptosis, stimulation of mitosis and cytoskeletal changes associated with motility^[3]. The family of VEGF

proteins binds to several different VEGFRs with distinct binding and signaling properties. VEGFR-1, VEGFR-2, and VEGFR-3 have similar structural features and form homodimers upon ligand binding. VEGFR-1 interacts with placental growth factor (PlGF), VEGF-B, and VEGF-A. VEGF has a higher affinity for VEGFR1, but VEGFR1 has relatively weaker tyrosine kinase activity. VEGFR-2 can interact with the processed forms of VEGF-C and D in addition to VEGF. This receptor is the major mediator of the mitogenic and angiogenic effects of VEGF. VEGFR-3 only interacts with VEGF-C and D and is involved in lymphangiogenesis^[2,4,5].

VEGF is an important regulator of angiogenesis and is principally stimulated by hypoxia. VEGF is over-expressed in several malignancies including gastro-intestinal tumors. Over expression has been associated with increased tumor vascularity, proliferation, progression, invasion, and metastasis^[6,7]. VEGF inhibition represents a promising venue for an anticancer approach. Furthermore, VEGF levels are elevated in patients with metastatic colorectal cancer, suggesting that VEGF induced vascular permeability could contribute to the formation of malignant ascites^[8]. VEGF antagonists have also been shown to increase the intratumoral delivery of cytotoxic chemotherapeutic agents thereby improving their anti-tumor efficacy without increasing toxicity^[9]. Previously, bevacizumab had shown promising results in patients with untreated metastatic colorectal cancer (mCRC). Hurwitz *et al.* showed the benefit of adding bevacizumab to standard chemotherapy in this first phase III trial of mCRC^[10].

Ziv-Aflibercept (Ziv) is a novel antiangiogenic agent which is a fusion protein consisting of human VEGF receptor extracellular domains fused to the Fc portion of human immunoglobulin G1 (IgG₁). It contains sequences encoding IGg domain 2 from VEGFR1 fused to Ig domain 3 from VEGFR2, which in turn is fused to the hinge region of the human IgG₁ Fc domain. Ziv complexes with VEGF in the blood and extravascular space and prevents VEGF from interacting with its receptors on endothelial cells. The VEGF trap binds with high affinity to soluble VEGF but also binds to the proangiogenic factors VEGF-B and PlGF 1 and 2. The combination of these actions may lead to the increased activity of Ziv as an antiangiogenic agent.

CLINICAL EFFICACY OF ZIV-AFLIBERCEPT

A phase I, dose escalation study of Ziv in combination with infusional fluorouracil, leucovorin and oxaliplatin, (FOLFOX4) was studied in 32 patients with advanced solid tumors^[11]. The types of cancer of these 32 patients were as follows: 8 pancreatic, 3 cholangiocarcinoma, 3 gastric, 5 breast, 4 ovarian and 7 other malignancies. In this study, 5 partial responses were noted in the pancreatic, cholangiocarcinoma and colorectal cancers while 10 patients had stable disease. Given the study results, Ziv 4

mg/kg combined with FOLFOX4 was selected for further investigations.

Another Japanese phase I study assessed the use of Ziv with infusional fluorouracil, leucovorin, and irinotecan (FOLFIRI) in metastatic colorectal cancer^[12]. Ziv, at two dose levels, was administered in combination with FOLFIRI in 16 patients who had received prior chemotherapy and the combination was found to be well tolerated. A phase III, randomized study published recently evaluated Ziv in combination with FOLFIRI in metastatic colorectal cancer patients^[13]. Six hundred and twelve patients were randomized to Ziv or placebo in combination with FOLFIRI with the primary endpoint of overall survival (OS). This study showed that the addition of Ziv to FOLFIRI significantly improved OS and progression free survival as compared to placebo. Response rates were also better in the Ziv group with the main side effects being anti-VEGF related effects.

ZIV-AFLIBERCEPT RELATED TOXICITIES

In the phase III trial of Ziv in colorectal cancer, grade 3 and 4 toxicities were increased in the Ziv arm as compared to the control arm. These included bleeding (2.9% *vs* 1.7%), arterial thromboembolic events (1.8% *vs* 0.5%) and venous thrombotic events (7.9% *vs* 6.3%) (Table 1). Grade 3 hypertension was seen in 19.1% of patients receiving Ziv *vs* 1.5% of patients in the control arm. Grade 4 hypertension was seen in only 1 patient (0.2%) in the Ziv arm. There was no difference in the development of gastrointestinal (GI) fistulae, other fistulae, or GI perforation between the groups. Acute infusion related reactions, grade 3 or 4, were no different and were reported to be 0.5% in both arms. The development of grade 3 or 4 proteinuria was found in the 7.9% of patients in the Ziv arm as compared to 1.2% in control arm. Two of the patients with grade 3 or 4 proteinuria in the Ziv arm developed nephrotic syndrome.

The incidence of chemotherapy related toxicities was also found to be increased with the addition of Ziv. The toxicities included diarrhea (19.3% *vs* 7.8%), asthenia (16.9% *vs* 10.6%), stomatitis and ulceration (13.7% *vs* 5%), infections (12.3% *vs* 6.9%) and palmar plantar erythrodysesthesias (2.8% *vs* 0.5%). Hematologic toxicity was seen more commonly with this agent. Grade 3 or 4 neutropenia occurring in 36.7% of the patients in Ziv arm *vs* 29.5% in the control arm; complicated neutropenia also occurred more frequently in the Ziv arm (0.7% *vs* 2.8%). Thrombocytopenia was also seen more commonly in Ziv arm (3.3% *vs* 1.7%). Permanent discontinuation of chemotherapy due to adverse events was also seen more commonly in the Ziv arm (26.8% *vs* 12.1%). The toxicities most frequently leading to discontinuation of chemotherapy in the study *vs* control arm were asthenic conditions (3.8% *vs* 1.3%), infections (3.4% *vs* 1.7%), diarrhea (2.3% *vs* 0.7%) and hypertension (2.3% *vs* 0%) (Table 2).

Hypertension

Grade 3 hypertension (HTN), defined as hypertension re-

Table 1 Selected grade 3/4 adverse events (%) with FOLFIRI/ziv-aflibercept in VELOUR study

Adverse event	Grade 3 %	Grade 4 %
Fatigue	11.9	0.7
Asthenia	4.9	0.2
Proteinuria	7.5	0.3
Urinary tract infections	0.8	0.0
Neutropenia	23.1	13.6

quiring the addition or modification of antihypertensive agents, is one of the more common toxicities associated with Ziv treatment. HTN can occur at any time during the course of treatment. There have been no deaths due to HTN reported. In the phase I Japanese study, any grade level of HTN was noted in 8 of 13 patients (61.5%) in 4 mg/kg arm and grade 3/4 in 4 patients (30.8%)^[14]. In the phase II study of Ziv administered at 4 mg/kg, the most common treatment related adverse events were HTN, proteinuria, fatigue and headache^[15]. In another phase I study of Ziv in advanced solid tumors, HTN was observed in 38.3% of patients^[16]. HTN of any grade occurred in 0%, 14.3%, 16.7%, 14.3%, 57.1%, 75.0%, and 61.5% of patients at the increasing dose levels of 0.7, 1, 2, 3, 4, 5 and 7 mg/kg respectively, and hypertension of grades 3 to 4 occurred in 0%, 0%, 16.7%, 0%, 42.9%, 75.0%, and 46.2% of patients respectively. The median time to onset of HTN in this study was 3.5 d.

In the phase III trial by Van Cutsem *et al*^[13], HTN in Ziv/FOLFIRI treatment group for all grades, grade 3 and 4 was 41.4%, 19.1% and 0.2%, respectively. In the FOLFIRI/Placebo group, HTN of all grades and grade 3 was 10.7 and 1.5% respectively. There was no grade 4 HTN in the chemotherapy alone arm^[13].

The variation in rates of higher grade of HTN among all of these trials may be due to several factors, including the differing sample sizes across the studies, differing thresholds among practitioners to initiate anti-hypertensive therapy, or variations in patient population across studies. In general, though, most studies do indicate that Ziv increases the risk of developing HTN.

Proteinuria

The risk of developing proteinuria has been shown in the VEGF inhibition pathway as demonstrated with previous bevacizumab related therapies. In the phase I study of Ziv at 2 dose levels, proteinuria of grade 2 or lower was observed in 3 patients (23.1 %) receiving the 4 mg/kg dose^[14]. In the phase I dose escalation study of Ziv in advanced cancers, proteinuria was observed in 10.6% of patients at different dose levels, but none of them were grade 4^[16]. The median time of onset of proteinuria was 15 d in this study.

In the phase III study, the incidence of proteinuria seen in Ziv/FOLFIRI treatment group in all grades, grade 3 and 4 was 62.2%, 7.5% and 0.3%, respectively. Two patients developed nephrotic syndrome. In the FOLFIRI/Placebo group, proteinuria of all grades and grade 3 was 40.7% and 1.2% respectively. There was no

grade 4 proteinuria reported in the placebo arm. There was no correlation between proteinuria and HTN.

Gastrointestinal perforation

There was no GI perforation noted at different dose levels of Ziv in the phase I study by Lockhart *et al*^[16]. In the phase III trial by Van Cutsem, the incidence of all grades, grade 3 and 4 toxicities were 0.5, 0.2 and 0.3%, respectively in Ziv + FOLFIRI treatment group compared to 0.5, 0.2 and 0.2%, respectively, in placebo + FOLFIRI group. The incidence of grade 3 or 4 GI fistula, other fistulae or GI perforation was less than 2% in both treatment groups^[13]. In the phase II study of recurrent ovarian cancer with malignant ascites, Ziv *vs* placebo showed a higher rate of intestinal perforation (three patients) than placebo (one intestinal fistula)^[17].

Thromboembolism

In the phase I study by Lockhart, there were no reported thromboembolic events^[16]. In a phase I dose escalation study of 18 patients treated with Ziv, pemetrexed, and cisplatin, pulmonary embolism was observed in 11% and deep vein thrombosis was seen in 6% of the patients^[18]. In the recent Phase III study, arterial thromboembolic events were seen in 1.8% in the Ziv + FOLFIRI arm compared to 0.5% in the placebo + FOLFIRI arm. Venous thromboembolic events were seen in 7.9% of patients receiving Ziv + FOLFIRI compared to 6.3% receiving placebo + FOLFIRI^[13].

Hemorrhage

In the previous phase III trials of bevacizumab, there was no significant increase in the risk of bleeding with bevacizumab^[10]. In the phase III VELOUR trial of Ziv with FOLFIRI, 37.8% patients had any grade of hemorrhage, 2.8% patients had grade 3 and 0.2% of patients had grade 4 hemorrhage in Ziv arm^[13]. In the chemotherapy only arm, 19% patients had any grade of hemorrhage and 1.7% patients had grade 3 hemorrhage. There was no reported grade 4 hemorrhage. A higher incidence of grade 3 and 4 hemorrhages was observed in the Ziv group (2.9%) as compared to the placebo group (1.7%).

PATHOGENESIS OF TOXICITIES ASSOCIATED WITH ZIV

The exact of mechanism underlying the toxicities related to Ziv is not fully known at this time.

Hypertension

The effect of Ziv on the development of hypertension is not completely understood. The control of blood pressure is complicated and is related to the factors affecting cardiac output and/or total peripheral vascular resistance^[19]. One explanation for Ziv-associated hypertension may be due to its effects on VEGF inhibition. Under normal circumstances VEGF stimulates nitrous oxide release which in turn causes vasodilation. Inhibition

Table 2 General guidelines for Ziv-aflibercept dosing and schedule modification due to adverse events per CTCAE 4.0

Event	Action to be taken
Hypertension	
Grade 3	If not controlled with medication, discontinue Ziv
Grade 4	Discontinue Ziv
Proteinuria	
> 2 g protein/24 h	Hold Ziv until proteinuria improves to < 2 g of protein/ 24 h
	Discontinue Ziv in a patient with > 2 g proteinuria/24 h that does not resolve in 3 mo time after holding Ziv. Work-up for proteinuria such as renal biopsy should be considered
grade 4 proteinuria (nephrotic syndrome)	Discontinue Ziv treatment
Gastrointestinal perforation	
Gastrointestinal perforation or dehiscence	Discontinue Ziv
Thromboembolic events	
Grade 3 venous thromboembolic event	Hold Ziv treatment
or incidentally discovered pulmonary embolus first occurrence	If the planned duration of therapeutic-dose anticoagulant therapy is ≤ 2 wk, Ziv should be held until the period of therapeutic-dose anticoagulant therapy is over
	If the planned duration of therapeutic-dose anticoagulant therapy is > 2 wk, Ziv should be held for 2 wk and then may be resumed during the period of therapeutic-dose anticoagulant therapy as soon as all of the following criteria are met:
	The patient must be on a stable dose of anticoagulant and, if on warfarin, have an INR within the target range (usually between 2 and 3) prior to restarting study drug treatment
	The patient has no history of Grade 3 or 4 hemorrhagic events before starting Ziv
	The patient has no evidence of tumor invading or abutting major blood vessels on any prior CT scan
Any grade arterial thromboembolic event or symptomatic	Discontinue Ziv
Grade 4 venous thromboembolic event first occurrence	
Hemorrhage	
Grade 1 and 2	No dose modification
Grade 3 or 4 (first occurrence)	Discontinue study treatment

of VEGF may reduce the amount of nitrous oxide available in turn causing vasoconstriction^[20]. This vasoconstriction leads to increased peripheral vascular resistance resulting in hypertension^[21]. VEGF may also have effects on the renin-angiotensin system, which plays a part in the regulation of homeostasis and blood pressure^[20]. The exact mechanism of the VEGF and the angiotensin system interaction is not well known at this time.

Proteinuria

The pathogenesis of proteinuria may be related to the effects of VEGF on the renal glomerulus. VEGF inhibition induces downregulation of nephrin, sometimes leading to nephritic syndrome or glomerular thrombotic microangiopathy^[22]. In this MEDLINE review, authors found that mild proteinuria ranged from 21%-63% and severe proteinuria in up to 6.5% of renal cell carcinoma patients. In some cases, discontinuation of the anti-VEGF agent induced improvement of proteinuria, but in many cases proteinuria persisted. Thus urinary protein should be checked periodically in these patients and if patients develop proteinuria they should be referred to nephrologists.

Neutropenia

Grade 3-4 neutropenia occurred in 30% of patients in the FOLFIRI arm and 37% of patients in the FOLFIRI + Ziv arm. Complications of grade 3 neutropenia occurred in 1.7% of patients in chemotherapy alone group and 4.4% in Ziv group. Complications of grade 4 neutropenia were seen similarly in both groups.

Wound healing

VEGF is associated with wound healing and VEGF inhibitors can affect dermal-wound angiogenesis causing delayed wound healing.

Gastrointestinal perforation

The mechanism underlying GI perforation is not known. However, it seems to be multi-factorial. The role of GI pathology related to the tumor, such as carcinomatosis, cannot be excluded in addition to the inhibition of VEGF. To date, however, no baseline risk factors for GI perforation have been identified.

Thromboembolism

Malignancies cause a hypercoagulable state from the procoagulant activity of cancer cells associated with tissue factor and fibrin generation^[23]. Thrombosis associated with VEGF inhibition was initially thought to be mainly arterial, but recent data have also reported venous thrombosis^[24]. The pathogenesis of thrombosis could be related to VEGF's role in vascular integrity. VEGF inhibition is speculated to cause apoptosis of endothelial cells thus resulting in the exposure of subendothelial cells which initiate the coagulation cascade causing the thrombosis^[25].

Hemorrhage

The exact mechanism of abnormal hemorrhage from VEGF inhibition is complex and yet not fully understood. The bleeding risk could be from VEGF's effect on the vascular endothelium promoting endothelial cell survival and vasculature integrity. VEGF inhibition may

decrease the renewal capacity of damaged endothelial cells causing bleeding^[26].

MANAGEMENT OF TOXICITIES

Hypertension

Patients receiving Ziv should have baseline documentation of blood pressure and frequent monitoring. Blood pressure should be monitored done at least once every 2 wk while on treatment. It is optimal for the measurement to be done after the patient has been in a resting/seated position for more than 5 min. If the initial reading is ≥ 140 mmHg systolic and/or ≥ 90 mmHg diastolic then a repeat measurement should be taken for verification.

For Grade I hypertension, defined as an asymptomatic, transient (< 24 h) increase by > 20 mmHg (diastolic) or to $> 150/100$ mmHg in a patient previously within normal range, no intervention is indicated. For Grade 2 hypertension, defined as recurrent or persistent (> 24 h) or a symptomatic increase by > 20 mmHg (diastolic) or an increase to $> 150/100$ mmHg if previously within normal range, anti-hypertensive medications such as calcium channel blockers, beta blockers, and/or ACE inhibitors should be considered. One can consider temporary discontinuation of Ziv if the blood pressure is not controlled with anti-hypertensive medications and once hypertension is controlled to $< 150/100$ mmHg, patients may continue on Ziv. For persistent or symptomatic hypertension or Grade 3 hypertension the Ziv should be held. Once the blood pressure is controlled subsequent cycles of Ziv should resume at a dose of 2 mg/kg. This dose should not be re-escalated. If blood pressure cannot be controlled with anti-hypertensive medications then the Ziv should be permanently discontinued.

Although ACE inhibitors seem more logical as they can control HTN and may also decrease the amount of proteinuria, no data to determine the best anti-hypertensive agent is available. Moreover, caution should be taken when using diuretics as these patients on cytotoxic agents are at risk for diarrhea, dehydration and volume depletion. In the event of grade 4 hypertension or hypertensive crisis or encephalopathy, Ziv should be permanently discontinued.

Proteinuria

Patients should be evaluated for proteinuria prior to starting the treatment and monitored regularly throughout treatment. We recommend a baseline urinalysis (dipstick or microscopic) to assess for protein before initiating Ziv. If the degree of proteinuria is \leq grade 1, Ziv can be given. If proteinuria is \geq grade 2, urinary protein creatinine ratio should be obtained. If protein creatinine ratio is more than 1, a 24-h urine collection to quantify protein should be sent for further evaluation. In a patient with urine protein > 2 g/24 h, as recommended in the package insert, we recommend holding Ziv until proteinuria improves to < 2 g/24 h of protein. If the 24-h urine does not improve within 3 mo, we recommend discontinuing Ziv permanently.

If proteinuria recurs, Ziv should be held till it improves to < 2 g/24 h and consider reducing Ziv dose to 2 mg/kg (rule of 2). Some clinicians elect to continue Ziv therapy until protein levels exceed 3.0 to 3.5 g/24 h. As there is no definitive data at this point, the decision of when to stop Ziv therapy requires clinical judgment. In patients with Grade 4 proteinuria (nephrotic syndrome) or thrombotic microangiopathy, Ziv should be permanently discontinued.

Neutropenia

Complete blood count should be monitored at baseline and prior to each cycle of therapy. If the neutrophil count is less than $1.5 \times 10^9/L$, chemotherapy + Ziv should be delayed until the neutrophil counts recover above $1.5 \times 10^9/L$.

Wound healing complications

Patients who undergo any invasive procedure while receiving Ziv may encounter problems with wound healing as seen previously with other VEGF inhibitors. Thus, we recommend holding Ziv at least 4 wk prior to and after the elective surgery. Ziv should be held until the surgical wound is fully healed. For minor surgeries like tooth extraction, biopsy or port placement, Ziv should be held till the wound is healed. If the wound is not healing well, Ziv should be discontinued permanently. For emergent surgery, the patient, surgeon, and nursing staff should be aware of the possible risks due to Ziv. In general, clear communication of the risks of Ziv should be done with the surgeon.

Gastrointestinal perforations

In the phase III trial of Ziv in colorectal cancer, grade 3 GI perforation was same in the chemotherapy and chemotherapy with Ziv arms^[13]. Grade 4 GI perforation was seen in 0.2% of patients in the FOLIRI arm and 0.3% of patients in the Ziv arm. Since previous VEGF inhibitors, such as bevacizumab, have shown a possible increase in the risk of GI perforation, Ziv should be held for 1-2 mo after surgery and till the surgical wound is completely healed. Patients should be closely monitored for signs and symptoms of GI perforation.

The exact interval for holding Ziv is unknown; however its long elimination half-life of 6 d should be taken into account. For elective operations, Ziv should be discontinued at least 30-60 d prior to a scheduled surgery. Even though Ziv's half-life is shorter than bevacizumab, we recommend holding Ziv for at least 4 wk prior to or after the surgery until more data is available. In cases of emergent procedures, Ziv should be held and patients followed closely for complications. Once patients develop GI perforation, Ziv should be discontinued.

Thrombosis

In patients who develop grade 3 thromboses, Ziv should be held. If a patient has grade 4 thrombosis, Ziv should be discontinued. We recommend that patients with a se-

vere arterial thromboembolic event during Ziv treatment should discontinue treatment permanently. Many patients with a prior arterial thromboembolic event are routinely treated with low dose aspirin. These patients can continue with Ziv. The use of high dose aspirin cannot be recommended due to the lack of safety data. Warfarin treatment during Ziv therapy should be followed very closely with serial INRs. If the INR is therapeutic, Ziv can be resumed as long as there is no history of severe bleeding associated with Ziv.

Hemorrhage

In patients who experience grade 3 hemorrhage, in the absence of any coagulation disorder that could increase their risk of bleeding, Ziv should be held till the bleeding resolves. Patients with grade 3 hemorrhage on full-dose anticoagulation, Ziv should be discontinued. If the patient experiences a repeat grade 3 or a new grade 4 hemorrhagic event, Ziv should be permanently discontinued. Brain metastases were excluded from the clinical trials and the use of Ziv in these patients could not be recommended.

CONCLUSION

Ziv in combination with FOLFIRI has shown a survival benefit in colorectal cancer^[13]. Ziv has been shown to be associated with the following higher ($\geq 5\%$) grade 3-4 adverse events including neutropenia, diarrhea, hypertension, leucopenia, stomatitis, fatigue, proteinuria and asthenia. Hypertension and proteinuria can occur at any time during treatment, while the risk of GI perforation is increased within 60 d of starting Ziv treatment. While hypertension is the most common adverse event, it is usually managed with oral agents. Wound healing difficulties, while infrequent, are most common when major surgery occurs while the patients are being treated with Ziv. There is potential for GI perforation, and while it is a rare complication, care must be given to patients who require surgery before, during or after Ziv treatment.

This review is mainly focused on Ziv in the treatment (Table 3) of colorectal cancers. Ziv has been studied in other malignancies including lung cancer and with other combinations of chemotherapy. The nature and rate of toxicities was similar in trials with other malignancies. Overall, the risk of grade 3 or 4 hypertension, hemorrhage and thromboembolism with Ziv appears to be low across all studies. In the colorectal cancer studies, GI perforations, while a serious event, have been relatively infrequent. In other malignancies this toxicity may be more of a concern. While the mechanism underlying this increased incidence of GI perforation is unknown, it may be related to the fact that these patients had very advanced disease and many patients had undergone multiple debulking surgeries.

To date, the efficacy and toxicity of Ziv has primarily been evaluated in patients with advanced disease, and the side effects of long-term therapy with Ziv in patients

with less advanced tumors is still unclear. This paper summarizes the guidelines by taking the information from the published data and extrapolating from related drugs like bevacizumab which has similar mechanism of action. As the trial data matures, we will have more clarity on the safety of Ziv. As more data becomes available, specific guidelines for the management of Ziv-related toxicities will become more detailed as well. This will be vital as anti-angiogenic agents become a more integral part of the standard care of patients with colorectal and other malignancies. Overall, though, the clear benefits of anti-angiogenic therapy vastly outweigh the small risks in the majority of patients. The key to administering treatment safely will be through education of patients, nurses and other healthcare providers.

EXPERT OPINION

Ziv is a recombinant fusion protein consisting of VEGF-binding portions from extracellular domains of human VEGF receptors 1 and 2 fused to the Fc portion of the human IgG1 that is designed to inhibit VEGF, thus inhibiting angiogenesis. Ziv has shown to extend survival in previously treated colorectal cancer. Ziv has provided an increase in median overall survival (13.5 mo *vs* 12.06 mo, $P = 0.0032$) when combined with FOLFIRI *vs* FOLFIRI alone. Clinical benefit, as measured by overall survival, was observed across all patients. Significant improvements in response rate and duration of response in combination with FOLFIRI were also achieved. The overall response rate was also improved in Ziv arm compared to FOLFIRI. Based on these data, Ziv was approved in combination with FOLFIRI by the FDA for the treatment of metastatic CRC, and is currently undergoing vigorous exploration in clinical trials for the treatment of many solid tumors, including non-small cell lung cancer.

In trials, Ziv was combined with conventional chemotherapy making it difficult to identify which toxicities are unique to it. However, since the adverse effects of chemotherapy are well characterized it is reasonable to assume that any new or unexpected adverse effects can be attributed to Ziv. It does not appear that any overlapping hematologic or gastrointestinal adverse effects exist between Ziv and conventional chemotherapy. Common adverse events associated with Ziv include HTN, proteinuria, and epistaxis (mainly mild). More patients in the Ziv arm of these studies experienced at least one grade 3 or 4 toxicity compared with chemotherapy alone, primarily attributable to HTN. Other frequent adverse events associated with Ziv are bleeding episodes and thrombotic events. The manufacturer has issued a black box warning regarding the risk of gastrointestinal perforation, wound dehiscence, and fatal hemoptysis.

The overall rate of grade 3-4 hypertension related to Ziv was approximately 19%. HTN can occur at any time during the course of treatment. No deaths from HTN have been reported and $< 1\%$ of patients has discontinued therapy due to HTN. Blood pressure is typically

Table 3 Recommendations for Dose Modification and Treatment Delay for Zaltrap

Discontinue ZALTRAP for:	Severe hemorrhage Gastrointestinal perforation Compromised wound healing Fistula formation Hypertensive crisis or hypertensive encephalopathy Arterial thromboembolic events Nephrotic syndrome or thrombotic microangiopathy (TMA) Reversible posterior leukoencephalopathy syndrome (RPLS)
Temporarily suspend ZALTRAP:	At least 4 wk prior to elective surgery For recurrent or severe hypertension, until controlled. Upon resumption, permanently reduce the ZALTRAP dose to 2 mg per kg For proteinuria of 2 grams per 24 h. Resume when proteinuria is less than 2 grams per 24 h. For recurrent proteinuria, suspend ZALTRAP until proteinuria is less than 2 grams per 24 h and then permanently reduce the ZALTRAP dose to 2 mg per kg (RULE OF 2)

Table 4 Special Situations about Toxicities of Zaltrap

Geriatric Use	The effect of ZALTRAP on overall survival was similar in patients < 65 yr old and ≥ 65 yr old who received ZALTRAP/FOLFIRI. No dose adjustment of ZALTRAP is recommended for patients ≥ 65 yr of age.
Paediatric Use	The safety in paediatric patients has not been established.
Hepatic Impairment	No dedicated clinical studies have been conducted to evaluate the effect of hepatic impairment on the pharmacokinetics of ziv-aflibercept.
Renal Impairment	No dedicated clinical studies have been conducted to evaluate the effect of renal impairment on the pharmacokinetics of ziv-aflibercept.
Contraception and Nursing Mothers	Females and males of reproductive potential should use highly effective contraception during and up to a minimum of 3 mo after the last dose of treatment.
Overdose	No information on the safety of aflibercept given at doses exceeding 7 mg/kg every 2 wk or 9 mg/kg every 3 wk is present. No specific antidote to ZALTRAP overdose exists. Patients of Zaltrap overdose should be managed with supportive measures.
Infections	The Velour study showed an increased incidence of infections in patients receiving ZALTRAP/FOLFIRI (46%, all grades; 12%, Grade 3-4) than in patients receiving placebo/FOLFIRI (33%, all grades; 7%, Grade 3-4), including urinary tract infection, nasopharyngitis, upper respiratory tract infection, pneumonia, catheter site infection, and tooth infection. Be vigilant about recognizing them. Treat them according to general guidelines.

controlled with an antihypertensive agent(s). The most common bleeding events in clinical trials were grade 1 and 2 epistaxis, which were transient. More serious bleeding events include central nervous system hemorrhage, hemoptysis, and gastrointestinal hemorrhage.

Ziv was associated with 2.6% of arterial thromboembolic events (ATE) including transient ischemic attack, cerebrovascular accident and angina pectoris compared to 1.7% of patients treated with placebo/FOLFIRI. In Ziv patients, grade 3-4 ATE occurred in 1.8% compared to 0.7% in placebo arm. Risk factors for arterial thromboembolic events included a history of prior arterial thromboembolic events such as stroke or heart attack, and age of 65 years or older.

There is paucity of data on drug interaction with other medications. However, based on the available data it is recommended that Ziv be used cautiously with medications that can increase the risk of bleeding, such as non-steroidal anti-inflammatory drugs, aspirin, and warfarin. Caution should be observed with the use of Ziv when administered to patients with a history of HTN, thromboembolism, bleeding, or preexisting proteinuria, as these conditions may be exacerbated by Ziv.

In elderly patients (above or 65 years), there was slightly increased (5%) incidence of diarrhea, dizziness,

asthenia, weight decrease and dehydration as compared to younger patients. Overall survival was same in all these patients. There is no dose adjustment recommended for elderly. Its safety profile in children is unknown. Animal studies in rabbits have shown that Ziv causes fetal abnormalities. Although there is no data on the effects of Ziv on human fetal development (Table 4), its use during pregnancy is not recommended based on experimental data in animals.

Currently there are no specific dose recommendations for patients receiving Ziv who have preexisting renal or hepatic dysfunction. Consideration of withholding Ziv should also be done in patients with low platelet counts or who are at an increased risk of bleeding. The manufacturer recommends permanent discontinuation of Ziv in patients with serious bleeding, gastrointestinal perforation or wound dehiscence requiring medical intervention. Patients who develop moderate to severe proteinuria while on treatment with Ziv should have treatment held until resolution or proteinuria.

Ziv was shown to be of benefit in metastatic colorectal cancer, while increasing patient response rates and prolonging survival. Additional information is needed to identify other diseases and stages most likely to benefit from anti-angiogenic agents and the optimal sequences

and therapeutic combinations also should be studied.

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Genomic era diagnosis and management of hereditary and sporadic colon cancer

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Abstract

The morbidity and mortality attributable to heritable and sporadic carcinomas of the colon are substantial and affect children and adults alike. Despite current colonoscopy screening recommendations colorectal adenocarcinoma (CRC) still accounts for almost 140000 cancer cases yearly. Familial adenomatous polyposis (FAP) is a colon cancer predisposition due to alterations in the adenomatous polyposis coli gene, which is mutated in most CRC. Since the beginning of the genomic era next-generation sequencing analyses of CRC continue to improve our understanding of the genetics of tumorigenesis and promise to expand our ability to identify and treat this disease. Advances in genome sequence analysis have facilitated the molecular diagnosis of individuals with FAP, which enables initiation of appropriate monitoring and timely intervention. Genome sequencing also has potential clinical impact for individuals with sporadic forms of CRC, providing means for molecular diagnosis of CRC tumor type, data guiding selection of tumor targeted therapies, and pharmacogenomic profiles specifying patient specific drug tolerances. There is even a potential role for genomic sequencing in surveillance for recurrence, and early de-

tection, of CRC. We review strategies for diagnostic assessment and management of FAP and sporadic CRC in the current genomic era, with emphasis on the current, and potential for future, impact of genome sequencing on the clinical care of these conditions.

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Key words: Colorectal adenocarcinoma; Familial adenomatous polyposis; Genome sequencing; Personalized medicine; Cancer genomics; Pharmacogenomics; Genomic medicine

Core tip: The era of genomic sequencing is beginning to make significant impact on the diagnosis and management of sporadic and inherited colorectal adenocarcinoma (CRC) such as familial adenomatous polyposis. This review will discuss the current guidelines for diagnosis and management of CRC and how genomic sequencing is enabling earlier definitive diagnosis with associated intensive surveillance and preventative interventions, molecular tumor characterization directing tumor specific therapy, germline patient genome analysis which informs individual drug tolerance and efficacy, and is evolving to develop post-treatment surveillance, with the potential to ultimately decrease the current prevalence and mortality of CRC, sporadic and hereditary.

Esplin ED, Snyder MP. Genomic era diagnosis and management of hereditary and sporadic colon cancer. *World J Clin Oncol* 2014; 5(5): 1036-1047 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i5/1036.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i5.1036>

INTRODUCTION

The annual incidence in the United States of colorectal adenocarcinoma (CRC) is almost 140000^[1]. The morbidi-

ty and mortality attributable to heritable and sporadic carcinomas of the colon are substantial and affect children and adults alike, with CRC being the third highest cause of cancer mortality among men and women alike. The average individual lifetime risk in the United States of developing sporadic CRC is estimated at 5%, with average onset being over 50 years of age. CRC mortality can be mitigated by early detection and intervention, such as by polypectomy^[2-4]. Accordingly, recommendations have been established for surveillance colonoscopy screening, the goal being to detect and remove adenomatous polyps at an early and curable stage.

However, according to the Centers for Disease Control and Prevention, only about 59% of adults aged 50-75 years undergo recommended colonoscopy^[5]. Though early detected CRC can be successfully removed, CRC which remains undetected until an advanced stage with metastases remains incurable^[6]. The molecular etiology of CRC has been studied extensively, revealing that it develops from an accumulation of genomic mutations. CRC has been associated with mutations in various genes that are altered in other forms of cancer, such as *ATM* in leukemia and lymphoma, *PPP2R1B* in lung and breast carcinoma, and *MYC* in hepatocellular carcinoma^[7-11], among others. However, the genes more commonly found mutated in CRC include the *APC* (approximately 80%), *KRAS*, *SMAD4*, and *p53*, and the cell signaling pathways most commonly impacted by mutations in CRC include the WNT, RAS, TGF-beta, PI3K and P53 pathways^[7,12].

While CRC often occurs sporadically, germline mutations in a number of genes can cause syndromes which predispose to the development of CRC. The heritable CRC syndromes are broadly categorized as polyposis associated [including familial adenomatous polyposis (FAP), *MUTYH*-associated polyposis or MAP, Gardner and Turcot syndromes] and non-polyposis associated (including Lynch, Peutz-Jeghers and PTEN hamartoma tumor syndromes)^[13,14]. This review will focus on polyposis associated heritable CRC, and FAP in particular. Inherited mutations in *APC* cause FAP, which is a colon cancer predisposition syndrome of autosomal dominant inheritance affecting children as early as 9 years of age, with reports of carcinoma in the first decade of life.

Characteristic features of FAP include development of hundreds to thousands of adenomatous polyps beginning in early adolescence, with development of CRC in the absence of treatment. About 7% of patients develop CRC by age 21, about 95% by age 50. In all FAP patients colon cancer is inexorable in the absence of colectomy. Though the classic course of FAP results in CRC, it can also become complicated by non-colonic expressions, in particular gastric and duodenal polyps, and is associated with elevated risk for duodenal, stomach, pancreatic, thyroid, liver, and CNS cancer^[15]. While FAP is known to be initiated by a germline mutation of *APC*, studies have yet to establish whether CRC in FAP requires a similar accumulation of genetic alterations as has been observed in

sporadic CRC. Since the beginning of the genomic era^[16] at the completion of the Human Genome Project^[17], the growth in capacity and availability of genomic sequencing has made it possible to more clearly elucidate the molecular etiology of conditions such as FAP related CRC. Genomic sequencing facilitates the identification of individuals with FAP, enabling and guiding appropriate intervention and is augmenting the ability to characterize CRC tumors on a molecular scale, for selection of targeted therapies that can be personalized per patient tolerance.

FAP DIAGNOSIS

Initial presentation and evaluation

FAP is second most common inherited CRC, with prevalence estimated at 1:10000, and is caused by mutations in *APC*^[13]. Patients will often present with occult blood in the stool, as polyps develop on average by 16 years of age^[18]. The average age at identification of CRC in untreated individuals is 39^[13]. Clinical diagnosis of classic FAP is established when 100 or more colonic polyps are observed on colonoscopy, or less than 100 colonic polyps are observed in a patient with a family history of FAP^[15]. A related syndrome, attenuated FAP, associates with a lower polyp burden (average of 30) and later age at diagnosis of CRC, though it is also caused by *APC* gene mutations^[19]. Identification of a mutation in *APC* provides molecular confirmation of FAP. The American College of Medical Genetics and Genomics guidelines recommend complete *APC* gene analysis be considered in any individual with 100 or more colonic polyps, autosomal dominant inheritance and/or extra-colonic manifestations of FAP (*e.g.*, congenital hypertrophy of retinal pigment epithelium, desmoids, gastric fundic gland polyps, among others), when no prior family member has undergone testing^[20]. If a familial mutation is identified, targeted *APC* analysis can be performed^[21]. This testing provides clinical confirmation necessary to guide predictive counseling and enable assessment of family members at increased risk, as *APC* alterations are found in as high as 90% of families with classic FAP. Testing is also important when the patient's presentation is not completely typical for classic FAP, such as demonstrating a lower than expected polyp burden or later age at onset. In some cases, *APC* gene analysis will confirm a mutation consistent with attenuated FAP, though *APC* alterations in the attenuated form are only discovered in 10%-56% of cases^[22].

Of particular importance in patients presenting with polyps not clearly identifiable as classic FAP, attenuated FAP, Gardener syndrome, Turcot syndrome, *MUTYH* associated polyposis (MAP) or one of the nonpolyposis syndromes, are the gene panels made possible by genomic sequencing. Three of these multi-gene sequencing panels are currently offered by Clinical Laboratory Improvement Amendments (CLIA) certified labs in the United States. They are performed with patient blood derived genomic DNA *via* next-generation sequenc-

ing of the coding regions of up to 19 different genes for point mutations associated with various hereditary colon cancer syndromes, are complemented by duplication/deletion analysis of the genes using microarray comparative genomic hybridization or multiplex ligation-dependent probe amplification, and point mutations are confirmed *via* Sanger sequencing. The OncoGene Dx gene panel offered by GeneDx analyses a total of 18 genes (including *APC*, *ATM*, *AXIN2*, *BLM*, *BMPR1A*, *CDH1*, *CHEK2*, *EPCAM*, *MLH1*, *MSH2*, *MSH6*, *MUTYH*, *PMS2*, *PTEN*, *SMAD4*, *STK11*, *p53*, *XRCC2*) and requires about 4 wk to complete the assay for a new patient (<http://www.genedx.com/test-catalog/disorders/colorectal-cancer>)^[23]. Ambry Genetics' ColoNext gene panel assays 15 genes (including *APC*, *BMPR1A*, *CDH1*, *CHEK2*, *EPCAM*, *MLH1*, *MSH2*, *MSH6*, *MUTYH*, *PMS2*, *PTEN*, *SMAD4*, *STK11*, *p53*) with analysis turnaround time approximately 12-16 wk for new patients (<http://www.ambrygen.com/tests/colonext>)^[24]. The University of Washington Genetics Laboratory offers ColoSeq which sequences the coding regions of 19 genes (*APC*, *AKT1*, *BMPR1A*, *CDH1*, *EPCAM*, *GALNT12*, *GREM1*, *MLH1*, *MSH2*, *MSH6*, *MUTYH*, *PIK3CA*, *POLD1*, *POLE*, *PMS2*, *PTEN*, *SMAD4*, *STK11*, *p53*), the estimated time to complete assessment is 12 wk for a new patient (<http://tests.labmed.washington.edu/COLOSEQ>)^[25]. Other assays are certain to be introduced. Through these genomic testing panels, patients with an ambiguous presentation can be tested simultaneously for multiple hereditary colon cancer syndromes, decreasing the time to molecular diagnosis and appropriately tailored familial testing and clinical surveillance.

Familial genetic testing

For unaffected individuals in families with FAP, testing is not generally offered until about 10-12 years of age, which would be the recommended age to initiate surveillance colonoscopy in an affected individual^[21]. Furthermore, efforts are made to identify an affected family member for whom testing has already confirmed an *APC* mutation, to allow targeted testing in the unaffected individual. If testing is performed in an affected family member and no mutation is found, unaffected individuals must undergo clinical surveillance empirically "as if" they were mutation carriers, because their carrier status cannot be ruled out.

Nondiagnostic and variants of unknown significance challenges

Approximately 90% of *APC* alterations in FAP introduce a stop codon causing truncation of the resulting protein at the C-terminus, with over 900 different germline *APC* alterations having been discovered in FAP individuals to date^[26-29]. Though clinical genetic testing of the *APC* gene has a 90% mutation detection rate^[30], approximately 10% of classic FAP cases do not have an identifiable mutation in *APC*, requiring greater reliance on clinical presentation and empiric surveillance screening in all at risk indi-

viduals in these families^[21,31]. The growing awareness of genetic and genomic testing and its utility in diagnosing FAP has also lead to the observation of non-truncating *APC* mutations, including missense and silent mutations in the coding sequence and splice-site mutations in less conserved intronic sequence, some of which have been correlated with FAP^[29,32,33]. The relevance of these variants of unknown significance (VUS) in some cases can be suggested by *in silico* prediction algorithms, however *in vivo* or *in vitro* functional analyses of these VUS provide more reliable data to predict the functional impact of *APC* variants. For example, a silent alteration in exon 14 (c.1869G > T) was reported to cause exon skipping due to its impact on splice enhancer sites^[34]. Also the p.I1307K missense mutation in *APC*, though not causing classic FAP, carries a 10%-20% increased lifelong risk of CRC, but the p.E1317Q missense mutation in *APC* has an, as yet, uncertain role in colon cancer^[20]. Though a minority of patients suspected to have FAP are found to carry VUS, with the increasing availability of *APC* gene testing and broader testing such as whole exome sequencing, its expected the number of patients with *APC* VUS will grow. Multiple groups have worked to better characterize the biological and clinical significance of these VUS^[29,35]. For example, *in vitro* and *in vivo* methods of evaluating VUS in MAP are being developed, providing additional information to facilitate characterization of VUS in an effort to clarify the diagnosis of patients suspected to have MAP^[36,37]. Further functional evaluation of VUS for FAP, and other hereditary CRC, is a critical step, with the increasing application of genomic sequencing technologies, for improving diagnostic accuracy of *APC* sequencing for patients with features concerning for FAP.

FAP MANAGEMENT

Surveillance strategies

Individuals with a family history of FAP meet criteria for additional assessment for high-risk syndromes if they present with > 10 adenomas in the same individual, per the most recent NCCN guidelines^[38]. Management of FAP includes surveillance colonoscopy every 1-2 years, starting at about 10 years of age. After polyp development is observed, annual colonoscopy is recommended. Colectomy is considered when more than 20 adenomatous polyps develop, when adenomas greater than 1 cm are noted or when concerning histology appears, and is recommended when polyp burden precludes safe colonoscopic surveillance^[15]. After colectomy surveillance continues. Annual colonoscopy is recommended if any rectal tissue remains. Upper GI endoscopy is recommended about every 1-3 years, starting at about age 25, as about 20% of patients will eventually require treatment of duodenal adenomas^[15,39].

Some correlation between genotype and clinical presentation of classic FAP has been suggested, for example patients with alterations in codon 1309 tend to have an increased number of colon adenomas at an early age,

with symptom onset at about 20 years of age. Those with alterations between codons 168 and 1580 had symptom onset about 30 years of age, and those with alterations affecting the 5' of codon 168 and the 3' of codon 1580 had symptom onset about 52 years of age^[40,41]. Profuse polyposis has been observed (average of 5000 polyps) in patients with mutations in codons 1250-1464^[42]. In contrast, attenuated FAP has been associated with the 5' portion of *APC*^[43], exon 9^[43-45] and the distal 3' portion of the gene^[43,46-48], interstitial deletions of chromosome 5q22 including *APC*^[49], partial and whole gene deletions^[50] and somatic mosaicism for *APC* mutations usually associated with classic FAP^[51-53]. While these correlations might help predict the course of polyposis in an individual, and suggest more or less aggressive surveillance and intervention strategies, these genotypes are not routinely applied to this purpose at present, though such applications could be clinically significant for management decisions in the future^[13].

Treatment options

Certain pharmacological treatments have attempted to abrogate the accumulation of polyps in FAP patients, however surgical resection remains the mainstay of intervention. Chemoprevention strategies, including non-steroidal anti-inflammatory drugs such as celecoxib and sulindac, have been shown to temporarily decrease size or quantity of polyps in FAP patients, however polyps may return while patients are still taking chemopreventive therapy^[54]. Currently, no chemopreventative strategy can replace regular surveillance, though this treatment may, in some cases, temporarily delay colectomy^[54-56].

Lifelong risk for CRC in FAP individuals approaches 100% by 50 years of age. Because of the significant increase in cancer incidence about the third decade, prophylactic colectomy is often recommended in the second decade^[57]. Generally three surgical strategies are available to FAP patients, including total proctocolectomy with ileal pouch anal anastomosis, total abdominal colectomy with ileorectal anastomosis and proctocolectomy with ileostomy^[58]. Selection of a surgical strategy takes into consideration rectal polyp burden, personal and familial phenotype, with classic FAP patients receiving proctocolectomy, if possible, due to the increased risk for rectal cancer^[58]. After surgical resection, surveillance continues, including endoscopic evaluation of any remaining rectal tissue and endoscopy of the upper gastrointestinal tract, as patients remain at increased risk for tumor formation despite colectomy^[57].

Molecular etiology

FAP is caused by germline alterations in *APC*, a tumor suppressor acting in the WNT signaling cascade^[59]. De novo *APC* mutations cause about 25% of FAP cases^[15]. Studies in sporadic CRC show mutations in *APC* appear to instigate the development of CRC^[12]. These studies show that *APC* acts in the colon to down regulate Wnt signaling by targeting beta catenin for degradation^[59].

Wild-type APC protein is an important component of the composite that includes Axin, GSK-3beta and casein kinase 1^[59]. This molecular composite regulates the phosphorylation of beta catenin necessary to target it for degradation^[59]. When *APC* is altered, beta catenin increases, translocates to the nucleus, and is thought to coactivate TCF-LEF, which is involved in the transcriptional activation of cell regulatory genes such as c-myc and cyclin D1^[59]. The multi-hit hypothesis of CRC tumor development suggests mutation or dysregulation of a number of genes occurs in the evolution of colon cancer, with *APC* mutations being present earlier in the process and *KRAS*, *SMAD4* and *p53* alterations being observed in later stages of cancer development^[12,15,60]. As FAP patients carry one germline *APC* mutation from birth, it is possible that the accumulation of molecular changes leading to malignancy in FAP recapitulates, to some degree, the molecular cascade observed in sporadic CRC (Figure 1), however no comprehensive analysis of the molecular etiology of tumorigenesis in FAP has been reported. Future genomic analyses of FAP may contribute to the understanding of FAP tumorigenesis as well as improving interventional and preventative strategies.

CRC DIAGNOSIS

Screening techniques-stool based

The morbidity and mortality of CRC can be minimized *via* prompt detection and appropriate intervention, such as polypectomy. Screening guidelines have been established to monitor individuals based on their estimated risk of developing CRC. An individual is stratified into a risk category based on their age, personal history (adenoma, CRC or inflammatory bowel disease) and family history^[38]. For patients of average risk (lifetime risk of approximately 5%), both structural and fecal based screening tests are available. Fecal occult blood tests (FOBT), both guaiac-based and immunochemical, are designed to detect blood in fecal matter as evidence suggestive of possible CRC. These are recommended annually alone, or in combination with flexible sigmoidoscopy every 5 years.

Positive FOBT results should prompt assessment by colonoscopy^[38]. Stool DNA tests are an evolving screening option which detect the presence of known CRC related DNA alterations in tumors cells excreted in stool. Single target stool DNA tests appear to have low sensitivity^[61], with multi-target stool DNA tests (assessing 21 alterations in genes such as *APC*, *KRAS* and *p53*) detecting up to 52% of CRC with sensitivity ranging from 20%-94% in different trials^[62,63]. ColoSure is the only stool DNA test available in the United States^[64], nevertheless the FDA has yet to approve stool DNA analysis. While it is not presently acknowledged as a first line assessment tool, additional stool DNA assays are under development^[65]. Stool screening tests have the advantage of being non-invasive and not requiring bowel clearance, which can enhance patient adherence to screening rec-

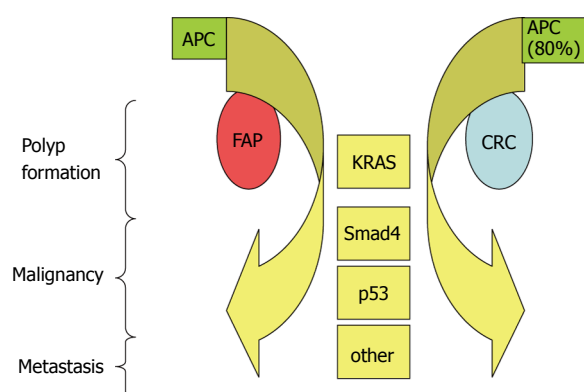


Figure 1 Hypothetical model of familial adenomatous polyposis molecular cascade. APC: Adenomatous polyposis coli; FAP: Familial adenomatous polyposis; CRC: Colorectal cancer; KRAS: Kirsten rat sarcoma viral oncogene homolog; Smad4-SMA: (Small) and MAD (mothers against decapentaplegic) related protein 4.

ommendations.

Screening techniques-colonoscopy

Structural based screening tests include colonoscopy, flexible sigmoidoscopy and computed tomography (CT) colonography. CT colonography, also referred to as virtual colonoscopy has the advantage of being non-invasive and not requiring sedation^[38]. Flexible sigmoidoscopy also proceeds without sedation and requires decreased bowel preparation, however is limited to evaluating the distal portion of the colon. In both cases positive findings (lesions greater than 1 cm in the case of flexible sigmoidoscopy) require follow up evaluation by colonoscopy and polypectomy as indicated^[38,66]. Colonoscopy remains the most complete screening procedure, permitting visualization of the complete colonic tract and simultaneous polypectomy, and is the gold standard against which other screening methods are measured. Studies have shown colonoscopy to reduce by an estimated 50% the incidence of CRC, and an inverse correlation between colonoscopy use and death from CRC^[67-72]. A recent study also found colonoscopy and sigmoidoscopy to be associated with a lower mortality from CRC of the distal colon, and colonoscopy associated with decreased mortality from proximal CRC^[69]. Their observations support the ten year assessment interval endorsed by current recommendations for individuals of average risk with a negative colonoscopy, documenting 1164 cases of CRC in individuals without endoscopy compared with 209 cases of CRC in individuals from 3 to 15 years post negative colonoscopy^[73].

Screening guidelines

Patients 50 years of age and older, without personal history of polyps, CRC or inflammatory bowel disease and without family history of CRC or advanced adenomatous polyps, are considered at average risk^[38]. A positive family history should prompt consideration of a CRC predisposition syndrome. The preferred screening strat-

egy in these individuals is colonoscopy. If no polyps are found, repeat colonoscopy is recommended in 10 years. If polyps are identified, polypectomy is performed and repeat colonoscopy recommendations depend on polyp characteristics. Patients with hyperplastic, non-sessile serrated and less than 1 cm polyps should have follow up colonoscopy in 10 years if polyps are left-sided or 5 years if right-sided^[38]. Adenomas or sessile serrated polyps are considered low risk if there are no more than 2 tubular polyps less than 1 cm and require repeat colonoscopy in 5 years. Follow up colonoscopy in 3 years is recommended if 3 or more villous polyps are observed, diameter is 1 cm or larger, or they demonstrate high-grade dysplasia^[38]. If more than 10 polyps are observed, one of the polyposis syndromes should be considered^[38].

CRC MANAGEMENT

Tumor characterization and resection

Current intervention for CRC includes diagnosis, staging, resection, adjuvant chemotherapy, treatment of recurrent disease and ongoing surveillance. Diagnostic determination depends in part on histological assessment of the resected polyp. All adenomatous polyps have some degree of dysplasia, falling on a spectrum from low to high. No specific definition of "high grade" dysplasia has been established, but a number of histological features are assessed in an effort to grade the level of dysplasia, which can include loss of glandular differentiation, cellular and nuclear pleomorphism, nuclear hyperchromatism, loss of nuclear polarity, multi-layered irregular nuclei and loss of mucin, nuclear atypia with prominent nuclei and focal cribriform patterns^[74,75]. Polyps with favorable histologic features are graded 1 or 2; those with less favorable histology are assigned grades 3 or 4. Histological grade, features such as angiolymphatic invasion and positive or negative resection margin help guide clinical decisions regarding the need for additional surgical resection. A polyp is considered malignant if cancer is observed infiltrating through the muscularis mucosa into the submucosa, and is designated T1^[76]. As nearly one third of CRC in the United States is associated with family clustering, once histological diagnosis of CRC has been confirmed, an individual should be counseled regarding the increased risk of CRC in their first degree relatives^[77].

Complete staging of malignant tumors is facilitated by pre-operative colonoscopy and CT scans of chest, abdomen and pelvis, is ultimately accomplished during surgical resection, and is categorized according to the TNM (tumor/node/metastasis) system^[76,77]. Staging takes into consideration local invasion of the primary tumor, evidence of lymph node metastasis and evidence of metastasis to other organ sites or the peritoneum, and is considered one of the most important indicators of post treatment outcome^[6]. Generally, stage I and II are assigned to lower and higher grade tumors, respectively, without nodal metastasis, stage III tumors have lymph node metastasis and stage IV tumors have metastasis to

other organ sites or the peritoneum, with stages II, III and IV having additional subclassifications^[76,78]. Though 20% of CRC is metastatic at presentation, 80% is localized to the colon wall or lymph nodes and surgical resection can be curative for localized CRC, accordingly treatment for CRC is primarily surgical. For resectable colon cancer, colectomy with regional lymph removal is the preferred surgical strategy, with the extent of colectomy based in part on tumor location as well as family history of polyposis^[77].

Adjuvant therapy and surveillance

For stage I CRC, post resection adjuvant chemotherapy is not recommended, however various studies have shown a role for adjuvant therapy in more advanced CRC. Current recommendations are for patients with high-risk stage II and stage III CRC to receive 6 mo of adjuvant chemotherapy after primary surgical resection, and for patients with low-risk stage II CRC to be considered for adjuvant chemotherapy, enrolled in a clinical trial or observed without adjuvant therapy^[77,79]. The specific chemotherapeutic regimens have been reviewed recently in detail^[77,80]. These recommendations are derived from various studies which have shown survival benefit in patients with resected early stage CRC who received adjuvant chemotherapy, with most of the benefit being seen with stage III CRC, but not with node negative stage II disease, suggesting the benefit is increased in patients at higher risk related to nodal status^[81-83]. Chemotherapy also has a role in patients presenting with more advanced CRC, including stage IV metastatic CRC. In individuals diagnosed with unresectable or medically inoperable CRC, chemotherapy is recommended in an effort to convert the lesion to a resectable state, and can be used to convert unresectable metastases, such as those in the liver, to resectable lesions, with the particular chemotherapeutic agents having been recently reviewed^[77,84,85].

Posttreatment surveillance includes application of a variety of tools in an effort to discover any recurrence that is potentially curatively resectable. These tools include serial history and physical examination by a physician, CEA testing, colonoscopy and in some cases CT scans of the chest, abdomen and pelvis^[77]. The advantages of more intensive surveillance regimens for patients with stage II and III CRC have been shown^[86-88] and current recommendations for patients with successfully treated stage I-III CRC include history and physical examination every 3-6 mo for 2 years, CEA testing every 3-6 mo for 2 years and colonoscopy 3-6 mo post-resection (if not performed preoperatively)^[77]. Surveillance colonoscopy is repeated based on findings (3 years if normal and 1 year if concerning adenomatous polyp removed), with CEA, history and physical exam spaced to every 6 mo to complete the first 5 years of posttreatment surveillance^[77].

Patients with a history of CRC have a particularly high risk of another cancer within 2 years after resection, and recommended surveillance frequencies for the first 5

years post treatment vary with stage of CRC and patient characteristics such as age of onset and history of hereditary CRC^[80]. Chest, abdominal and pelvic CT scans are recommended yearly for 3-5 years for stage II-III CRC patients at high risk for recurrence and every 3-6 mo for 2 years spaced to every 6-12 mo for a total of 5 years for individuals with stage IV CRC^[77]. If disease recurrence is observed, the steps of diagnosis, staging, treatment *via* resection and/or adjuvant chemotherapy are revisited as with a primary presentation, with the potential complications of a more advanced presentation and chemotherapy resistant lesions.

CRC genomics

In the more than ten years since the completion of the Human Genome Project^[17], advances in the capacity, speed and accuracy of genomic DNA sequencing have rapidly increased our knowledge of the molecular basis of multiple diseases, and ushered in the genomic era^[16]. These powerful tools of genomic analysis have been recently trained on CRC, the results of which are just beginning to exert what is expected to be a substantial effect on the diagnosis and treatment of this disease.

Genome scale sequencing (including whole exome and whole genome sequencing) of over 200 CRC samples was recently completed by The Cancer Genome Atlas Network (TCGA), with a primary goal of characterizing somatic mutations in these lesions. The 24 genes which they found to be significantly mutated included somatic alterations in genes known to act in CRC, namely *APC*, *TP53*, *SMAD4*, *PIK3CA* and *KRAS* (Figure 1), as well as *ARID1A*, *SOX9* and *FAM123B*^[7]. They observed significantly different somatic mutations rates among the tumors assessed, classifying them into two categories: hypermutated and non-hypermutated. As a potential etiology for the elevated mutation rate, they tested and found 77% of hypermutated tumors to have elevated levels of micro-satellite instability (MSI), which is caused by DNA mismatch repair (MMR) deficiency^[77], and can occur due to deleterious mutations to the genes *MLH1*, *MLH3*, *MSH2*, *MSH3*, *MSH5* and *PMS2*^[7]. In fact, in the majority of these same hypermutated lesions with high MSI were found evidence of epigenetic silencing of *MLH1* and frameshift/nonsense/missense mutations in *MLH1*, *MLH3*, *MSH2*, *MSH3*, *MSH5* and *PMS2*^[7]. They proposed that the higher survival rate of patients with high MSI-related cancers, with these tumors being hypermutated, the mutation rate may be a prognostic indicator^[7].

Increasing understanding of the role of MMR in tumorigenesis has already impacted the clinical approach to CRC. Current recommendations include assessment of new CRC for evidence of MMR deficiency for patients younger than 50 years old, though many centers assess for MMR deficiency, and sometime MSI, on all patients with CRC. This is done for two reasons: (1) it can be used as a screening tool to identify individuals at risk to have Lynch syndrome, causing hereditary colon and endometrial cancer^[14], for whom genomic sequence analysis

Table 1 Evolving genomic tools for management of colon cancer

	Detection	Diagnosis	Management
Clinically available	Fecal occult blood test	Single gene sequencing (APC) Multigene panel next generation sequencing	Targeted gene analysis for therapeutic contraindications (<i>KRAS</i> , <i>UGT1A1</i>)
Research basis	Fecal genomic DNA analysis	Tumor genome sequencing for prognosis	
Future application	Cell free genomic DNA sequencing		Development of gene pathway directed therapeutics (<i>e.g.</i> , small molecule inhibitors)

APC: Adenomatous polyposis coli; *KRAS*: Kirsten rat sarcoma viral oncogene homolog; *UGT1A1*: Uridine diphosphate-glucuronosyl transferase 1A1.

for mutations in *MLH1*, *MSH2*, *MSH6* or *PMS2* would be diagnostic; (2) deficiency in tumor MMR (as measured by protein immunohistochemistry) or high MSI tumor status is suggested to indicate decreased likelihood to metastasize^[80] and be a prognostic indicator of more favorable outcome^[89,90].

The application of genome sequencing technology has also led to an evolving array of clinical tools to augment the diagnosis and treatment of CRC (Table 1). For example, multigene assays have been developed to provide prognostic and predictive information about individual CRC tumors, as well having the potential to guide tumor specific therapeutic choices. Several such multigene panels are currently available (Oncotype DX Colon, ColoPrint, ColoDX), simultaneously assessing 18 genes or more, using the data to predict an individual tumor's risk of recurrence^[80]. While early trials have found such multigene panels can help predict recurrence risk for stage I - III CRC, they do not appear to predict the benefit of adjuvant therapy^[80], and further studies are necessary before such genomic assessment tools will have clinical relevance for choice of adjuvant therapy.

With detailed genomic information now more easily obtainable for individual tumors, the potential for delivering treatments more specifically targeted to a tumor's molecular signature is becoming a reality. For example, MSI observed in CRC tumors by the TCGA study, has been found to be a potential predictor of benefit from adjuvant therapy, with fluoropyrimidine specifically^[89]. MSI in CRC has been shown to be a predictor of more favorable outcome, and studies suggest high MSI, or deficient MMR, to be a marker predicting decreased benefit, and potential deleterious effect, of adjuvant treatment with fluoropyrimidine alone in individuals with stage II CRC^[89,90]. It is currently recommended that MMR tumor analysis be considered for individuals with stage II disease and planned fluoropyrimidine adjuvant treatment alone^[77].

In another example, TCGA genomic analysis of CRC further confirmed the presence of a significant number of *KRAS* mutations (in 43% of non-hypermutable CRC), consistent with its role in the molecular etiology of CRC (Figure 1)^[7]. Additional studies have shown up to 40% of CRC tumors have alterations in codons 12 and 13 of exon 2 in the coding region of *KRAS*^[91,92]. These particular *KRAS* alterations have been shown to be predictive of a lack of response to specific chemotherapies, anti-EG-

FR drugs cetuximab and panitumumab in particular^[93-97], and the FDA has stated that these drugs should not be used for management of CRC with these specific *KRAS* alterations [Cetuximab (package insert). Branchburg, NJ: ImClone Systems Incorporated; 2009; Vectibix (package insert). Thousand Oaks, CA: Amgen Inc.; 2009].

Furthermore, downstream of activated *KRAS*, the *BRAF* gene protein product is activated. *BRAF* mutations were observed in the TCGA CRC genomic analysis (46% of hypermutated tumors)^[7] and up to 9% of CRC contain the *BRAF* gene V600E mutation^[98]. Retrospective studies have suggested that mutated *BRAF*, in the presence of wild-type *KRAS*, also confers a lower response rate to cetuximab^[99]. For this reason, patients with stage IV CRC whose tumor has tested wild-type for *KRAS*, should have the option of *BRAF* tumor genotyping, in an effort to avoid potentially ineffective therapy choices^[77]. With these potential opportunities to positively impact the selection of chemotherapeutics on a tumor specific basis, genotyping of CRC tumor tissue in all individuals with metastatic CRC diagnosed as stage IV is now strongly recommended (Table 1)^[77].

Pharmacogenomic data represents another advance in CRC therapy with the ability to personalize the selection of agents for CRC treatment to each patient's tolerance. Germline genomic sequencing of individual CRC patients is enabling the identification of individuals who have increased susceptibility to the side effects of particular drugs as well as increased or decreased metabolism of specific pharmacological agents. Each of these characteristics can positively, or negatively, impact the efficacy of a selected therapeutic agent in treating the patient's CRC. Data is growing to provide clinical guidance in the selection of chemotherapeutics for CRC based in part on a patient's germline genomic sequence, to avoid excessively toxic drugs and promote optimal dosing. For example, patients with a particular variant of the uridine diphosphate-glucuronosyl transferase 1A1 (*UGT1A1*) gene are at higher risk of developing neutropenia and diarrhea when treated with irinotecan for CRC^[100-102]. Accordingly, the FDA currently recommends genotyping of patients under consideration of irinotecan treatment for CRC prior to initiation (Table 1)^[103].

Genomic analysis of CRC by TCGA continued the expansion of our understanding of the molecular etiology of CRC by identifying somatic mutations in novel genes not previously associated with CRC, such as *SOX9*^[7].

Their analysis also found a majority of recurrent CRC mutations could be grouped into several major cellular networks, specifically the WNT, MAPK, PI3K, TGF-beta and p53 pathways. For example, though alterations in *APC*, a component of the WNT pathway commonly altered in CRC, was mutated in 81% of non-hypermuted and 53% of hypermutated CRC, the overall WNT signaling pathway was altered in 93% of non-hypermuted and 97% of hypermutated CRC^[7]. This observation suggests that almost all CRC are driven in part by a very similar molecular mechanism to that which initiates CRC in patients with FAP (Figure 1). This also suggests that pathway or network-level convergence would be an important methodology whereby the functional impact of non-synonymous point mutations in CRC might be predicted^[104]. The TCGA data also suggest various potential targets for therapeutic intervention including proteins in the WNT, RTK-RAS and PI3K pathways which could be targets for inhibition^[7]. Some of these targets are already under investigation and have shown initial potential (Table 1), such as inhibitors of WNT signaling and beta-catenin inhibitors^[105-107].

The diagnosis of CRC, both at initial presentation and during surveillance for recurrence, has yet to be significantly impacted by genomic sequencing applications. However, the developing ability to more accurately sequence cell free genomic DNA isolated from the serum, as has been done for prenatal diagnostics^[108], and the detection of significant levels of tumor DNA in the blood of cancer patients^[109], suggest there is significant potential for genomic advances in the diagnosis of CRC (Table 1). Many exciting discoveries remain on the horizon for the diagnosis and management of CRC as the genomic era continues to unfold.

CONCLUSION

We have discussed the current diagnosis and management of sporadic and FAP related CRC, and the initial impacts genomic sequencing is having on these diseases. The diagnosis of FAP continues to rely on the performance of a thorough patient history and physical examination, which includes a detailed familial medical history. Clinical diagnosis *via* colonoscopy provides the gold standard for identifying the physical manifestations of FAP related CRC. Genomic sequencing has begun to manifest its impact on the diagnosis of FAP in the availability of *APC* gene sequencing and next generation sequencing based multi-gene panels, through which patients without a significant family history or with an ambiguous presentation, can be tested simultaneously for several hereditary cancer syndromes, minimizing the time to confirmed diagnosis and initiation of recommended surveillance protocols, testing of at risk family members. Molecular testing of at risk but asymptomatic family members has the added benefit of confirming individuals as being unaffected by an *APC* gene mutation, and allowing them to safely avoid unnecessary colonoscopic surveillance beyond that recommended

for individuals of average risk^[38]. With the increasing availability of these genomic sequencing assays to assess the molecular status of *APC*, a small but growing number of VUS are accumulating, which complicates the diagnostic capacity of these tests and leaves patients having to undergo empiric intensive FAP CRC screening protocols in the absence of a definitive “positive” or “negative” result. Further functional characterization of VUS remains an important area of research to improve the accuracy and applicability of diagnostic genomic sequencing.

Sporadic CRC has also begun to benefit from the tools of genome sequencing analysis. Recent whole exome and whole genome sequencing of hundreds of CRCs have confirmed the presence of the canonical genetic mutations contributing to its pathogenesis, while also identifying novel genes with potential roles in CRC tumor development^[7]. The genomic era has begun to contribute new tools to facilitate both diagnosis and guide management of sporadic CRC. Fecal derived DNA sequencing assays are being tested as a precursor to colonoscopic assessment, providing a vast amount more information than its antecedent the fecal occult blood test, though these remain unready at this time for clinical application. Direct genomic analysis of patient CRC tumors is being actively pursued in the research setting, with growing evidence that such information may provide prognostic and predictive information about clinical course and response to intervention, with the current standard of care already requiring certain CRC genes be assessed prior to treatment with specific chemotherapeutic agents^[7]. Germline genomic sequencing of CRC patients has the potential to allow personalization of treatment to patient tolerances, and some pharmacogenomic studies have already lead to screening of patients prior to initiation of specific regimens in order to avoid deleterious side effects. These advances, brought about in the short time since the advent of the genomic era^[16], have already significantly impacted the clinical management of sporadic and FAP related CRC, with the promise of further discoveries on the horizon with the potential to ultimately decrease the current prevalence and mortality of CRC, both sporadic and hereditary.

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Therapeutic strategy for postoperative recurrence in patients with non-small cell lung cancer

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Abstract

Postoperative recurrence occurs in approximately half of patients with non-small cell lung cancer (NSCLC), even after complete resection. Disease recurrence after surgical resection reduces the patient's life expectancy sharply. The prognosis after postoperative recurrence is considered to largely depend on both the mode of first recurrence (distant, locoregional or combined) and the treatment modality: (1) The majority of cases of postoperative recurrence involve distant metastasis with or without locoregional recurrence. Platinum-based systemic chemotherapy is practically accepted as the treatment for these diseases on the basis of evidence for original stage IV disease. The advent of both pemetrexed and molecular-targeted drugs has improved the survival of nonsquamous NSCLC and changed the chemotherapeutic algorithm for NSCLC; (2) Among patients with distant metastatic recurrence without locoregional recurrence at the primary tumor site, the metastasis is often limited in both organ and number. Such metastases are referred to as oligometastases. Local therapy, such as surgical resection and radiotherapy, has been suggested to be the first-line treatment of choice for

oligometastatic recurrence; and (3) While locoregional recurrence is likely to cause troublesome symptoms, it is a potentially limited disease. Therefore, providing local control is important, and radiation is usually beneficial for treating local recurrence. In order to obtain better control of the disease and provide treatment with curative intent in patients with limited disease, the administration of concurrent platinum-based chemoradiotherapy is recommended according to the results of originally nonresectable stage IIIA and IIIB disease.

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Key words: Non-small cell lung cancer; Postoperative recurrence; Distant metastasis; Oligometastases; Local treatment; Locoregional recurrence

Core tip: The postrecurrence survival in non-small cell lung cancer (NSCLC) is considered to largely depend on both the mode of first recurrence (distant, locoregional or combined) and the treatment modality. Therefore, the therapeutic strategy for treating postoperative recurrence in patients with NSCLC should be considered according to the mode of first recurrence. In this way, proper treatment specific to the mode of recurrence will be developed and improvements of the postrecurrence survival can be obtained.

Yano T, Okamoto T, Fukuyama S, Maehara Y. Therapeutic strategy for postoperative recurrence in patients with non-small cell lung cancer. *World J Clin Oncol* 2014; 5(5): 1048-1054 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i5/1048.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i5.1048>

INTRODUCTION

Primary lung cancer is currently the leading cause of cancer-related mortality worldwide. Despite progress in

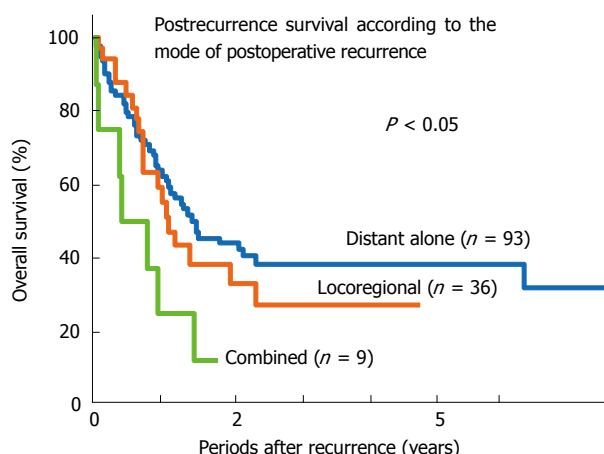


Figure 1 Postrecurrence survival according to the mode of postoperative recurrence. From 1994 through 2004, 418 consecutive patients with NSCLC underwent complete resection at Kyushu University Hospital. Of these patients, 138 experienced postoperative recurrence by December 2005, and were followed until the end of 2009.

both chemotherapy regimens and radiotherapy, surgical resection still remains the first choice of treatment for locally limited non-small cell lung cancer (NSCLC). Although the results of surgery for NSCLC have improved, this is mostly attributed to improvements in diagnostic techniques and early detection of the disease. In fact, the outcomes of surgical resection for locally advanced stages (II or IIIA) of disease are not acceptable, when a complete resection can be performed. According to the international database of the IASLC Lung Cancer Staging Project in 2007, the five-year-survival rate after complete resection is 73% for pathologic stage I A disease, 58% for I B disease, 46% for II A disease, 36% for II B disease, and 24% for IIIA disease^[1].

Postoperative recurrence occurs in approximately 45% of patients, even after complete resection of NSCLC^[2]. Disease recurrence after curative surgery reduces the patient's life expectancy sharply. The median postrecurrent survival time ranges from 8.1 to 17.7 mo in the literature^[3-5]. The postrecurrence survival is considered to largely depend on both the mode of recurrence (Figure 1) and treatment modality^[6,7]. To date, however, there have been few studies regarding the treatment of postoperative recurrence according to the mode of recurrence.

In this review article, the authors would like to propose a perspective treatment strategy according to the mode of postoperative recurrence, which is expected to prolong postrecurrence survival in patients with a good performance status.

FIRST SITE OF POSTOPERATIVE RECURRENCE

Information regarding the first recurrence site after surgery is useful for patient management. The mode of postoperative recurrence is usually classified into distant recurrence, locoregional recurrence and combined recur-

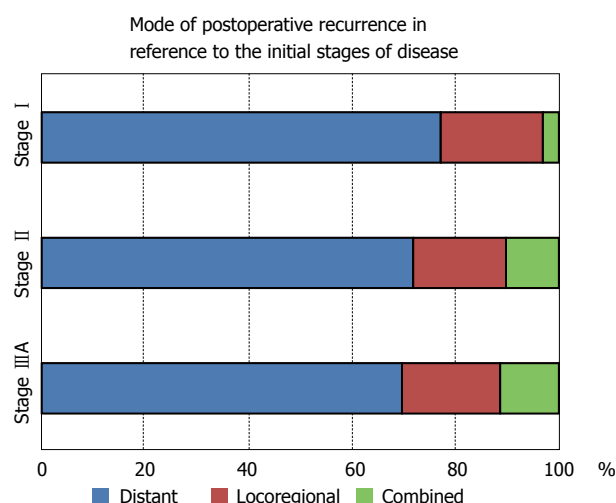


Figure 2 Mode of postoperative recurrence in reference to the initial stages of disease.

rence. From the point of view of the selection of treatment modality, local recurrence is defined as recurrent disease within the ipsilateral hemithorax and mediastinum, excluding pulmonary lesions. Postoperative recurrence in the ipsilateral lung or even the contralateral lung was previously classified as intrathoracic local failure. However, in view of the pathophysiological analysis and selection of treatment modalities, pulmonary lesions appearing after surgery should be differentiated into hematogenous distant metastases, locoregional recurrent tissues at the surgical margin or second primary cancer tumors. Therefore, ipsilateral pulmonary lesions including the surgical margin should be diagnosed as lesions of local recurrence.

It was first reported in 1994 that the mode of recurrence does not differ with respect to the pathological stage at the time of surgery (Figure 2) and that the first site of recurrence is distant organs in 73.4% of cases, locoregional sites in 19.0% of cases, and combined sites in 7.6% of cases^[2]. Common sites of distant metastasis include the brain, bone and lungs^[8]. Recently, both whole-body ¹⁸fluorine deoxy-²fluoro-*D*-glucose positron emission tomography (FDG-PET) and brain magnetic resonance imaging have become commonly included for meticulous preoperative screening. Consequently, the incidence of distant metastasis after surgery has decreased due to better preoperative staging and improved selection of surgical patients. Hence, the incidence of distant metastases as the first site of recurrence site has decreased substantially^[6,9].

TREATMENT OF POSTOPERATIVE DISTANT METASTASIS WITH OR WITHOUT LOCOREGIONAL RECURRENCE

The majority of recurrent NSCLC patients after surgery involve distant metastasis with or without locoregional recurrence^[2,6,8,10]. Although there are no definitive therapeutic guidelines for the treatment of recurrent disease

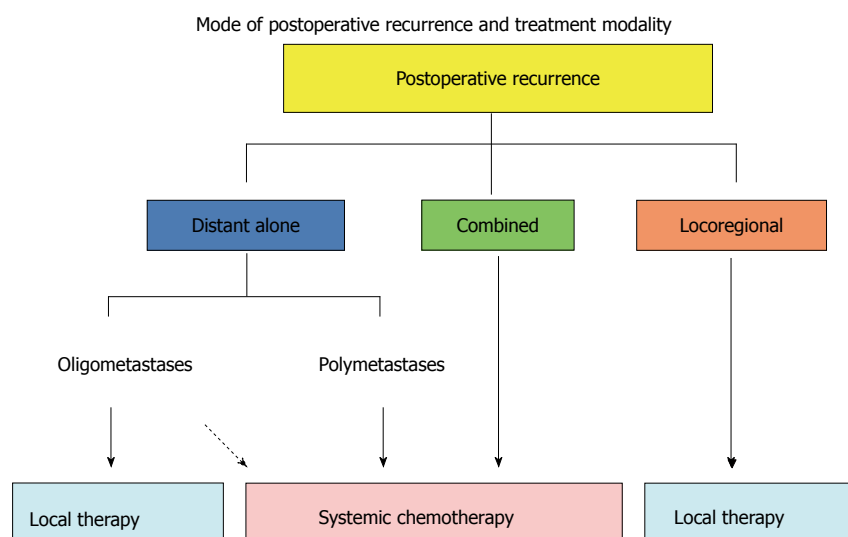


Figure 3 Mode of postoperative recurrence and treatment modality.

after complete resection, the administration of systemic chemotherapy is practically accepted as the treatment for these diseases on the basis of evidence for original stage IV disease (Figure 3).

As to the first-line treatment for distantly metastatic stage IV disease, platinum-based chemotherapy is known to prolong survival, compared with the administration of best supportive care alone^[11]. The median overall survival (OS) of NSCLC patients with clinical stage IV disease has been reported to be about one year with a median progression-free survival (PFS) of about five months in those treated with platinum-doublets chemotherapy consisting of platinum and third-generation cytotoxic drugs including paclitaxel, docetaxel, gemcitabine, vinorelbine and CPT-11^[12,13]. The advent of both pemetrexed and molecular-targeted drugs has improved the survival of patients with nonsquamous NSCLC and drastically changed the chemotherapeutic treatment algorithm for NSCLC (Figure 4). Since pemetrexed has been identified to be more effective for nonsquamous NSCLC than squamous cell carcinoma^[14], regimens of platinum plus pemetrexed are now standard first-line treatments for nonsquamous NSCLC. Furthermore, it is evident that both the PFS and OS are prolonged by treatment with platinum plus pemetrexed followed by continuous maintenance chemotherapy with pemetrexed alone^[15]. In addition, the combined use of bevacizumab, anti-VEGF (vascular endothelial growth factor) antibodies prolongs the PFS in both the induction phase of platinum-doublet regimens and the continuous maintenance phase in the setting of nonsquamous NSCLC^[16-19].

Recently, on the other hand, various types of molecular-targeted drugs have been developed in addition to conventional cytotoxic agents. It is now well-known that the response to epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs), such as erlotinib and gefitinib, is largely limited to specific mutations of the EGFR gene (*EGFR*) at exons 18 through 21^[20-22] and that EGFR-TKIs thereby achieve a longer PFS (9.2-13.7 mo) than can be obtained with standard platinum-

based chemotherapy (4.6-5.4 mo) for *EGFR* mutational NSCLC^[23-26]. In patients with *EGFR* mutations, EGFR-TKIs are now preferentially administered as first-line treatment (Figure 4). In the subgroup analysis of a phase II study of first-line erlotinib, the MST of the patients with postoperative recurrence who exhibited *EGFR* mutations was 18.2 mo^[27].

Following the identification of the *EGFR* mutation, the echinoderm microtubule-associated protein-like 4 (EML4)-anaplastic lymphoma kinase (ALK) fusion gene was discovered to be a driver oncogene for nonsquamous NSCLC in 2007^[28]. Crizotinib, an ALK inhibitor, has been identified to be effective for EML4-ALK-positive NSCLC, with both a response rate of 60.8% and a PFS of 9.7 mo^[29,30].

Since novel driver oncogenes have been extensively explored, it is essential to properly preserve surgical specimens for the future evaluation of biomarkers of molecular-targeted therapy.

TREATMENT OF OLIGOMETASTATIC RECURRENCE

Among patients with distant metastatic recurrence without locoregional recurrence at the primary tumor site, the metastasis is often limited in both organ and number. Such limited metastases are referred to as oligometastases. Local therapy, such as surgery and radiotherapy, has been applied successfully in appropriately selected patients, especially for patients with either brain metastasis alone or those with adrenal metastasis alone^[31-34]. Recently, Yano et al. reported a retrospective study reviewing their therapeutic experience with postoperatively recurrent NSCLC patients and demonstrated that a histology of adenocarcinoma, a longer disease-free interval (≥ 1 year) and the use of local therapy are significantly preferable prognostic factors for the postrecurrence OS of patients with distant metastasis alone^[6]. It has been suggested that local control of the metastatic tumor prolongs both the

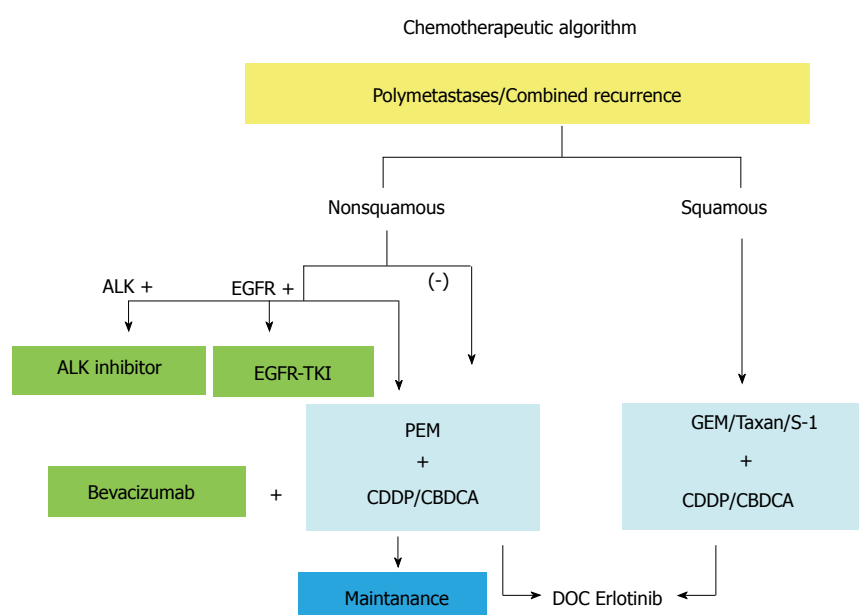


Figure 4 Chemotherapeutic algorithm. PEM: Pemetrexed; GEM: Gemcitabine; CDDP: Cisplatin; CBDCA: Carboplatin; DOC: Docetaxel; EGFR-TKI: Epidermal growth factor receptor tyrosine kinase inhibitor; ALK: Anaplastic lymphoma kinase fusion gene.

PFS and OS when distant metastases are limited in organ and number without local relapse at the primary site. These investigators subsequently reported findings of a prospective observational study that showed that 54.8% of postoperatively recurrent patients with distant metastasis alone exhibit oligometastatic metastasis without primary site recurrence and that the administration of local therapy, such as surgical resection or radiotherapy, results in a relatively long PFS of the patients with oligometastasis^[35]. In that study, patients with only brain metastasis were excluded from the survival analysis since stereotactic radiotherapy is already practically accepted as the standard treatment for these limited brain metastases. In the oligometastatic patients who received local treatment, the median PFS was 20 mo. In that series, patients with metastasis to the lungs or bone were present among the long-term progression-free survivors.

Prior to application of local treatment for postoperative oligometastatic recurrence, it is essential to rule out both locoregional recurrence at primary site (in the locoregional lymph nodes) and other systemic metastasis. Therefore, for an accurate clinical diagnosis of oligometastases, FDG-PET examinations should be performed at the time of postoperative recurrence, as this modality has a high ability to detect asymptomatic recurrence^[36].

TREATMENT OF LOCOREGIONAL RECURRENCE

While locoregional recurrence is likely to cause troublesome symptoms, it is a potentially limited disease. Therefore, providing local control is important, and the administration of local treatment, such as radiotherapy, is usually beneficial for local recurrence after complete resection in patients without pleural dissemination or effusion (Figure 3). In a study by Yano *et al.*^[2], half of the locoregionally recurrent patients who received radiation

treatment exhibited a good local response, resulting in a prolonged survival, with a median survival time (MST) of 27 mo. On the other hand, the MST of the patients with uncontrolled disease was only six months. The administration of modern three-dimensional conformal radiotherapy with a curative dose of 60–66 Gy has been reported to achieve approximately 90% response rate (65% complete response and 24% partial response) for postoperative thoracic lymph node recurrence^[37]. As a result, the five-year PFS and OS rates are 22.2% and 36.1%, respectively.

Postoperative locoregional recurrence is considered to be pathophysiologically the same as originally nonresectable stage IIIA and IIIB diseases, although the MST after treatment of a curative dose of radiation is longer for patients with postoperative locoregional recurrence (ranging from 14 mo to 19 mo^[38–40]) than for nonresectable stage IIIA and IIIB diseases (ranging from 8.5 to 14.1 mo^[41]). The therapeutic outcomes of the nonresectable stage IIIA and IIIB disease have been improved with recent developments in chemoradiotherapy, particularly platinum-based regimens, compared with that achieved with radiation alone^[41]. In patients with a good performance status, the administration of concurrent chemoradiotherapy improves survival compared with the use of sequential chemoradiotherapy. Therefore, postoperative locoregional recurrence should be treated with concurrent chemoradiotherapy in order to obtain better control of the disease and provide curative treatment in patients with limited disease.

The potential of radiotherapy to control localized lesions is clearly best with small-volume disease^[37]. Furthermore, in patients with small-volume disease which potentially remains localized without any hematogenous distant metastasis, curative radiotherapy is considered to be the treatment of choice. When deemed feasible, surgical resection is also another potential treatment of choice. However, early small-volume locoregional recur-

rence, especially in the hilar and mediastinal lymph nodes, is rarely detected on chest X-rays. Therefore, in addition to periodically obtaining chest X-rays, chest computed tomography should be performed annually for the first two years during the postoperative period^[42].

CONCLUSION

In conclusion, the therapeutic strategy for treating postoperative recurrence in patients with NSCLC should be considered according to the mode of first recurrence. In this way, proper treatment specific to the mode of recurrence will be developed and improvements of the postrecurrence survival can be obtained. Based on the treatment algorithm shown in Figures 3 and 4, a multi-institutional prospective cohort study on treatment modalities for postoperative recurrence is currently proceeded by the Kyushu University Lung Surgery Study Group.

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Using shape contexts method for registration of contra lateral breasts in thermal images

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transformation of boundaries points has been applied successfully to map the two breasts interior points. Some of advantages for using shape context method in this work are as follows: (1) no special land marks or key points are needed; (2) it is tolerant to all common shape deformation; and (3) although it is uncomplicated and straightforward to use, it gives remarkably powerful descriptor for point sets significantly upgrading point set registration. Results are very promising. The proposed algorithm was implemented for 32 cases. Boundary registration is done perfectly for 28 cases.

CONCLUSION: We used shape contexts method that is simple and easy to implement to achieve symmetric boundaries for left and right breasts boundaries in thermal images.

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Abstract

AIM: To achieve symmetric boundaries for left and right breasts boundaries in thermal images by registration.

METHODS: The proposed method for registration consists of two steps. In the first step, shape context, an approach as presented by Belongie and Malik was applied for registration of two breast boundaries. The shape context is an approach to measure shape similarity. Two sets of finite sample points from shape contours of two breasts are then presented. Consequently, the correspondences between the two shapes are found. By finding correspondences, the sample point which has the most similar shape context is obtained.

RESULTS: In this study, a line up transformation which maps one shape onto the other has been estimated in order to complete shape. The used of a thin plate spline permitted good estimation of a plane transformation which has capability to map unselective points from one shape onto the other. The obtained aligning

Key words: Breast thermal images; Shape contexts; Registration; Cancer detection; Infrared

Core tip: Breast thermography has shown to be as a non-invasive safe method to detect breast abnormalities. Comparison between two breast temperature distributions is helpful to identify irregularities. Since in many cases real thermal breast images do not have symmetric boundaries, a registration is needed. A registration shape context approach is applied in this work.

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INTRODUCTION

Breast abnormalities are major concerns in health issues

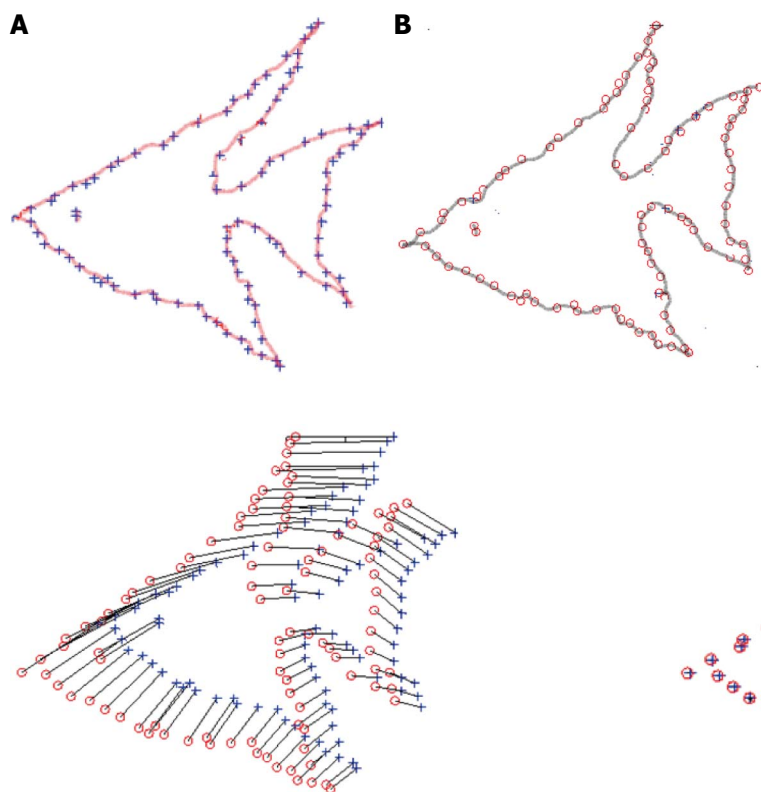


Figure 1 An example of known and unknown objects as well as their points sets. A: Known object; B: Unknown object.

Figure 2 The correspondences between the two shapes.

for women today. Identification of potentially malignant cells has a great significance for physician. Breast thermal imaging for early detection has an arguable history. With developing high sensitive infrared cameras besides great knowledge of advanced image processing and advanced computer algorithms have renewed attention to breast infrared imaging. The level of blood perfusion can affect the surface temperature that results infrared radiation emitted from skin breasts^[1]. By utilizing a sensitive infrared camera, the skin temperature changes can be captured. Since cancerous cells are hotter than normal cells, they are appeared as hot spots in thermal images^[2]. Usually healthy subjects are symmetric^[3]. Consequently, asymmetrical temperature patterns between two contra lateral breasts could be a sign of abnormality^[4-12]. In practice, most of the real IR breast images do not have symmetric boundaries. Hence, in order to compare the thermal distribution of two contra lateral breasts, a registration for two breasts is needed. In this work, first the shape context method is applied for registration of two contra lateral breasts boundaries. Then the obtained transformation function of boundaries points is utilized to transfer breasts interior points. The paper is organized as follows: The shape context method and the thin plate spline (TPS) are introduced in section 1. Section 2 explains dataset and describes the proposed algorithm steps. The experimental results are presented in section 3. Section 4 concludes the findings.

MATERIALS AND METHODS

Shape context

The shape context is an algorithm was proposed by Var-

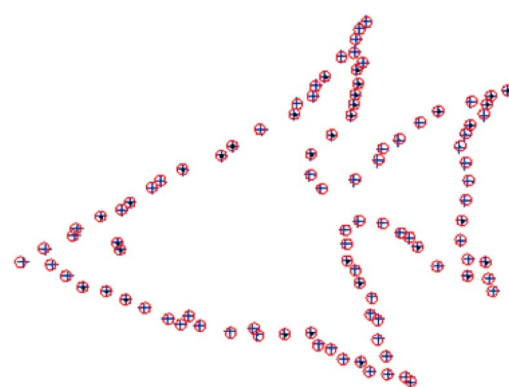


Figure 3 Alignment of the two shapes after applying alignment transformation.

dasca *et al* in 2002^[13]. It is an approach to describe shapes in measuring shape alikeness and improving of point similarities. In this way, there are two objects, known and unknown. Assuming the shape of the known object is captured by points set $p = \{p_1, | p_n\}$ and the unknown object by $q = \{q_1, | q_n\}$. The expansion of relative places is vigorous, dense, and thoroughly differential descriptor. Hence, the coarse histogram of relative coordinates of $n - 1$ points are being left, for point p_i , is described as the shape context of p_i which is expressed in Eq. (1)

$$h_i(k) = \#\{q \neq p_i : (q - p_i) \in \text{bin}(k)\} \quad (1)$$

First, the correspondences between two shapes are found. A set of finite representative points from shape boundaries illustrates shapes. They are not required to be special points such as curvature extrema or landmarks, *etc.* The more samples are used, the better approximations to the underlying shape are obtained. Using the shape context, we outline the coarse expanding of the remaining of the shape regarding to a specified point on the shape by utilizing the shape context. By discovering similarities between two shapes, for each representative point on one shape, the representative point on the other shape that has the most sameness shape context will be ascertained. The calculation of a mapping function that transfers one shape onto the other is extended by given correspondences at representative points. Euclidean, affine, and regularized thin plate splines can be used for transformation. Aligning shapes can be considered as a measure of shape similarity. We are able to obtain the differences between two shapes by calculating a sum of fitting errors

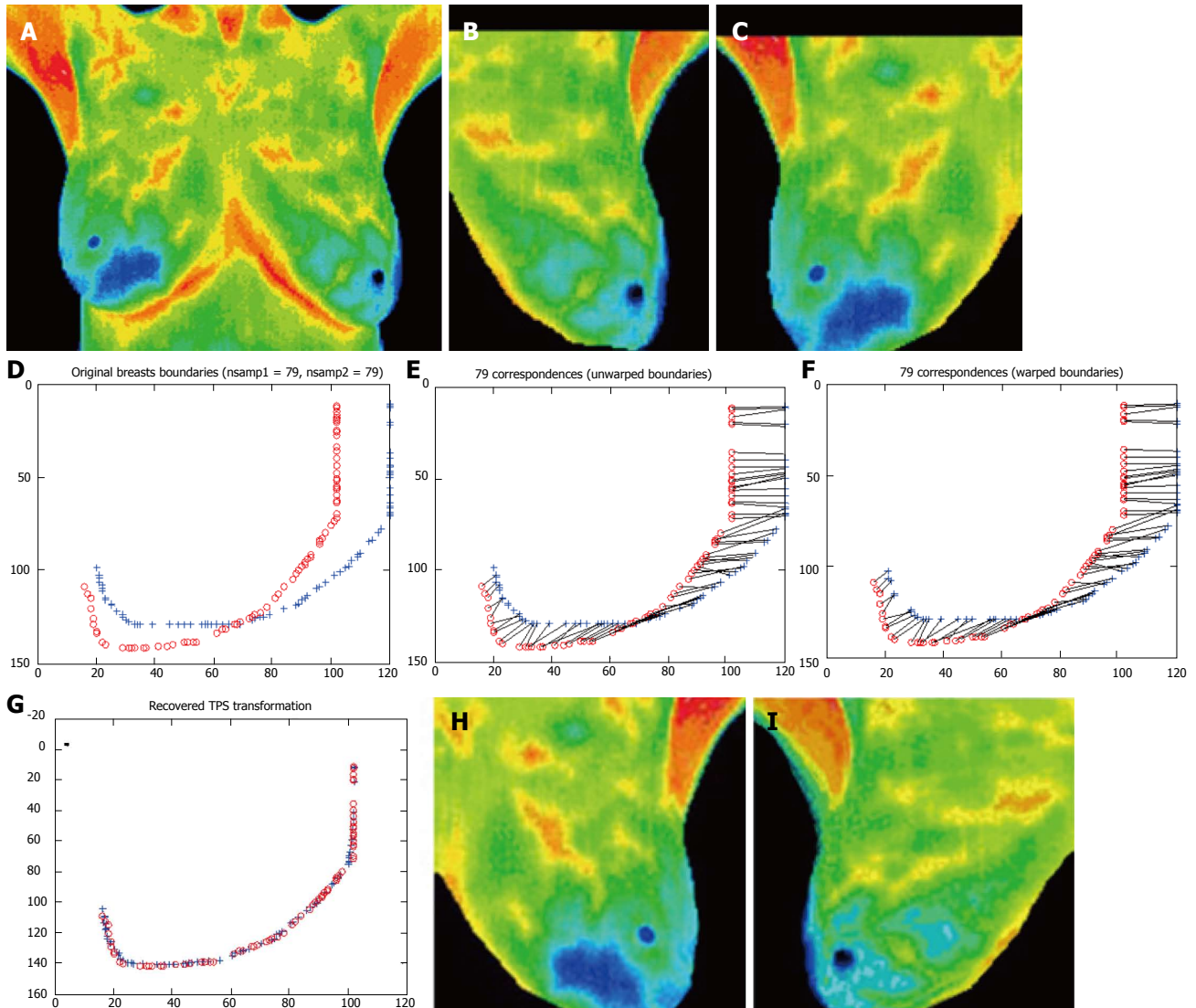


Figure 4 First sample. A: Original image; B: Left breast; C: Right breast; D: Points set of boundaries of right breast (+ in blue), points set of boundaries of left breast (o in red); E: Unwarped boundaries; F: Warped boundaries; G: Boundaries of two breasts after using the mapping function; H: Interpolated left breast; I: Interpolated left breast.

between similar points, in conjunction with an expression obtaining the magnitude of the mapping function.

Considering p_i a point on the known shape and q_j a point on the unknown shape. Supposing $C(p_i, q_j)$ is the cost of matching p_i with q_j . We represent shape contexts as histogram which is X^2 test statistics

$$C(p_i, q_j) = 1/2 \sum_{k=1}^K \{ [h_i(k) - h_j(k)]^2 / [h_i(k) + h_j(k)] \} \quad (2)$$

where $h_i(k)$ and $h_j(k)$ show the k bin normalized histogram at p_i and q_j respectively. It denotes the set of costs c_{ij} between all pairs of points p_i on the known shape and q_j on the unknown shape. It is desired to minimize the total cost of matching expressed by Eq. (3)

$$H(n) = \sum_i c(p_i, q_{\pi(i)}) \quad (3)$$

where π is a permutation. By using Hungarian technique it can be solved in $O(N^3)$ time. Examples of known object as well as unknown object with their point sets are shown in Figure 1A, Figure 1B respectively. Besides, the correspondences between the two shapes are depicted in Figure 2.

Moreover, Figure 3 shows the alignment of two shapes after applying alignment transformation. By evaluating a finite set of similarities between points on two shapes, we obtain a plane transformation. In this work, TPS was used. In the following, a brief description of TPS is provided.

Thin plate spline

Bookstein^[14] realized the model changes in biological forms where TPS is the mostly powerful candidate. When working with shape contexts TRS is greatly preferable. It is applied to map randomly points from one shape to another. TPS is 2D generalization of the cubic spline. Supposing that position (x_i, y_i) are not collinear and are all different. The Bending energy is minimized by the TPS interpolant $f(x, y)$ and has the form I_f .

$$I_f = \iint (\partial^2 f / \partial x^2)^2 + 2(\partial^2 f / \partial x \partial y)^2 + (\partial^2 f / \partial y^2)^2 dx dy$$

$$f(x, y) = a_0 + a_1 x + a_2 y + \sum_{i=1}^n w_i U(|(x_i, y_i) - (x, y)|) \quad (4)$$

where $U(r)$ is defined as Eq. (5)

$$U(r) = r^2 \log r^2 \quad (5)$$

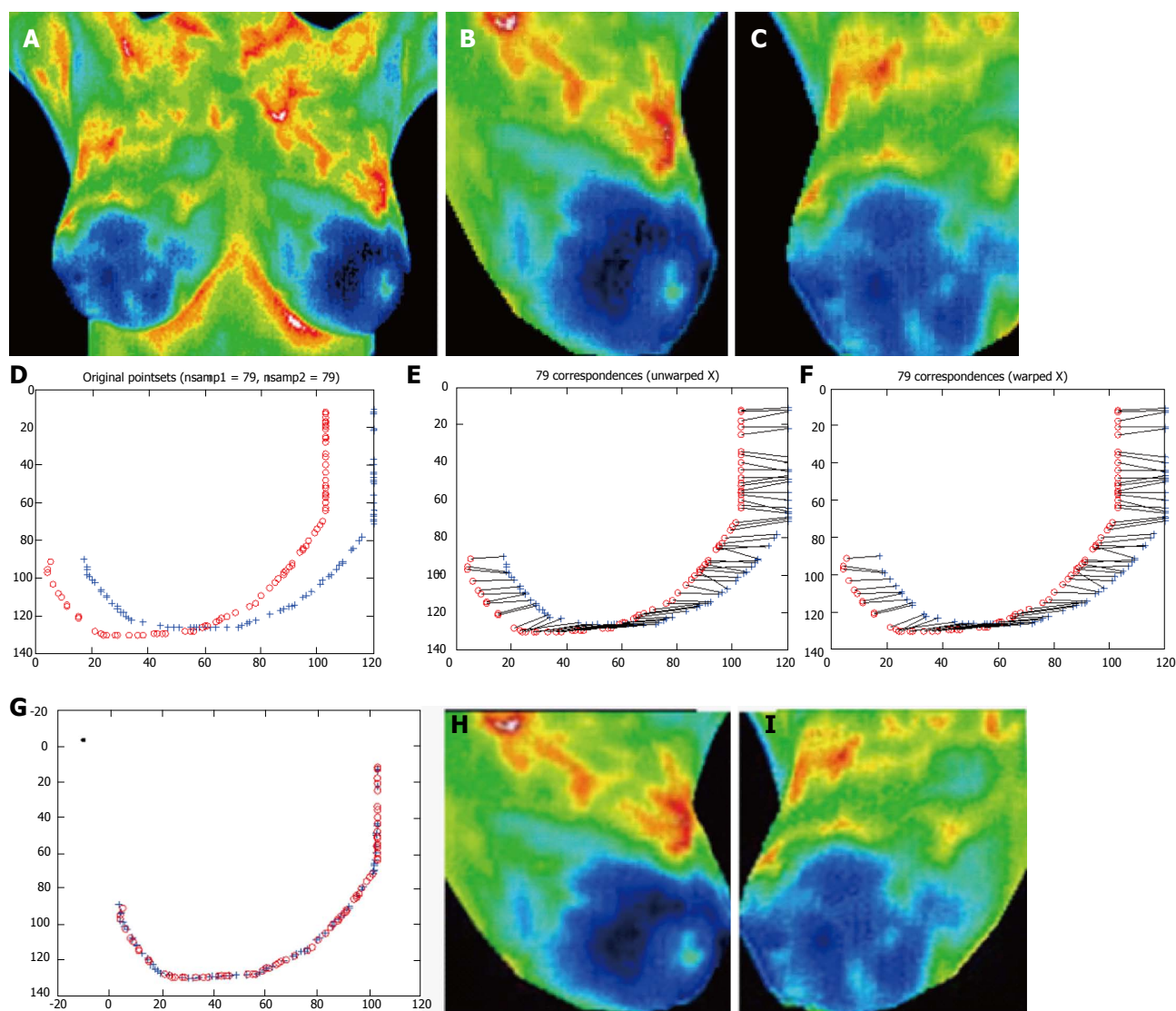


Figure 5 Second sample. A: Original image; B: Left breast; C: Right breast; D: Points set of boundaries of right breast (+ in blue), points set of boundaries of left breast (o in red); E: Unwarped boundaries; F: Warped boundaries; G: Boundaries of two breasts after using the mapping function; H: Interpolated left breast; I: Interpolated right breast.

In this work, thermal images, available from Thermal imaging lab in the San Francisco Bay Area^[15], Ann Arbor thermography center^[16], American College of Clinical Thermology^[17], Sun State thermal imaging center in Australia^[18], and Thermography of Iowa^[19] were studied^[20].

From the Department of Diagnostic Radiology, Singapore General Hospital, some data were also collected^[21]. For imaging the room temperature was selected in the range of 20 °C–22 °C (within ± 0.1 °C) in addition with humidity at $60\% \pm 5\%$. To stabilize and reduce basal metabolic rate, patients were required to rest at least 15 min. Since the vascularization is at basal level with least engorgement of blood vessels during the period of the 5th to 12th and 21th day after the onset of menstrual cycle, the patients were recommended to have examinations within these periods. The algorithm follows the following steps: (1) Randomly choosing a set of points of left breast boundaries as well as a set of points of right breast boundaries; (2) Evaluating similarities between points on

two breasts boundaries; and (3) Utilizing the similarities to obtain mapping function. The obtained mapping function is applied to map the interior points. In digital images, pixels are limited to set on a sampling grid, to occupy an integer lattice. Usually the output grid is not correlated with the integer lattice. However, the place of grid points may be any continuous values that are obtained by utilizing the mapping function. Consequently, an interpolation is required to fit a continuous surface through the data. Hence, non-integer places of output grid are attainable by sampling the continuous surface. The output image quality is very depended on the accuracy of interpolation; and (4) Determining the R,G,B amounts of the output grid by applying cubic spline properly.

RESULTS

The algorithm was implemented in 32 cases. It works perfectly for 28 cases. Implementations of the algorithm

for two cases are shown in Figure 4 and Figure 5. Left breasts are shown in Figure 4A and Figure 5A as well as right breasts in Figure 4B and Figure 5B. Points set of boundaries of right breasts and points set of boundaries of left breasts are depicted in Figure 4D and Figure 5D. Unwarped boundaries are shown in Figure 4E and Figure 5E and warped boundaries in Figure 4F and Figure 5F respectively. The boundaries of two breasts after employing the mapping function are presented in Figure 4G and Figure 5G. Moreover interpolated left and right breasts giving the mapping of the interior points are shown in Figure 4H and Figure 5H.

In conclusion, we have proposed an approach to achieve symmetric boundaries for left and right breasts boundaries in thermal images. The algorithm is based on using shape contexts method. Then, to map the interior points of two breasts, the calculated mapping function of boundaries points is used. Some of advantages for using shape context method in this work are as follows: (1) no special land marks or key points are needed; (2) it is tolerant to all common shape deformation; and (3) although it is uncomplicated and straightforward to use, it gives remarkably powerful descriptor for point sets significantly upgrading point set registration. Results are very promising. The algorithm was implemented for 32 cases. It works perfectly for 28 cases.

In future work, we can use the two obtained symmetric breasts in thermal images to compare their thermal profiles by comparing some extracted features from contra lateral breasts in order to identify thermal dissimilarities.

COMMENTS

Background

Breast health is one of major concerns of women's health today. Moreover safe modality can play an important role to identify breast irregularities. Breast thermography has shown that is a non-invasive, safe method to detect breast abnormalities.

Research frontiers

In practice, most of the real thermal breast images do not have symmetric boundaries. Hence, in order to compare thermal distribution of two breasts by comparing extracted features, a registration is needed.

Innovations and breakthroughs

Since symmetry usually indicates healthy subjects, asymmetrical temperature distribution between left breast and right breast could be a strong sign of abnormality. In practice, most of the real thermal breast images do not have symmetric boundaries. Therefore, in order to compare thermal distribution of two breasts by comparing extracted features, a registration is needed. In this work, a registration method based on shape context algorithm is introduced.

Applications

The study results suggest a registration method based on shape context algorithm for comparison between contra lateral breast temperature distributions.

Terminology

Shape context: an approach to measure shape similarity. First, two sets of finite sample points from shape contours of two breasts are introduced. Second, the correspondences between the two shapes are found. Third, by finding correspondences, the sample point which has the most similar shape context is obtained. Forth, an aligning transformation that maps one shape onto the other in order to complete shape is estimated.

Peer review

This article shows promising outcomes and it very technical and tough for common physicians.

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Clinicopathological features and treatment outcomes of brain stem gliomas in Saudi population

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RESULTS: We analyzed 49 brain stem glioma (BSG) patients from July 2001 to December 2012; 31 of them were males (63.3%) with a median age of 12.6 years (range: 8-64 mo). Twenty-two patients (44.9%) had diffuse intrinsic pontine gliomas (DIPG) and 15 (30.6%) presented with focal/tectal BSG. Histopathology was available in 30 patients (61.2%). Median survival time for the whole cohort was 1.5 years. One and two year OS rates were 51.1% and 41.9% respectively. Two year OS rates for focal/tectal, dorsally exophytic, cervicomedullary and DIPG tumors were 60%, 33.3%, 33.3% and 13.6% respectively ($P < 0.0001$). Significant prognostic factors related to OS were age at diagnosis (worse for > 18 years) $P = 0.01$, KPS < 70 $P = 0.02$, duration of symptoms (< 60 d) $P = 0.002$, histology (better for favorable) $P = 0.002$, surgery (maximal resection) $P = 0.002$, and concurrent chemotherapy with radiation therapy in DIPG (better if given) $P = 0.01$.

CONCLUSION: BSG, especially the DIPG subgroup, had a dismal prognosis, needing more aggressive neurosurgical, radiation and chemotherapy techniques, while focal and tectal tumors were found to have a better prognosis.

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Key words: Brain stem glioma; Children; Adults; Saudi Arabia; Treatment outcomes

Abstract

AIM: To analyze experiences to identify treatment outcomes and prognostic factors in a Saudi population.

METHODS: Medical records of patients with brainstem gliomas treated from July 2001 to December 2012 were reviewed to identify treatment outcomes of surgery, radiation therapy and chemotherapy and associated prognostic factors in a Saudi population.

Core tip: Brain stem gliomas (BSG) are a heterogeneous group of tumors with a poor prognosis. We analyzed 49 BSG patients from July 2001 to December 2012 with a median age of 12.6 years (range: 8-64 mo). Twenty-two patients (44.9%) had diffuse intrinsic pontine gliomas (DIPG) and 15 (30.6%) presented with focal/tectal BSG. Histopathology was available in 30 patients (61.2%). Median survival time for the whole cohort was 1.5 years. One and two year OS rates were 51.1% and 41.9% respectively. Two year OS rates for

focal/tectal, dorsally exophytic, cervicomedullary and DIPG tumors were 60%, 33.3%, 33.3% and 13.6% respectively ($P < 0.0001$). We concluded that BSG, especially the DIPG subgroup, had a dismal prognosis, needing more aggressive neurosurgical, radiation and chemotherapy techniques, while focal and tectal tumors were found to have a better prognosis.

Bayoumi Y, Sabbagh AJ, Mohamed R, ElShokhaiby UM, Maklad AM, Tunio MA, Balbaid AAO. Clinicopathological features and treatment outcomes of brain stem gliomas in Saudi population. *World J Clin Oncol* 2014; 5(5): 1060-1067 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i5/1060.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i5.1060>

INTRODUCTION

Brain stem gliomas (BSG) account for about 10%-20% of all central nervous system tumors in children and 1%-2% in adults^[1,2]. Traditionally, the term “brain stem glioma” was designed as a clinical diagnosis without histological confirmation because the morbidity for surgical intervention within the pons was high and the relevance of a histological diagnosis was low. With the advent of newer diagnostic modalities, BSG are now considered a heterogeneous group of tumors which are mainly divided into three categories according to treatment and prognosis^[3]: (1) the dorsally exophytic and cervicomedullary tumors appear to benefit significantly from surgical resection^[3]; (2) focal tectum glioma (solid or cystic) may be associated with a long history of symptoms and with neurofibromatosis type I^[4]; and (3) the largest subgroup of diffuse intrinsic pontine glioma (DIPG), in contrast, have a poor prognosis^[5]. The DIPG subgroup clearly differs from focal, dorsally exophytic and cervicomedullary tumors on various points as DIPG is typically seen with rapidly progressing symptoms and signs comprising multiple erratic cranial nerve palsies, long track deficits, cerebellar symptoms and/or raised intracranial pressure with a median survival of 9 mo^[6,7]. Gadolinium enhanced magnetic resonance imaging (MRI) allows easy confirmation of diagnosis for DIPG and high-grade BSG^[8].

Surgery is the mainstay of therapy for focal, dorsally exophytic and cervicomedullary BSG; however, for DIPG the radiation therapy remains the standard treatment option^[9,10]. Various chemotherapeutic agents investigated as monotherapy neoadjuvant agents (carboplatin or irinotecan) or as combination neoadjuvant chemotherapeutic agents (carboplatin, etoposide and vincristine or cisplatin, cyclophosphamide, etoposide and vincristine) have offered no significant improvements^[11,12]. Similarly, concurrent chemotherapeutic agents (etanidazole, topotecan, carboplatin and temozolomide) or high-dose chemotherapy followed by stem cell support have not shown any significant improvements in overall and progression free survival rates^[13,14].

Our aim was to evaluate the frequency of BSG and to

identify treatment outcomes of surgery, radiation therapy and chemotherapy and associated prognostic factors in a Saudi population.

MATERIALS AND METHODS

After formal approval from the institutional ethical committee, medical charts of patients with confirmed brainstem gliomas who were treated in our hospital were reviewed. Patients were selected if they met the following criteria.

Availability of a complete medical record: (1) demographic data [age at diagnosis, gender, main symptoms and duration, performance status according to Karnofsky Performance Scale (KPS) and main neurological signs]; (2) radiological characteristics of the tumors on MRI (T1 and T2-weighted images); and (3) surgical procedures including histopathological characteristics and other treatment modalities (radiation therapy and chemotherapy).

The epicenter (main bulk) of the tumor was located in the brainstem (midbrain, pons and medulla oblongata) and diagnosis was either based on clinical history and characteristic MRI features or histopathological confirmation.

Exclusion criteria were: (1) the epicenter of the tumor was located in the thalamus, cerebellar peduncles or cervical spinal cord; and (2) suspicion of infection could not be ruled out on MRI in the absence of biopsy results.

Toxicity

The National Cancer Institute Common Toxicity Criteria (NCI-CTC) version 2.0 was used to score acute radiation and chemotherapy toxicity (< 90 d from the start of radiation therapy). The Radiation Therapy Oncology Group Late Radiation Morbidity Scoring Criteria was used to score radiation toxicity persisting beyond 90 d from the completion of radiotherapy.

Follow-up

Functional recovery after surgical and other treatment modalities was assessed. Radiological response to radiotherapy and chemotherapy was reported according to Response Evaluation Criteria in Solid Tumors (RECIST): (1) a complete response (CR), *i.e.*, disappearance of all visible tumor; (2) a partial response (PR), *i.e.*, a decrease of > 50% in the axial cross-section of the greatest surface area; (3) progressive disease (PD), *i.e.*, > 25% increase in axial cross-section of the greatest surface area; or (4) stable disease (SD), *i.e.*, all other situations.

Statistical analysis

The primary endpoints were functional recovery, response rates and the overall survival. Progression-free survival (PFS) was defined as the duration between the completion of treatment and the date of documented disease progression, death resulting from the cancer and/or last follow-up visit (censored). Overall survival (OS) was defined as the duration between the completion of

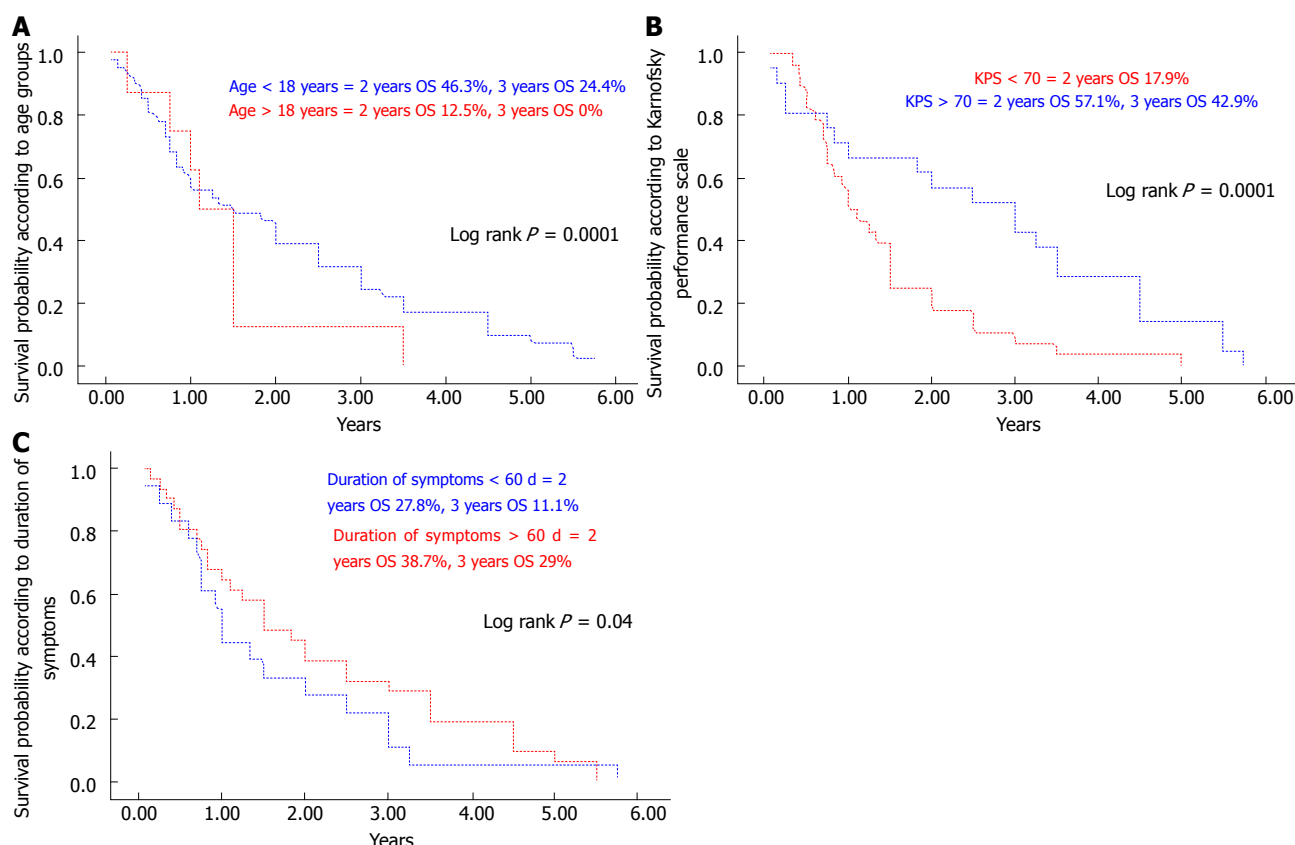


Figure 1 Kaplan-Meier curve. A: Showing overall survival probability according to age groups (< 18 years vs > 18 years); B: Showing overall survival probability according to Karnofsky performance scale (< 70 vs > 70); C: Showing overall survival probability according to duration of symptoms (< 60 d vs > 60 d). OS: Overall survival.

treatment and the date of patient death or last follow-up visit (censored). The probabilities of OS were determined with the Kaplan-Meier method and its 95%CI by the Rothman method. The comparisons for various endpoints were performed using the log-rank test. A P value of 0.05 was considered statistically significant. The multivariate analysis was used to test prognostic factors in multivariate analysis. Results are expressed with relative risk and its 95%CI. Statistical analyses were performed using the computer program SPSS (Statistical Package for the Social Sciences, version 17.0, SPSS Inc., Chicago, IL).

RESULTS

Study population

Between July 2001 and December 2012, 49 patients with BSG from the institutional database fulfilled the criteria and were analyzed.

Clinicopathological characteristics

Among forty-nine patients, the majority of the cohort consisted of children and adolescents (81.6%) with a median age of 12.62 years (range: 0.8-64). An age of > 18 years at diagnosis was associated with a significantly shorter OS compared with a younger age ($P = 0.0001$) (Figure 1A). Median Karnofsky performance status (KPS) at diagnosis was 80 (range: 40-100). KPS < 70 was related to a shorter OS ($P = 0.0001$) (Figure 1B). Main

symptoms at time of diagnosis were headaches (42.8%), diplopia or squint (38.8%), gait disturbance (34.7%), nystagmus (37.7%) and difficulty in swallowing or choking (26.5%). Mean duration of symptoms before diagnosis was 83.4 ± 47.5 d. Patients with short duration of symptoms (< 2 mo) had poor OS ($P = 0.04$) (Figure 1C). Main neurological signs were cranial nerve palsies, mainly VI, VII, IX, X (65.3%), cerebellar dysfunction (51%), bilateral papilledema (38.8%), nystagmus (37.7%) and motor weakness (28.6%). Histopathological diagnosis was available in 30 patients (61.2%), mainly of astrocytic origin (23/28) and high grade (63.3%) (Table1).

MRI characteristics at time of diagnosis

The main MRI characteristics are illustrated in Table 2.

On MRI, patterns were identified representing non-enhancing diffusely infiltrative tumors (54.5%), contrast-enhancing localized masses (33.3%) and tectal tumors (33.3%). Presumed necrosis on MRI, defined as a zone of irregularly shaped T1 hyposignal surrounded by contrast enhancement, was found in 5 (10.2%) patients.

Treatment characteristics

Thirty patients (61.2%) had surgery. Complete resection was done in dorsally exophytic (83.3%), focal tectal (66.7%) and focal (50%) tumors. Cerebrospinal fluid (CSF) shunts, including ventriculoperitoneal shunt, endoscopic third ventriculostomy and endoscopic ven-

Table 1 Clinicopathological characteristics of cohort

Mean age at diagnosis (yr)	12.62 (0.8-64) SD \pm 13.42	Diffuse astrocytoma grade II	4/30 (13.3%)
Gender		Anaplastic astrocytoma	10/30 (33.3%)
Male	31 (63.3%)	Glioblastoma multiforme	4/30 (13.3%)
Female	18 (36.7%)	Astroblastoma	1/30 (3.3%)
According to age		Nonspecified glioma	5/30 (16.7%)
Children and adolescents	40 (81.6%)	No	19 (38.8%)
Adults	9 (18.4%)	Radiological diagnosis	
Duration of symptoms (d)	83.4, SD \pm 47.5	Focal	12 (24.5%)
Karnofsky Performance Status	80 (50-100)	Tectal	3 (6.1%)
Symptoms at time of presentation		Dorsally exophytic	6 (12.2%)
Headache	21 (42.8%)	Cervicomedullary	3 (6.1%)
Vomiting	11 (22.4%)	DIPG	22 (44.9%)
Diplopia/ squint	19 (38.8%)	Others	3 (6.1%)
Unsteady gait	17 (34.7%)	Surgery	30/49 (61.2%)
Difficulty in swallowing or choking	13 (26.5%)	Total/ maximal resection	14/30 (36.7%)
Motor weakness/ paresis	10 (20.4%)	Subtotal resection	14/30 (36.7%)
Convulsions	4 (8.2%)	Biopsy only	2/30 (6.1%)
Dysphonia/ dysarthria	11 (22.4%)	VP shunt	19/30 (63.3%)
Altered consciousness	5 (10.2%)	ETV	4/30 (13.3%)
Isolated facial paresis	6 (12.2%)	EDV	5/30 (16.7%)
Hearing problems	3 (6.1%)	IONP	14/30 (36.7%)
Fever	2 (4.1%)	Radiation therapy	32/49 (65.3%)
Failure to thrive	1 (2.0%)	Postoperative	14/32 (43.7%)
Neurological signs at time of presentation		Radiotherapy alone	18/32 (56.3%)
Mental status change	8 (16.3%)	Total dose (Gy)	50.4-59.4
Cranial nerve palsies	32 (65.3%)	Fractions	30-33
Trigeminal	2 (6.3%)	Duration (wk)	6-6.5
Abducens	18 (56.3%)	Technique	
Facial	12 (37.5%)	3DCRT	15 (46.9%)
Vestibulocochlear	2 (6.3%)	IMRT	17 (53.1%)
Glossopharyngeal	12 (37.5%)	Chemotherapy	23/49 (46.9%)
Vagus	8 (25.0%)	Concurrent	12/23 (52.2%)
Motor deficit	14 (28.6%)	TMZ	12/12 (100%)
Sensory deficit	6 (12.2%)	Neoadjuvant	7/23 (30.4%)
Bilateral Babinski sign	13 (26.5%)	Vincristine + carboplatin	4/7 (51.1%)
Cerebellar signs	25 (51.0%)	High dose chemotherapy with stem cell rescue	2/7 (28.7%)
Nystagmus	17 (34.7%)	Cyclophosphamide	1/7 (14.3%)
Bilateral papilledema	19 (38.8%)	Adjuvant/ salvage	11/23 (47.8%)
Pathological diagnosis		BCNU + procarbazine + vincristine	5/11 (45.7%)
Yes	30 (61.2%)	Vincristine + carboplatin	4/11 (36.2%)
Pilocytic astrocytoma	6/30 (20.0%)	Irinotecan + bevacizumab	2/11 (18.1%)

DIPG: Diffuse intrinsic pontine glioma; VP shunt: Ventriculoperitoneal; ETV: Endoscopic third ventriculostomy; EDV: Endoscopic ventricular drain; IONP: Intra-operative neurophysiology; 3DCRT: Three dimensional conformal radiation therapy; IMRT: Intensity modulated radiation therapy; TMZ: Temozolomide; BCNU: 1,2-bis (2-chloroethyl) 1-nitrosourea.

Table 2 Magnetic resonance imaging characteristics in our cohort of brain stem glioma *n* (%)

Subgroups	Enhancement enhancing	Non-enhancing	T2W image hyper	Intensity mixed	Character		
					Cystic	Solid	Mixed
Focal (12)	17 (100)	-	10 (83.3)	2 (16.7)	3 (25)	6 (50)	3 (25)
Focal tectal (3)	2 (66.7)	1 (33.3)	1 (33.3)	2 (66.7)	3 (100)		
Dorsally exophytic (6)	4 (66.7)	2 (33.3)	3 (50.0)	3 (50.0)		4 (66.7)	2 (33.3)
Cervicomedullary (3)	2 (66.7)	1 (33.3)	-	3 (100)	3 (100)		
DIPG (22)	10 (45.5)	12 (54.5)	10 (45.4%)	12 (54.5)		19 (86.4)	3 (13.6)

DIPG: Diffuse intrinsic pontine glioma.

tricular drain was performed in 28/49 patients (57.1%) to control raised intracranial pressure. Interestingly, 6/22 patients (27.3%) with DIPG underwent surgical debulking (Table 3). Postoperative radiation therapy was given

in 14/32 patients (43.7%) and radical radiation therapy with and without chemotherapy was given in 18/32 patients (56.3%). Mean duration of time between surgery and starting radiation therapy was 25 d (range: 21-28).

Table 3 Surgical resection in our cohort of brain stem glioma *n* (%)

Subgroups	Resection		VP shunt	ETV	EVD	IONP
	Complete	Incomplete/biopsy				
Focal (12)	6 (50.0)	6 (50.0)	3 (25.0)	1 (8.3)	2 (16.6)	5 (41.7)
Focal tectal (3)	2 (66.7)	1 (33.3)	2 (66.7)	1 (33.3)	1 (33.3)	2 (66.7)
Dorsally exophytic (6)	5 (83.3)	1 (16.7)	3 (50.0)	-	2 (33.3)	5 (83.3)
Cervicomedullary (3)	1 (33.3)	2 (66.7)	-	-	-	2 (33.3)
DIPG (22)	-	6 (27.3)	11 (50)	2 (9.0)	-	1 (4.5)

VP shunt: Ventriculoperitoneal; ETV: Endoscopic third ventriculostomy; EDV: Endoscopic ventricular drain; IONP: Intra-operative neurophysiology.

Table 4 Radiation therapy in our cohort of brain stem glioma *n* (%)

Subgroups	Indication		Technique		Total dose (Gy)
	Postoperative	Radical	3DCRT	IMRT	
Focal (12)	6 (50)	-	3 (50)	3 (50)	50.4-54
Focal tectal (3)	1 (33.3)	-	-	1 (100)	54
Dorsally exophytic (6)	1 (16.7)	-	1 (100)	-	54
Cervicomedullary (3)	2 (66.7)	-	2 (100)	-	50.4-54
DIPG (22)	6 (27.3)	16 (72.7)	9 (40.9)	13 (59.1)	54-59.4

3DCRT: Three dimensional conformal radiation therapy; IMRT: Intensity modulated radiation therapy; Gy: Gray.

Table 5 Chemotherapy in our cohort of brain stem glioma *n* (%)

Subgroups	Neoadjuvant	Concurrent	Adjuvant/salvage
Focal (12)	-	-	3 (25.0)
Focal tectal (3)	-	-	-
Dorsally exophytic (6)	-	-	-
Cervicomedullary (3)	1 (33.3)	-	2 (66.6)
DIPG (22)	6 (27.3)	12 (54.5)	6 (27.3)

DIPG: Diffuse intrinsic pontine glioma.

The majority of cases (17/32) were treated with intensity modulated radiation therapy (Table 4). Among all 32 patients who received radiation therapy, the treatment protocol completion rate was 90% (95%CI, 85-100). Chemotherapy in an adjuvant or salvage setting was given mainly for the DIPG subgroup and patients with leptomeningeal dissemination which was seen in 5/49 patients (10.2%) (Table 5).

Toxicity profile

Common acute grade 2 radiation induced toxicities were nausea and vomiting (30/32) and worsening of weakness (21/32). Grade 3 toxicities were nausea and vomiting (2/32) and worsening of weakness (4/32) and were treated with antiemetics and corticosteroids. Acute grade 2 otitis media was seen in one patient. Late toxicities at time of analysis were minimal and grade 2 skin pigmentation was seen in one patient. Common acute grade 3 chemotherapy induced toxicities were myelosuppression (5/23), thrombocytopenia (2/23), rash (1/23) and febrile neutropenia (5/23), of whom three had repeated episodes. No treatment related death was seen.

Response rates

Clinical response of radiotherapy \pm chemotherapy (defined as regression of cranial nerve palsies or weakness of the limbs or cerebellar symptoms for > 3 mo) was seen in 16/32 (50%) patients, confirmed by a neurologist. Radiological response was also evaluated in all patients and response rates according to RECIST were CR (0/32), PR (16/32), SD (6/32) and PD (11/32). The mean response time was 12 ± 8 mo (range: 7-30). The mean reduction of tumor volume was 50% and clinical benefit (PR + SD) was 68.7% for all patients. Clinical response of adjuvant chemotherapy was seen in 2/11 (18.1%) at the mean time of 5 mo (range: 4-18). Three months after chemotherapy, radiological PR was seen in two patients, SD in five (45.7%) and progressive disease in four cases (36.2%).

Progression free survival and overall survival

Median survival time for the whole cohort was 1.5 years and 1, 2, 3 year OS rates for the whole cohort were 51.1%, 41.9% (29/49 died) and 23.1% (Figure 2). PFS rates at 1 and 2 years were 57.3% and 38.2% respectively.

At 2 years, the OS rate for radiologically low grade (favorable) tumors was clearly high (57.1%) compared to high grade (unfavorable) in which the OS rate was 17.9% ($P < 0.001$) (Figure 3A). Furthermore, among the subgroups, two year OS rates for focal/tectal, dorsally exophytic, cervicomedullary and DIPG tumors were 60%, 33.3%, 33.3% and 13.6% respectively ($P < 0.0001$) (Figure 3B).

Two year OS rates for patients (14/30) with complete or maximal resection and patients (16/30) with incomplete resection or biopsy only were 53.8% and 27.8% respectively ($P = 0.002$) (Figure 3C).

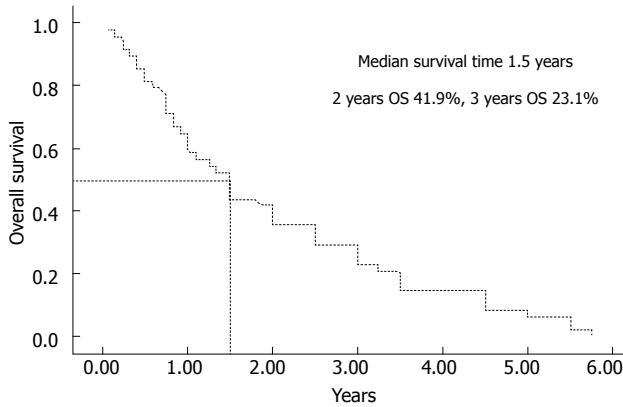


Figure 2 Median survival time, one, two and three years overall survival rates for whole cohort. OS: Overall survival.

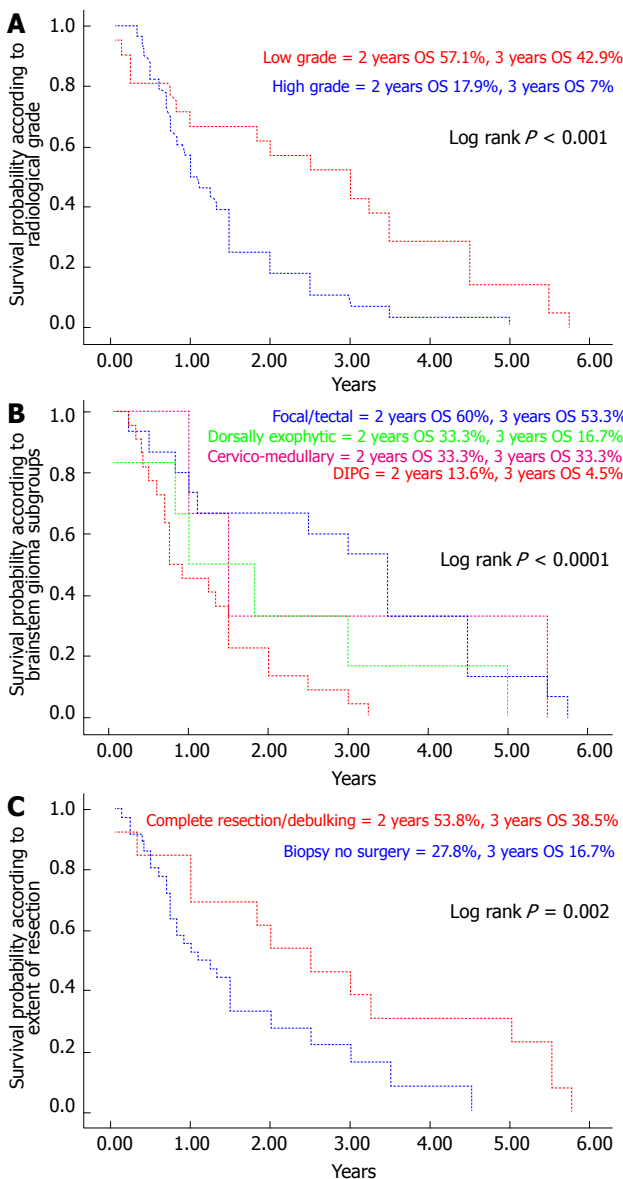


Figure 3 Kaplan-Meier curve. A: Showing overall survival probability according to grade of tumors (< low grade vs high grade); B: Showing overall survival probability according to tumor subgroups (focal/tectal vs dorsally exophytic vs cervicomedullary vs diffuse intrinsic pontine glioma); C: Showing overall survival probability according to type of resection (complete/maximal vs incomplete/biopsy). OS: Overall survival.

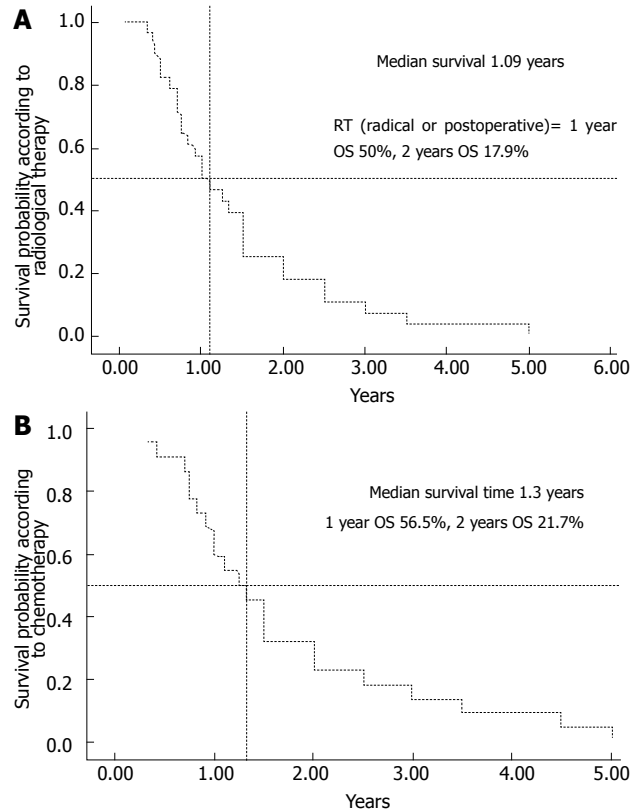


Figure 4 Median time of survival, one and two year overall survival rates for patients. A: Treated with radiation therapy (postoperative or radical radiation therapy); B: Treated with chemotherapy (neoadjuvant, concurrent or adjuvant/salvage). OS: Overall survival.

Median time of survival, one and two year OS rates for patients who were treated with postoperative (16/32) or radical radiation therapy (16/32) were 1.09 years, 50% and 17.9% respectively (Figure 4A). Patients treated with chemotherapy (neoadjuvant, concurrent or adjuvant/salvage) had a median survival time of 1.3 years and one and two year OS rates of 56.5% and 21.7% respectively (Figure 4B).

Multivariate analysis showed that for favorable tumors, important prognostic factors were: (1) age at diagnosis (worse for > 18 years); (2) KPS < 70; (3) histopathologically high grade; and (4) incomplete resection. Important prognostic factors for unfavorable tumors including DIPG were: (1) age at diagnosis (worse for > 18 years); (2) KPS < 70; (3) histopathologically high grade; (4) radiological high grade (necrosis on MRI); and (5) no concurrent chemotherapy, as shown in Table 6.

DISCUSSION

BSG remains a therapeutic dilemma because of the location and heterogeneous biological behavior of these tumors, as seen in our cohort, comprised mainly of children and adolescents (81.6%). A median survival time of 1.5 years, one and two year OS rates of 51.1% and 41.9%, and clinical prognostic factors in our cohort were found to be in agreement with previously reported data^[15,16]. We found no significant difference between

Table 6 Multivariate analysis of various prognostic factors in brainstem glioma

Variables	RR (95%CI)	P value
Age at diagnosis (> 18 yr)	3.0 (1.8-6.0)	0.01
KPS < 80	3.3 (1.7-5.3)	0.02
Duration of symptoms (< 60 d)	6.7 (4.3-9.4)	0.002
Histopathology (high grade)	6.1 (3.5-10.2)	0.002
MRI characteristics (presence of necrosis)	3.0 (1.9-5.9)	0.01
Incomplete resection for favorable tumors	6.6 (3.9-12.2)	0.002
No concurrent chemotherapy with RT	3.1 (2.2- 8.2)	0.01

RR: Relative risk; CI: Confidence interval; KPS: Karnofsky performance scale; MRI: Magnetic resonance imaging; RT: Radiation therapy.

children, adolescent and adult BSG in clinical presentation, MRI characteristics and treatment course, but there was a significant difference in median survival times (1.8 years in children/adolescents *vs* 1.2 years in adults) which is similar to other previous pediatric studies, although clearly shorter than reported by previous studies in adult BSG. The possible explanation for shorter median survival rates in adults could be high grade histology in our cohort. Similar findings were reported by Kaplan *et al*^[15] and Reithmeier *et al*^[16].

In our cohort, the most common subgroup was DIPG, of whom 27.3% underwent biopsy and subtotal resection, which is far from routine practice. The majority of biopsy proven DIPG had high grade astrocytoma on histology, which reflects the poor prognosis and shorter median survival in DIPG cases without histopathological confirmation. In addition, DIPG were also found to be more responsive to radiotherapy with concurrent chemotherapy in our cohort, suggesting that OS rates differ with different treatment strategies. However, trials of dose escalation (> 54Gy), hyperfractionated radiation therapy and incorporation of novel chemotherapeutic agents have failed to produce any meaningful change in the outcomes^[11-14,16]. These findings are confirmatory for the heterogeneous nature of DIPG^[17].

The subgroup of focal gliomas was the second most predominant in our cohort and these tumors were clearly found to be different from DIPG; however, the clinical picture was similar to DIPG. Complete removal in the majority of cases in our cohort reflected the improvement in median survival for such cases. Focal tectal gliomas constituted a small subgroup and these cases required CSF shunts for raised intracranial pressure, as reported by other studies^[18]. However, adjuvant radiotherapy can be criticized in children as such patients have been managed with a CSF shunt or observation alone for long periods^[19].

The third most common subgroup of dorsally exophytic gliomas in our cohort were managed successfully with complete resection in the majority of patients. However, in contradiction to the literature, our patients had a shorter median survival. A possible explanation could be high grade histology and no adjuvant radiotherapy^[20,21]. A similar explanation for shorter median survival was also

justified in our cohort of cervicomedullary glioma. Non-specific BSG, including medullary astroblastoma and pontomedullary BSG, had a similar clinical behavior and treatment outcome to DIPG^[22].

In conclusion, brain stem gliomas have heterogeneous biological behavior. The DIPG subgroup had a dismal prognosis, needing more aggressive neurosurgical, radiation and chemotherapy techniques, while focal and tectal tumors were found to have a better prognosis.

COMMENTS

Background

BSG remains a therapeutic dilemma because of the location and heterogeneous biological behavior of these tumors and treatment is mainly through a multidisciplinary approach.

Research frontiers

The present study focused on a Saudi population and found that the DIPG subgroup had a dismal prognosis, requiring more aggressive neurosurgical, radiation and chemotherapy techniques.

Innovations and breakthroughs

The present study revealed that stereotactic biopsy is feasible in DIPG and radiation therapy is associated with improvement of survival in patients with DIPG.

Applications

This study provides a treatment algorithm in brainstem glioma.

Peer review

The article addressed an important disease with a poor prognosis.

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MiR-210 expression reverses radioresistance of stem-like cells of oesophageal squamous cell carcinoma

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Abstract

AIM: To investigate the expression of miR-210 and the role it plays in the cell cycle to regulate radioresistance in oesophageal squamous cell carcinoma (ESCC).

METHODS: MiR-210 expression was evaluated in 37 pairs of ESCC tissues and matched para-tumorous normal oesophageal tissues from surgical patients who had not received neoadjuvant therapy, and in the cells of two novel radioresistant cell lines, TE-1R and Eca-109R, using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The transient up-regulation of miR-210 expression in TE-1R and Eca-109R cells was studied using liposomes and was confirmed using qRT-PCR. The rate of cell survival after a series of radio-treatment doses was evaluated using the clone

formation assay. Flow cytometry was used to detect the changes to the cell cycle patterns due to radiation treatment. RT-PCR and Western blot were used to detect the expression of ataxia telangiectasia mutated (ATM) and DNA dependent protein kinase (DNA-PKcs) after irradiation, and the cell sphere formation assay was used to evaluate the proliferative ability of the cancer stem-like cells.

RESULTS: The level of miR-210 expression was significantly decreased, by 21.3% to 97.2%, with the average being $39.2\% \pm 16.1\%$, in the ESCC tissues of most patients (81.1%, 30 of 37 *vs* patients with high miR-210 expression, $P < 0.05$). A low level of expression of miR-210 was correlated with a poorly differentiated pathological type ($P < 0.01$) but was not correlated with the T-stage or lymph node infiltration (both $P > 0.05$). Early local recurrences (< 18 mo, $n = 19$) after radiotherapy were significantly related with low miR-210 expression ($n = 13$, $P < 0.05$). The level of miR-210 was decreased by approximately 73% (*vs* TE-1, 0.27 ± 0.10 , $P < 0.01$) in the established radioresistant TE-1R cell line and by 52% (*vs* Eca-109, 0.48 ± 0.17 , $P < 0.05$) in the corresponding Eca-109R line. Transient transfection with a miR-210 precursor increased the level of miR-210 expression, leading to a significant increase in cell survival after radiotherapy ($P < 0.05$). Twenty-four hours after radiation, the proportion of pmiR-210 cells in S phase was increased (*vs* control cells, $30.4\% \pm 0.4\%$, and *vs* untreated TE-1R cells, $23.3\% \pm 0.7\%$, $P < 0.05$ for both). The levels of DNA-PKcs (0.21 ± 0.07) and ATM (0.12 ± 0.03 , $P < 0.05$) proteins were significantly lower in the PmiR-210 cells than in control cells, but no differences were found in the levels of the corresponding mRNAs in the two cell types ($P > 0.05$ for all). Exogenous miR-210 expression decreased the diameter of pmiR-210 cell spheres (*vs* control cells, 0.60 ± 0.14 , $P < 0.05$).

CONCLUSION: MiR-210 expression is negatively correlated with the pathological type and the local survival

rate after radiotherapy, and high expression of miR-210 may reverse the radioresistance of ESCC stem-like cells.

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Key words: MiR-210; Oesophageal squamous cell carcinoma; Radiation resistance; Cell cycle arrest; Stem-like cells

Core tip: A low level of miR-210 expression, which is common in oesophageal squamous cell carcinoma (ESCC) tissues, was found to be negatively correlated with the tumour pathological type and the prognosis in ESCC patients after radiotherapy, although the sample size was small. A relatively high level of *in vitro* miR-210 expression reversed the radioresistance of ESCC stem-like cells by decreasing the extent of ataxia telangiectasia mutated/DNA dependent protein kinase-dependent cell cycle arrest, failure of DNA double-strand break repair and stem cell proliferation.

Chen X, Guo J, Xi RX, Chang YW, Pan FY, Zhang XZ. MiR-210 expression reverses radioresistance of stem-like cells of oesophageal squamous cell carcinoma. *World J Clin Oncol* 2014; 5(5): 1068-1077 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i5/1068.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i5.1068>

INTRODUCTION

Oesophageal squamous cell carcinoma (ESCC) has occult symptoms and signs and is difficult to diagnose in the early stages. Radiation therapy is currently one of the main treatments for ESCC, particularly in the case of cervical and upper thoracic lesions. Even with concurrent chemoradiotherapy, the 5-year survival rate is still less than 30%, worse than those of many other squamous cell carcinomas. Local recurrences and the apparently increased radioresistance of recurrent tumours are the main reasons for treatment failure.

The mechanism of tumour resistance to radiotherapy is still unclear. There is a growing body of evidence that microRNAs (miRNAs) involved in the regulation of multiple cellular pathways are associated with radiation resistance. A number of miR-210 target genes have been identified that play roles in the cell cycle^[1], DNA repair^[2], vascular generation^[3] and tumour stem cell survival^[4]. MiR-210 was shown to be involved in the radiosensitivity of tumour cells^[5,6]. Ataxia telangiectasia mutated (ATM) is a key signalling gene in the early reaction to irradiation, which causes the double-strand break (DSB)-induced DNA damage response^[7]. ATM is a Ser/Thr kinase that phosphorylates more than a hundred proteins to orchestrate cell cycle checkpoint activity^[8-10].

However, there is no evidence that miR-210 affects the radiosensitivity in ESCC. Thus, the purpose of this study was to evaluate miR-210 expression in oesophageal cancer tissues, to explore the possibility that it participates in regulating cellular radioresistance, and to study its pos-

sible role in cell cycle regulation to explore the feasibility of miR-210 as a radiation-sensitive therapeutic target.

MATERIALS AND METHODS

Patients

This study included 37 male patients with a median age of 54 (range, 42-71) years. All of the patients had been diagnosed with ESCC by biopsy. The para-tumorous normal oesophageal tissues, which comprised the oesophageal mucosa 5 cm from the cancer tissue collection site and close to the resection margin, were normal in appearance. The tissue specimens were collected less than 15 min after resection, fixed for 30 min in liquid nitrogen and stored at -80 °C. All of the selected patients received radiotherapy or concurrent chemoradiation in 2008-2009. All of the patients received radiotherapy no more than 3 mo after surgery and were followed until a local recurrence arose or for at least 35 mo. The median follow-up time was 23.4 (range, 8.7-35.3) mo. This study was approved by the Institutional Review Board of the First Affiliated Hospital of the Medical School of Xi'an Jiaotong University.

Cell lines and cell culture

The human ESCC cell lines Eca-109 and TE-1 (a gift of the Department of Cardiothoracic Surgery, Second Military Medical University, Shanghai, China) were cultured using high-glucose Dulbecco's modification of Eagle's medium (DMEM) that was supplemented with 10% foetal bovine serum (10000 units of penicillin and 10000 µg of streptomycin per mL, all of which were purchased from Gibco Invitrogen, CA, United States). The stem-like radioresistant cell lines were created using fractionated radiation of up to 100 Gy, as previously described^[11], and were named TE-1R and Eca-109R. Cell spheres were cultured using DMEM/F12 medium (Gibco Invitrogen) that was supplemented with 2 ng/mL of epidermal growth factor and basic fibroblast growth factor (b-FGF) (all obtained from Pepro Tech Inc., NJ, United States) on 50 g/L agarose-phosphate Buffered Saline (PBS) coated plates. All of the cells were cultivated in a humidified atmosphere containing 50 g/L of CO₂ at 37 °C.

MiRNA precursor transfection

Pre-miR-210 (50 pmol; Genetimes Tech Inc., Shanghai, China) and a scrambled control (50 pmol; Genetimes Tech Inc.) were transfected into TE-1R cells growing in six-well dishes (plated at 2×10^5 cells per well 24 h before transfection), which were called PmiR-210 cells and Ctrl cells, respectively. Transfection was conducted using Lipofectamine 2000 (Invitrogen). The transfection efficiency (> 200%) at 24, 36, 48 and 72 h after transfection was determined using quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

RNA extraction and real-time PCR profiling of the content of mature miRNA

The total RNA was extracted from cell lysates according

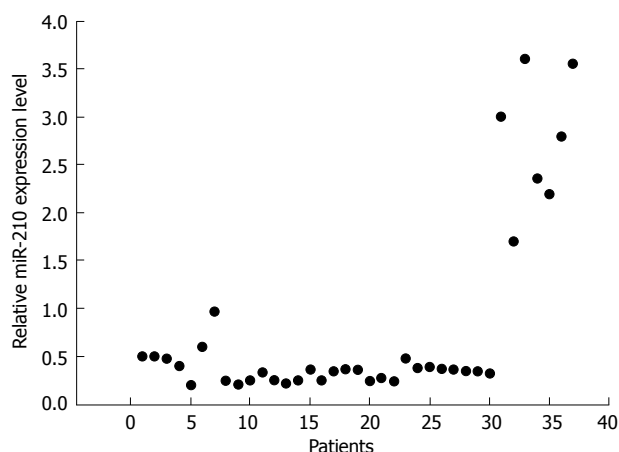


Figure 1 MiR-210 expression in selected oesophageal squamous cell carcinoma patients. The level of MiR-210 expression in an oesophageal squamous cell carcinoma tumour tissue was related to the level in that patient's para-tumorous normal oesophageal tissue.

to the manufacturer's protocol. The concentration of the RNA was determined using Ultraviolet Rays spectrophotometry (NanoDrop® ND-8000 Spectrophotometer, Thermo Fisher Scientific, CA, United States). The quality of RNA was determined by electrophoresis on a 10 g/L agarose gel. All of the RNA samples were confirmed to be non-degraded by visualisation of the distinct 28S and 18S rRNA species. The total RNA was used to synthesise first-strand cDNA. The expression of mature miRNA-210 was profiled using a real-time quantitative PCR assay (all of the kits were obtained from Fermentas, Thermo Scientific, CA, United States) as previously described^[11].

Cell irradiation and measurement of cell viability

Cells (3×10^3 cells per well in a 12-well plate) were irradiated at room temperature with 10 mV photons from a linear accelerator (Electa, WI, United States) at doses of 2, 4, 6 or 8 Gy (a uni-dose of 6 Gy for the cells that were used in the cell-sphere formation and cell-cycle analyses). The controls were handled identically as were the irradiated cells with the exception of the radiation treatment. The cells were allowed to grow for 7 d before analysis of the clones or spheres that formed.

Analysis of the cell cycle distribution

Cells were seeded in 100-mL culture flasks at a density of 5×10^5 cells per flask. After 24 h, the cells were treated with uni-dose irradiation at 6 Gy. The attached and floating cells were harvested at different time points. The cells were then suspended at 1×10^6 /mL of propidium iodide solution (3.8 mmol/L of sodium citrate; 0.05 g/L of propidium iodide and 1 g/L of Triton X-100) supplemented with RNaseB and were maintained in the dark at 4 °C. The cells were then analysed using flow cytometry.

Western blot

The cells were twice washed with phosphate-buffered solution and then directly lysed for 30 min on ice using

radio-immunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology). After centrifugation at $12000 \times g$ for 26 min, the protein concentrations were determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). The proteins were transferred onto nitrocellulose membranes. After blocking with 5% milk in Tris-buffered saline containing 0.5 g/L Tween-20, the membranes were incubated with anti-ATM (rabbit monoclonal antibody, 1:500, Epitomics, CA, United States), anti-DNA-PKcs (rabbit monoclonal antibody, 1:500, Cell Signaling Technology, MA, United States) and anti-glyceraldehyde-3-phosphate dehydrogenase (rabbit monoclonal antibody, 1:1000, Cell Signaling Technology). The immune complexes were detected using horseradish peroxidase-conjugated immunoglobulin G (goat anti-rabbit antibody, Cell Signaling Technology). The labelled antibodies were visualised using an enhanced chemiluminescent substrate (34079, Thermo Fisher Scientific). All of the membranes were exposed to Kodak X-OMAT X-ray film. The details of the method were previously described^[11].

Statistical analysis

The data are expressed as mean and standard deviation or standard error of the mean of the results of two or three independent experiments. Normalisation of the miRNA expression levels was obtained using the comparative DCt method (2-delta-delta computed tomography using the miR-210 expression levels in normal tissues and that of RNAU6B as references). The difference between the times of local recurrence of the groups was tested for significance using the log rank test. The clones were counted using Photoshop cs3 as previously described^[12]. After filtration through 100-µm pores and suspension in 100 µL of cold PBS, the cell spheres in three randomised images taken at $100 \times$ were compared using Photoshop cs3. Student's *t*-test was used to analyse the significance of the differences between the different treatment groups whenever applicable with the ^a*P*-value set at < 0.05 and the ^b*P*-value set at < 0.01 . The statistical analyses were performed using SPSS version 13 software.

RESULTS

A low level of miR-210 expression in ESCC tissues is related to early recurrence

In most of the ESCC patients, the level of miR-210 expression in the ESCC tissue was significantly lower than that in the para-tumorous normal oesophageal tissue from the same patient (81.1%, 30 of 37 patients *vs* patients with high miR-210 expression, $P < 0.05$). The expression levels ranged from 21.3% to 97.2%, and the average level was $39.2\% \pm 16.1\%$ in the patients with low miR-210 expression. Seven patients had a higher level of miR-210 expression in their ESCC tissues than in their normal tissues. The level of high expression ranged from 170.4%-360.8%, and the average level was $193.5\% \pm 36.2\%$ (Figure 1). A poor pathological type was significantly related to a low level of miR-210 expression in

Table 1 Clinical and pathological data and levels of miR-210 expression in the selected oesophageal squamous cell carcinoma patients

Clinical feature		n	MiR-210 expression level		Local recurrence time	
			Low	High	Early	Late and not
Pathological grade	II	11	7	6	2	9
	III	26	23	1 ^b	17	9 ^b
T-stage	II	13	11	2	3	10 ^a
	III	24	19	5	16	8
Lymph node infiltration	+	16	12	4	11	5
	-	21	18	3	8	13

^a $P < 0.05$, ^b $P < 0.01$, patients *vs* the patients with miR-210 expression. All of the patients were male, with a median age of 54 (range, 42-71) years. The level of miR-210 expression was determined using quantitative reverse transcription-polymerase chain reaction. The level of expression in the oesophageal squamous cell carcinoma tissue was compared with that in the same patient's para-tumorous normal oesophageal tissue. The endpoint of the follow-up period was when a local recurrence arose or at least 35 mo, and 18 mo was chosen to distinguish early and non-early recurrences.

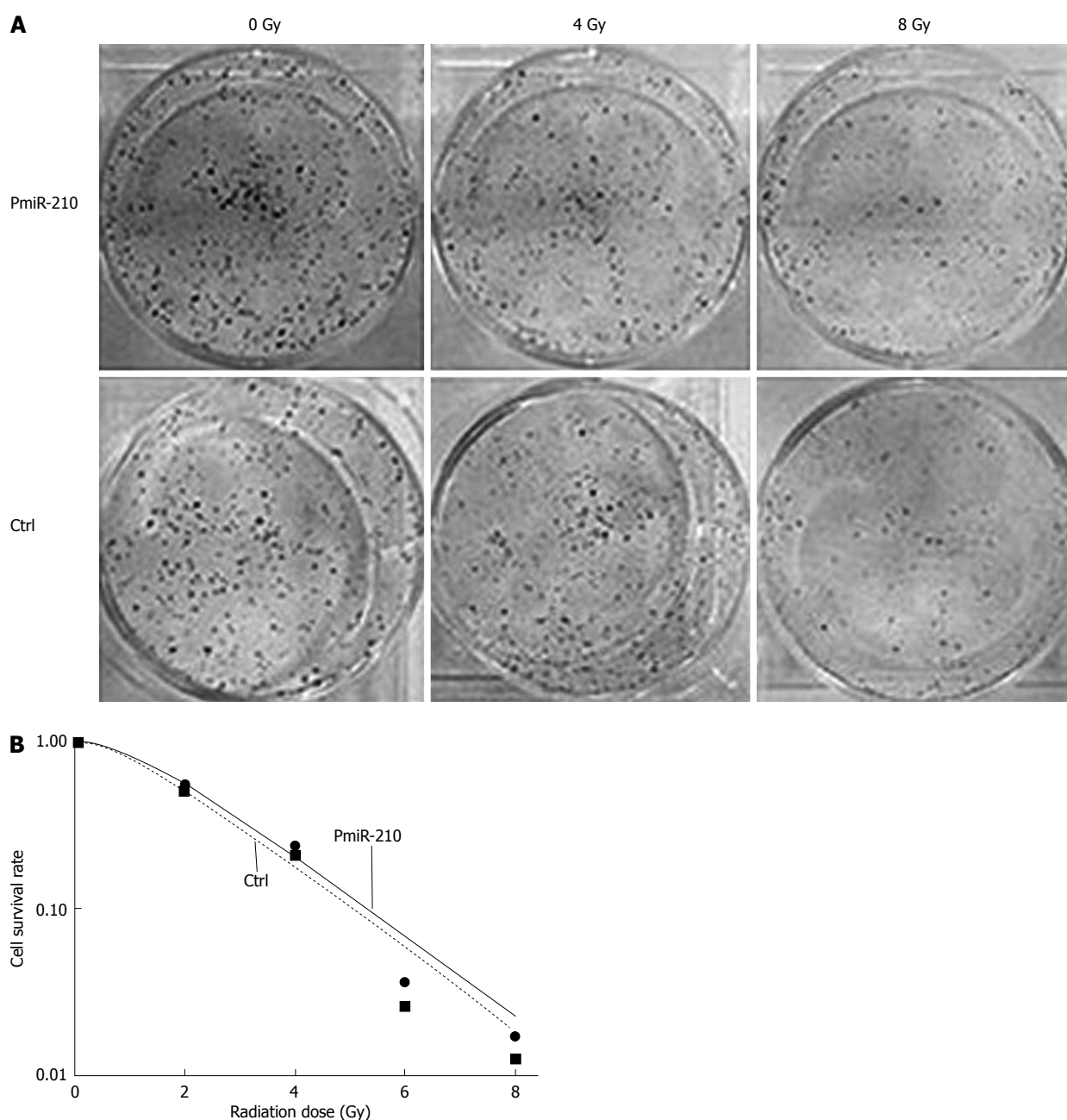


Figure 2 MiR-210 reverses the radioresistance of TE-1R cells. A: Clonal formation of TE-1R cells that were transfected with the miR-210 mimic (pmiR-210) or with a nonsense sequence (Ctrl) after irradiation at 0, 4 or 8 Gy of 10 mV X-rays. The experiments were performed in triplicate; B: Cell survival curve for TE-1R cells that were transfected with the miR-210 mimic (pmiR-210) or with a nonsense sequence (Ctrl). The cell survival rates were plotted on a logarithmic scale.

Table 2 Rates of clone formation of irradiated TE-1R cells

Cell type	0 Gy	2 Gy	4 Gy	6 Gy	8 Gy
PmiR-210	100.00% ± 0.77%	52.49% ± 0.22%	21.82% ± 0.17% ^a	3.17% ± 0.11% ^a	1.27% ± 0.03% ^a
Ctrl	100.00% ± 0.14%	58.17% ± 0.16%	26.88% ± 0.36%	7.51% ± 0.09%	2.49% ± 0.01%

^a $P < 0.05$, *vs* Ctrl. PmiR-210: TE-1R cells transfected with an miR-210 mimic; Ctrl: TE-1R cells transfected with a nonsense sequence. The experiments were performed in triplicate.

Table 3 Radiobiological indices of the pmiR-210 and Ctrl cells

	D ₀	D _q	N	SF ₂
PmiR-210	1.73% ± 0.01%	1.56% ± 0.02% ^a	1.97% ± 0.03% ^a	43.62% ± 0.14% ^a
Ctrl	1.75% ± 0.02%	1.89% ± 0.13%	3.13% ± 0.07%	67.83% ± 0.16%

^a $P < 0.05$, *vs* Ctrl. PmiR-210: TE-1R cells transfected with the miR-210 mimic; Ctrl: TE-1R cells transfected with a nonsense sequence. The experiments were performed in triplicate. D₀: Mean lethal dose; D_q: Quasi-threshold dose; N: Extrapolated number; SF₂: Survival fraction after 2-Gy irradiation. The lower D₀ and N of the pmiR-210 cells compared with those of the Ctrl cells indicate a less efficient repair of radiation damage. A low SF₂ value indicates a level of high sensitivity to radiotherapy.

the ESCC tissue ($P < 0.01$, Table 1). However, there was no significant correlation between the level of miR-210 expression and the T stage ($P > 0.05$) or lymph-node infiltration ($P > 0.05$). However, early local recurrences (< 18 mo, $n = 19$) were significantly correlated with low miR-210 expression ($n = 13$, $P < 0.05$). Four patients did not experience local recurrence over the 35-mo observation period.

Up-regulated miR-210 expression reverses the radioresistance of TE-1R cells

The level of miR-210 expression was decreased by approximately 73% (*vs* TE-1, 0.27 ± 0.10 , $P < 0.01$) in radioresistant TE-1R cells and by approximately 52% (*vs* Eca-109, 0.48 ± 0.17 , $P < 0.05$) in radioresistant Eca-109R cells. Transient transfection of an miR-210 precursor into TE-1R cells led to a high level of miR-210 expression at 72 h (*vs* TE-1, > 120 times higher, $P < 0.001$), compared with that of Ctrl cells, which remained high even at 5 d (approximately 19.4 times higher, $P < 0.05$). PmiR-210 and Ctrl cells were irradiated with a series of Gy doses and a significant difference in cell clone formation at 7 d of culture was observed in the cells that had been irradiated with high dosages (Table 2, Figure 2A). A significant left-downward trend in the pmiR-210 cell-survival curve compared with that of the Ctrl cells was observed (Figure 2B). The radiobiological indices (D₀, N, SF₂) of the pmiR-210 cells that were obtained by regression of their survival curve data were lower than those of the Ctrl cells (Table 2, $P < 0.05$ for all; Table 3), indicating their higher sensitivity to irradiation.

MiR-210 transfection induces S-phase arrest caused by radiation stress

PmiR-210 and Ctrl cells were uni-irradiated with 6 Gy and then the cell cycle distribution was analysed using flow cytometry (Figure 3). There were no significant differences between the cell cycle distributions of the

Ctrl and untreated cells in terms of the proportion of cells in G₀/G₁ phase ($64.2\% \pm 0.5\%$ *vs* $61.8\% \pm 0.7\%$, $P > 0.05$), in S-phase ($23.3\% \pm 0.8\%$ *vs* $27.6\% \pm 0.5\%$, $P > 0.05$) or in G₂/M phase ($13.4\% \pm 0.4\%$ *vs* $12.1\% \pm 0.5\%$, $P > 0.05$). However, compared with that of the Ctrl cells, a significantly larger proportion of the pmiR-210-transfected cells were in S phase ($30.4\% \pm 0.4\%$ *vs* $23.3\% \pm 0.7\%$, $P < 0.05$), although no significant differences in the proportion of cells in the G₀/G₁ ($69.2\% \pm 0.7\%$ *vs* $62.5\% \pm 0.6\%$, $P > 0.05$) or G₂/M ($12.4\% \pm 0.5\%$ *vs* $20.2\% \pm 0.3\%$, $P > 0.05$) phases were observed. The levels of DNA-pkcs and ATM mRNAs and proteins were also analysed using RT-PCR and Western blot, respectively, (Figure 4) and no differences in the relevant mRNA levels in the two cell types were found ($P > 0.05$ for all), but the levels of the proteins were significantly lower in the pmiR-210 cells than in the Ctrl cells, with the DNA-pkcs level at 0.21 ± 0.07 and the ATM expression level at 0.12 ± 0.03 ($P < 0.05$ for both) in the former cells.

Up-regulated miR-210 expression reduces the size of the spheres formed by the stem-like cells

The spheres that had the various cell types had developed in serum-free medium at 7 d after irradiation were compared (Figure 5). The diameters of the cell spheres that pmiR-210 transfected TE-1R cells formed were smaller than those formed by Ctrl cells (0.60 ± 0.14 , $P < 0.05$) or by untreated TE-1R cells (0.25 ± 0.08 , $P < 0.01$).

DISCUSSION

MiR-210 is highly expressed in glioma^[13], melanoma^[14,15], renal cell carcinoma^[16], pancreatic cancer^[17,18], breast cancer^[19-21] and lung cancer^[22] that are generally associated with a poor disease-free survival rate or poor overall survival rate. The up-regulation of miR-210 expression directly suppressed Bcl-2 adenovirus E1B 19 kDa-inter-

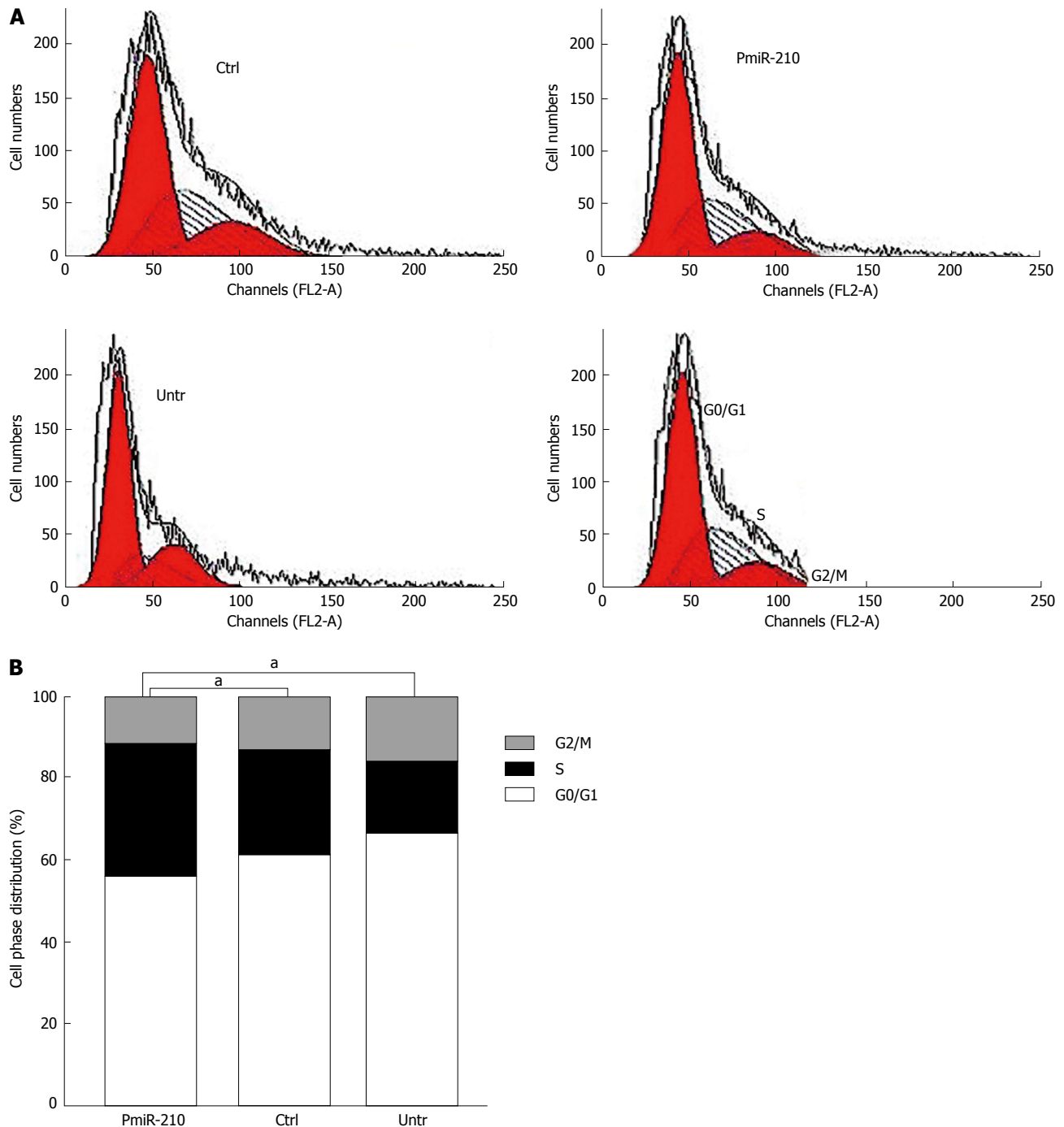


Figure 3 S-phase arrest in miR-210 up-regulated radioresistant oesophageal squamous cell carcinoma cells. A: Flow cytometry was used to determine the distribution of the cells according to the phases of the cell cycle. All of the cells were uni-irradiated at 6 Gy and analysed 24 h later. The experiments were performed in triplicate. PmiR-210: Transfected with pmiR-210. Ctrl: Transfected with a nonsense sequence. Untr: Untreated; B: Histogram showing the cell cycle distribution of the cells. The fractions of cells in S phase were compared. ^a $P < 0.05$, pmiR-210 vs Ctrl or Untr.

acting protein 3 (BNIP3) expression to maintain the survival of neural progenitor cells^[4] and knocking down the expression of miR-210 in combination with radiotherapy was found to have enhanced its anti-tumour effect in human hepatoma xenografts^[5]; and moreover, miR-210 expression promoted more efficient DSB repair^[7]. Unexpectedly, the significantly lower expression of miR-210 in ESCC tissues was observed in paired tumour/normal tissue sets in our study. In this study, we observed a low level of miR-210 expression in 81.1% of the tumours of

male ESCC patients than in the isogenically paired tissues ($P < 0.01$), which was correlated with the early local recurrence after surgery followed by radiotherapy ($P < 0.05$). Our results were consistent with those of some of the other ESCC studies^[23,24] in which compared to that of normal oesophageal tissue, a low level of miR-210 expression in ESCC was correlated with either poorly differentiated carcinoma or a poor prognosis. Although the sample size ($n = 37$) is not large, all of our selected patients received radiotherapy after surgery, which most

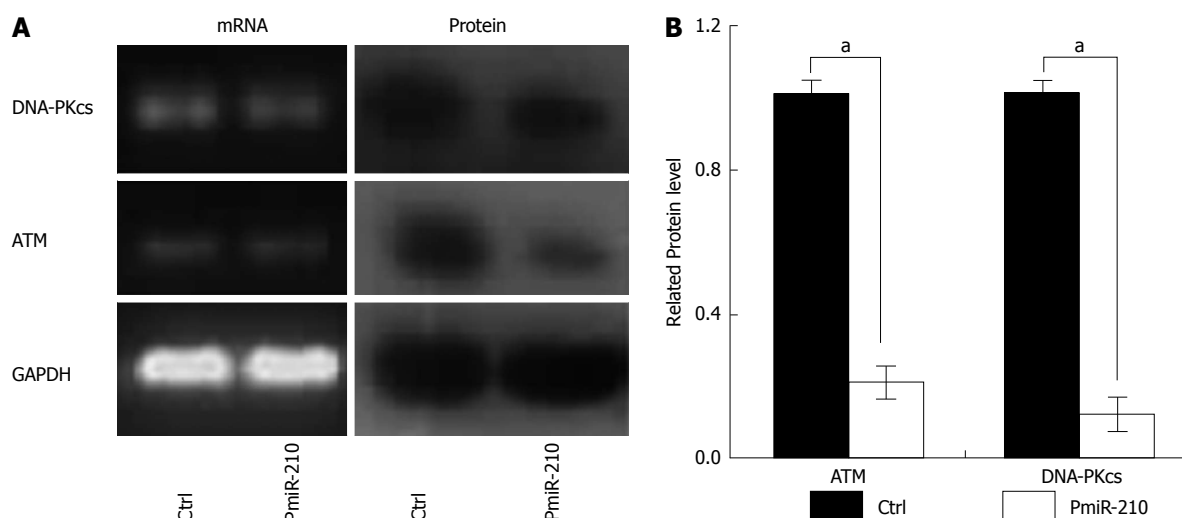


Figure 4 Double-strand break-related changes in protein expression in miR-210 up-regulated radioresistant oesophageal squamous cell carcinoma cells. **A:** The expression levels of ataxia telangiectasia mutated (ATM) and DNA dependent protein kinase (DNA-PKcs) in transfected and control TE-1R cells. The mRNA levels were determined using reverse transcription-polymerase chain reaction. The protein levels were determined using Western blot. The experiments were performed in triplicate. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal reference; **B:** Histogram showing the levels of ATM and DNA-PKcs protein expression in transfected and control TE-1R cells. ^a $P < 0.05$, pmiR-210 vs Ctrl.

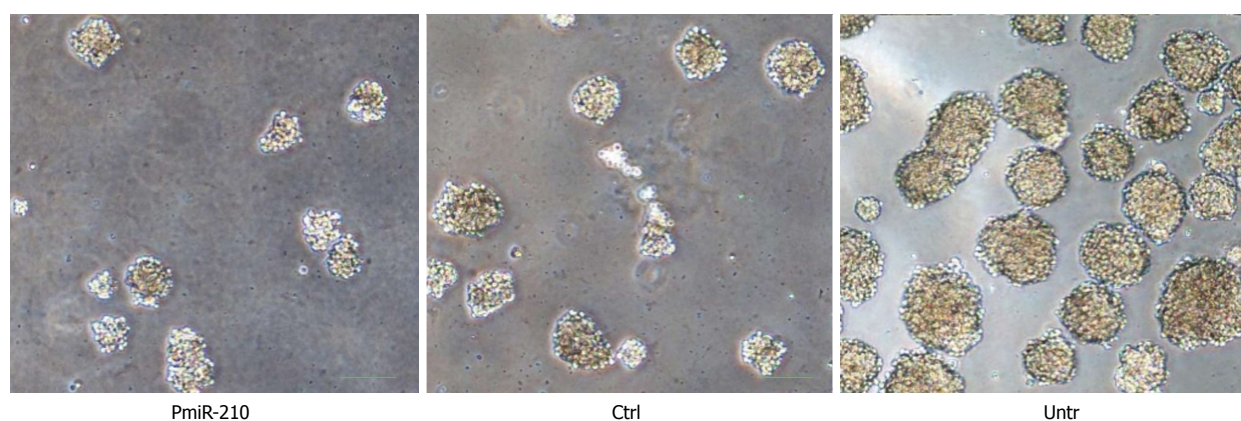


Figure 5 Cell sphere formation of pmiR-210 cells after irradiation (bar: 100 μ m). PmiR-210: Transfected with pmiR-210; Ctrl: Transfected with a nonsense sequence; Untr: Untreated. Cell spheres were allowed to form in serum-free medium for 7 d after 4-Gy radiation. The diameter and number of pmiR-210 cell spheres were the smallest among those of the three types of cells. Bar, 100 μ m.

closely resembles the clinical reality. Obviously, the non-randomised patient selection will lead to some bias in our observations and might have caused false-positive outcomes in this study. Therefore, we used two radioresistant cell lines, TE-1R and Eca-109R, to verify the relationship between radioresistance and the level of miR-210 expression.

We found that miR-210 expression was decreased in both of the radioresistant cell lines, by 73% (0.27 ± 0.10 , $P < 0.01$) in TE-1R cells and by 52% (0.48 ± 0.17 , $P < 0.05$) in Eca-109R cells. These results confirmed our result that the down-regulation of miR-210 expression was correlated with bad local tumour control after surgery followed by radiotherapy. We then up-regulated the expression of miR-210 by transiently transfecting the miRNA-210 precursor in the more radioresistant TE-1R cells in which miRNA-210 expression was more down-regulated to analyse the relationship between miR-210

expression and radiosensitivity. The results showed that up-regulated miR-210 expression could reverse the radioresistance of TE-1R cells compared with the effect of transfection with the scrambled sequence (Ctrl cells, Figure 2, Tables 2 and 3). MiR-210 has been previously shown to repress or stimulate cell proliferation, depending on the cellular model. MiR-210 targets proteins that are crucial for cell cycle progression, such as E2F3, FGFR139, or HOXA1, to inhibit cellular growth. In contrast, miR-210 also targets the Myc-antagonist MNT41 to promote cell cycle progression in some types of cancer cells. Lung adenocarcinoma (A549 or H1975) cells that stably expressed miR-210 did not show any alteration in their proliferation rate but a high level of miR-210 expression after irradiation significantly reduced the apoptosis rate of A549 cells and improved their DSB repair rate^[6]. The lack of radiosensitisation in prostate cancer cells treated with an miR-210 inhibitor under an-

oxic conditions suggested that the extent of inhibition of miR-210 expression may depend on the type of cancer and/or the degree and duration of hypoxia^[6]. Our results regarding ESCC were consistent with those of another study, in which miR-210 was found to inhibit cancer cell survival and proliferation by inducing cell death^[24].

Consistent with the observed miR-210-mediated sensitivity to radiation, analysis of the cell cycle distribution of the cell populations revealed an increase in S-phase arrest in pmiR-210 cells compared with Ctrl TE-1R and untreated TE-1R cells after irradiation (Figure 3). In contrast, in another study, no significant changes were observed in the cell viability rate or the cell cycle profile when the expression of miR-210 was suppressed^[3]. Transfection with an miR-210 inhibitor was shown to decrease the rates of cell viability and accumulation of CAKI-2 cells in the G₂ phase of the cell cycle^[25]. However, it was previously reported that miR-210 could induce cell-type specific proliferation. In certain transformed cells, inhibiting the expression of the c-Myc antagonist and miR-210 revealed a direct target, MAX-binding protein (MNT), which was demonstrated to be functionally important in controlling the progression of the cell cycle through the reciprocal up-regulation of c-Myc activity. Increasing the level of miR-210 expression in various tumours could, through targeting E2F3 and activin receptor 1b (ACVR1b), activate G₁/S-phase cell cycle progression and increase the rate of cellular proliferation^[26]. In other types of tumours, depending on the contextual cues, miR-210 could target a different set of mRNAs, such as HOXA3 and HOXA9, and contribute to the reduction of the rate of cell proliferation^[27]. The S-phase arresting effect observed under our conditions, which contradicted the results of another study of cell cycle arrest in the G₁/G₀ and G₂/M phases^[24], might be explained by the fact that the function of miR-210 has to date been studied within 3 d following transient transfection using a relatively high concentration of an miRNA precursor. The true effect of increased miR-210 expression on the cell cycle may be observed during shorter periods, as was found in arsenic trioxide-treated tumour cells that were arrested at the G₂/M phase of the cell cycle at 30 h post-treatment and which overrode the G₂/M boundary at 48 h^[28].

To understand the potential of increased miR-210 content on the DSB repair function of TE-1R cells, changes in the ATM and DNA-PKcs levels were evaluated in pmiR-210 and Ctrl cells after irradiation. ATM and DNA-PKcs play different roles in the DNA damage response pathway (DDR), but both of them are activated by the occurrence of DSB; they have common targets in the DDR pathway and the absence of either kinase results in faulty DSB repair. The absence of ATM allows timely repair, which nevertheless, is incomplete. In contrast, the absence of DNA-PKcs leads to slower repair, which in turn gives rise to the accumulation of simple and complex chromosomal reorganisations^[7]. Consistent with the miR-210-mediated sensitivity to radiation, the levels of ATM and DNA-PKcs proteins were surprisingly significantly lower in PmiR-210 cells that were sub-

jected to 6 Gy compared with those of Ctrl cells (DNA-PKcs, 0.21 ± 0.07 and ATM, 0.12 ± 0.03 , $P < 0.05$ for all), but no differences were found in the corresponding mRNA levels of the two cell types ($P > 0.05$ for all) (Figure 4) under our conditions. In contrast, it has been shown that miR-210 expression promoted more efficient DSB repair in A549 cells^[6]. MiR-210 has been shown to repress mitochondrial metabolism by targeting a number of proteins that are crucial for normal tricarboxylic acid cycle and electron transport chain activity^[29]. To date, there is no evidence that miR-210 targets the 3'-UTR of ATM or DNA-PKcs mRNAs. MiR-101 can bind to the 3'-UTR of DNA-PKcs and ATM mRNAs^[3]. However, this miRNA targeted not only the 3'-UTR but also the 5'-UTR and coding sequences, which were still present in the expression construct in which only the 3'-UTR of ATM had been substituted, allowing ATM expression to be regulated by miR-181a, miR-326, and miR-345^[30]. Thus, there is a potential for miR-210 to target the 5'-UTR or coding sequences of their mRNAs to regulate the expression of the ATM or DNA-PKcs proteins.

A radioresistant cell is considered a type of cancer stem-like cell. Thus, we examined the effect of miR-210 expression on the sphere-formation ability of these stem cells, which is a widely accepted method to determine the proliferative ability of stem cells. The up-regulation of miR-210 content directly suppressed the expression of BNIP3 to maintain the survival of neural progenitor cells under hypoxic conditions^[4]. In contrast, it was observed that miR-210 expression inhibited the formation of cell spheres by ESCC radioresistant TE-1R cells (Figure 5), which is first reported here. This result suggested that miR-210 expression reversed the radioresistance of ESCC cancer stem cells.

Taken together, the results of this study demonstrated that a low level of miR-210 expression was common in the tumours of ESCC patients and that the level of miR-210 expression was negatively correlated with a poorly differentiated pathological type and rate of local control after radiotherapy. Increased miR-210 expression reversed the radioresistance of stem cell-like cells of ESCC by decreasing the ATM and DNA-PKcs-dependent cell cycle arrest and the rates of DSB repair and stem cell proliferation. The mechanisms underlying these processes must be determined in future investigations.

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COMMENTS

Background

The mechanism underlying tumour resistance to radiotherapy is unclear. There is a growing body of evidence showing that microRNAs are involved in the

regulation of multiple cellular pathways that are associated with radiation resistance. A number of miR-210 target genes that have been identified are involved in the cell cycle, DNA repair, vascular generation and tumour stem cell survival, most of which are important processes in the development of radioresistance.

Research frontiers

MiR-210 appears to be involved in radiosensitivity of tumour cells. However, there is no evidence that miR-210 affects the radiosensitivity of oesophageal squamous cell carcinoma (ESCC) cells. In this study, the authors demonstrated that miR-210 may affect the prognosis of radiotherapy and participate in the regulation of radioresistance in ESCC cells.

Innovations and breakthroughs

Recent reports have highlighted the importance of miR-210 expression in hepatomas and lung cancer. This is the first study to report that miR-210 is expressed at a low level in ESCC and that the level of its expression is correlated with the prognosis of ESCC patients treated with radiotherapy. Furthermore, the results of the authors *in vitro* studies suggested that this miRNA may be an important regulator of radioresistance in ESCC.

Applications

By demonstrating that miR-210 regulates the radioresistance of ESCC cells, this study presents a novel target for reversing the radioresistance in relapsed ESCC.

Terminology

The radioresistance that develops during radiotherapy leads to an increased number of cancer stem cells, which appear to be concentrated by therapeutic selection or radiation induction. Radioresistant cells are also called cancer stem-like cells. These cells have been found to have a distinctive cell cycle distribution pattern and a high efficient mechanism of DNA double-strand break repair.

Peer review

It is meaningful in that miR-210 is explored in ESCC cells with radiation resistance.

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Blood classical monocytes phenotype is not altered in primary non-small cell lung cancer

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Abstract

AIM: To evaluate the M1 and M2 monocyte phenotype in patients with non-small cell lung cancer (NSCLC) compared to controls. Also, to examine the expression of Th1 and Th2 cytokines in plasma of NSCLC vs controls.

METHODS: Freshly prepared peripheral blood mononuclear cells samples were obtained from patients with NSCLC (lung adenocarcinoma and squamous cell lung carcinoma) and from non-cancer controls. Flow cytometry was performed to investigate M1 and M2 phenotypes in peripheral monocytes (classical monocytes CD14+, CD45+ and CD16-) using conventional surface markers. Th1 and Th2 cytokine production was also

analysed in the plasma using cytometric bead array technique.

RESULTS: There were no significant difference in expression of M1 (HLA-DR) and/or M2 markers (CD163 and CD36) markers on classical monocytes in patients with NSCLC compared to non-cancer controls. Expression of CD11b, CD11c, CD71 and CD44 was also shown to be similar in patients with NSCLC compared to non-cancer controls. Th1 and Th2 cytokines [interleukin (IL)-1 β , IL-2, IL-4, IL-5, IL-8, IL-10, IL-12 (p70), tumor necrosis factor (TNF)- α , TNF- β , and interferon- γ] analysis revealed no significant difference between patients with NSCLC and non-cancer controls.

CONCLUSION: This study shows no alteration in peripheral monocyte phenotype in circulating classical monocytes in patients with NSCLC compared to non-cancer controls. No difference in Th1 and Th2 cytokine levels were noted in the plasma of these patients.

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Key words: Lung cancer; Monocyte; Phenotype; Polarisation; Tumour progression; Tumour regression

Core tip: Monocytes perform a critical role in immune system and have similar phenotype as seen in M1 (classically activated) and M2 (alternatively activated) tumour-associated macrophage. Nevertheless, monocyte phenotypes in human lung cancer patients are not fully understood and further investigation are really needed. Our study examines the M1 and M2 monocyte phenotypes in patients with non-small lung carcinoma [non-small cell lung cancer (NSCLC)] compared to non-cancer controls. This study indicated that freshly isolated peripheral blood monocytes from patients with NSCLC do not show an altered phenotype and/or cytokines secretion. These outcomes might enhance the knowledge regarding the connections between monocyte-macrophage phenotype and tumour progression.

Almatroodi SA, McDonald CF, Collins AL, Darby IA, Pouniotis DS. Blood classical monocytes phenotype is not altered in primary non-small cell lung cancer. *World J Clin Oncol* 2014; 5(5): 1078-1087 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i5/1078.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i5.1078>

INTRODUCTION

Lung cancer is now one of the most common cancers in the world and, as the leading cause of cancer mortality, is responsible for about 1.4 million deaths worldwide annually^[1]. Despite incremental advances in treatment strategies, the prognosis for lung cancer remains poor, with only 10%-15% of patients surviving five years or longer^[1,2]. Further understanding of the immunology of lung cancer may enable the development of immune-modulatory strategies beyond those in current use, such as targeted monoclonal antibodies to specific cellular receptors.

Monocytes are an important part of the innate immune response to cancer. The notion that the immune system has a protective role in tumour development is well established^[3], with recent work also suggesting a converse role in promoting tumour initiation and progression^[4]. Previous studies looking at monocytes in a range of different cancer types have demonstrated conflicting results regarding monocyte phenotype and function in different cancer microenvironments. Studies in patients with lung, breast and other cancers have described hindered monocyte function^[5,6], whereas Mariotta *et al*^[7] suggested that non-small lung carcinoma (NSCLC) does not affect monocyte adherence and phagocytosis in lung cancer patients compared to healthy controls. Other studies demonstrated that monocytes are capable of both inhibiting and stimulating tumour growth^[8].

Monocytes can be characterised into classical monocyte (pro-inflammatory) and non-classical monocyte (anti-inflammatory) phenotype, both of which have been detected in circulating peripheral blood mononuclear cells (PBMC)^[9,10]. Monocytes are known to differentiate into tissue macrophages. Classical monocytes (CD14⁺⁺/CD16) identified to differentiate into M1 macrophage, while non-classical monocytes (CD14⁺/CD16⁺⁺) differentiate into M2 macrophage^[11]. M1 works as an antigen-presenting cell and has a vital role in immune activation and function^[12]. In contrast, M2 is known to be associated with poor antigen presentation producing factors that suppress T cell proliferation and activity^[12]. Although, the main source of tissue macrophages is classical monocytes, the majority of the macrophages within the tumour area have been identified as M2 macrophage^[11]. However, a study has reported that classical monocytes can differentiate into M2 macrophage^[13].

There is a controversy regarding monocyte differentiation and effect in tumour microenvironment. Therefore in this current study, freshly isolated un-stimulated classical monocytes were used to ascertain the phenotypic changes in patients with NSCLC compared to non-

cancer controls. To the best of our knowledge this is the first study that has analysed classical monocytes from freshly isolated PBMC to give a better understanding of the NSCLC, monocyte phenotype and function and Th1/Th2 plasma expression.

MATERIALS AND METHODS

Study participants

Blood was obtained from 30 patients undergoing diagnostic bronchoscopy for investigation of NSCLC recruited through the Department of Respiratory and Sleep Medicine, Austin Health, Heidelberg, VIC, Australia. Twenty non-cancer control samples were obtained from subjects undergoing diagnostic bronchoscopy for investigation of breathlessness, or chronic cough and haemoptysis, or benign lung lesions. Ethics committee approval was received from Austin Health Ethics Committee, and informed consent of all participating subjects was obtained. Patient demographic information is presented in Table 1. Staging was applied in this study using the new TNM (tumour, node, metastases) staging system (7th edition) for lung cancer^[14].

PBMC

Venous blood was collected in heparinised tubes and PBMC isolated by Ficoll-Paque density gradient centrifugation (GE Healthcare Bio-sciences, Uppsala, Sweden). Complete blood count (CBC) was completed for all blood samples using Beckman Coulter (AcT 5 blood differentiation, RMIT Hematology Department, Melbourne) (Fullerton, CA, United States). PBMC washed twice with PBS (phosphate buffered saline) and then resuspended in RPMI completed media (2% Hepes buffer, 0.1 mmol/L 2-mercaptoethanol, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mmol/L glutamine and 10% fetal calf serum) (Sigma, St. Louis, MO). All samples were stored at -80 °C until use.

Monocytes phenotype analysis by flow cytometry

Flow cytometry was used to assess classical monocytes expression of CD14, CD45, CD71, CD11b, CD44, CD16 and CD11c (BD Pharmingen™, United States) as well as the M1 marker HLA-DR (BD Pharmingen™, United States) and the M2 markers CD163 and CD36 (BD Pharmingen™, United States). Cells were stained with antibodies directly conjugated to fluorescent probes for 30 min at 4 °C in 2% (w/v) BSA/PBS. Cells were washed and analyzed by flow cytometry (FACS Canto, BD Biosciences, San Jose, CA, United States). 10⁶ cells were stained with various combinations of antibodies for 40 min in the dark on ice. Purity of classical monocytes was assessed according to CD14, CD45 and CD16 expression by flow cytometry. At least 5000 cells were collected and analysed (BD FACS Canto BD Biosciences, San Jose, CA, United States). All analysis was completed within the RMIT Flow Cytometry Facility, Bundoora, Melbourne. All quadrants were set up according to matched isotype

Table 1 Demographic details of lung cancer and non-cancer control subjects

	<i>n</i>	Age (yr) Mean \pm SD	Gender M/F	Smoking status N/Ex/S	Stages I / II / III / IV	Subtypes N/A/S
Control	20	60.45 \pm 19.31	10/10	7/11/2		
Cancer	30	67.1 \pm 10.68	17/13	3/16/11	8/3/7/12	9/11/10

Demographic details of the participants and staging details of lung cancer patients. This table shows the total number of patient samples, age, gender, smoking status and lung tumour subtypes and stages. n: Number; SD: Standard deviation; M: Male; F: Female; N: Non-smoker; Ex: Ex-smoker; S: Smoker; N: Non-small cell lung cancer; A: Lung adenocarcinoma; S: Squamous cell lung carcinoma.

Table 2 Complete blood count details of non-small cell lung cancer and non-cancer control patients

Groups	Total number	WBC	NE	LY	MO	EO	BA	RBC
Normal value		4-11 $\times 10^9$ /L	2-7.5 $\times 10^9$ /L	1.5-4 $\times 10^9$ /L	0.2-0.80 $\times 10^9$ /L	0.04-0.40 $\times 10^9$ /L	0.02-0.10 $\times 10^9$ /L	3.80-6.50 $\times 10^9$ /L
Control	20	4.35 \pm 1.82	2.11 \pm 1.07	1.31 \pm 0.47	0.23 \pm 0.14	0.13 \pm 0.06	0.38 \pm 0.5	4.85 \pm 0.91
Cancer	30	6.76 \pm 3.9	3.4095 \pm 3.46	1.68 \pm 1.31	0.34 \pm 0.22	0.2 \pm 0.17	0.29 \pm 0.2	5.04 \pm 1.08

Complete blood count (CBC) analysis of study participants. CBC analysis was performed on whole blood collected from patients with primary non-small cell lung cancer and non-cancer controls using Beckman Coulter (Act 5 blood differentiation) (Fullerton, CA, United States). WBC: White blood cell; NE: Neutrophil; LY: Lymphocyte; MO: Monocyte; EO: Eosinophil; BA: Basophile; RBC: Red blood cell.

control antibodies and all results are shown as surface expression (%SE) and mean fluorescence intensity (MFI).

Cytometric bead array

The Human Th1/Th2 11plex Ready-to-Use FlowCytomix Multiplex (eBiosciences, United States) was applied to detect the level (pg/mL) of Th1/ Th2 cytokines including interleukin (IL)-1 β , IL-2, IL-4, IL-5, IL-8, IL-10, IL-12 (p70), tumor necrosis factor (TNF)- α , TNF- β , and interferon (IFN)- γ in plasma samples of patients with primary lung cancer and non-cancer controls, according to the manufacturer's instructions (Human Th1/Th2 11plex RTU FlowCytomix Kit). Fluorescence was analysed using flow cytometry (FACS Canto, BD Biosciences, San Jose, CA, United States) and cytokine levels were determined using the BMS FlowCytomix Software manual within the RMIT Flow Cytometry Facility, Bundoora, Melbourne.

Statistical analysis

Experiments were performed in triplicate. Mean values \pm standard error (SEM) were compared using the one way ANOVA (GraphPad Prism 6) with *P*-values \leq 0.05 was considered to be significant.

RESULTS

Underlying CBC was investigated in patient groups to verify that the patients has significant underlying medical condition *e.g.*, infection, which could results in monocyte phenotype alteration. The total mean number of white blood cells (WBC) in NSCLC patients was significantly higher than non-cancer controls. The mean values of all WBC types (except basophile cells) and RBCs were higher in patients with NSCLC compared to non-cancer controls. However, the mean values of all WBC types and RBCs were within

the normal range in both groups (Table 2). In flow cytometry results, there were no statistically significant differences in M1 marker (HLA-DR), M2 markers (CD163 and CD36) and (CD11c and CD44) in patients with NSCLC compared to non-cancer controls. Both %SE and MFI expression of surface markers showed similar values. CD11b and CD71 expression was also shown to be similar between patient groups.

No difference in HLA-DR, CD163 and CD36 expression in patients with primary lung cancer compared to non-cancer controls

Classical monocytes were gated based on forward scatter (FSC) and side scatter (SSC) profiles within patient groups and based on the expression of CD14, CD45 and CD16 markers. Results show that there were no significant differences in %SE (*P* = 0.155) and MFI (*P* = 0.51) of HLA-DR (M1 marker) expression in patients with NSCLC compared to non-cancer controls. The expression of HLA-DR did not differ depending on tumour progression, as there were no significance differences between early and/or advanced lung cancer criteria (Figure 1). In addition, flow cytometry analysis showed there were no significant differences in the %SE (*P* = 0.505) and MFI (*P* = 0.39) of CD163 (M2 marker) in NSCLC patients compared to non-cancer controls. We also showed that there were no significant differences in the %SE (*P* = 0.160) and MFI (*P* = 0.17) of CD36 (M2 marker) staining between the two groups. Again, the expression of CD163 and CD36 showed no difference in patients with more advanced compared to early lung cancer (Figure 2).

No difference in CD11b, CD71, CD11c and CD44 expression

The %SE and MFI of CD11b, CD71, CD11c and CD44

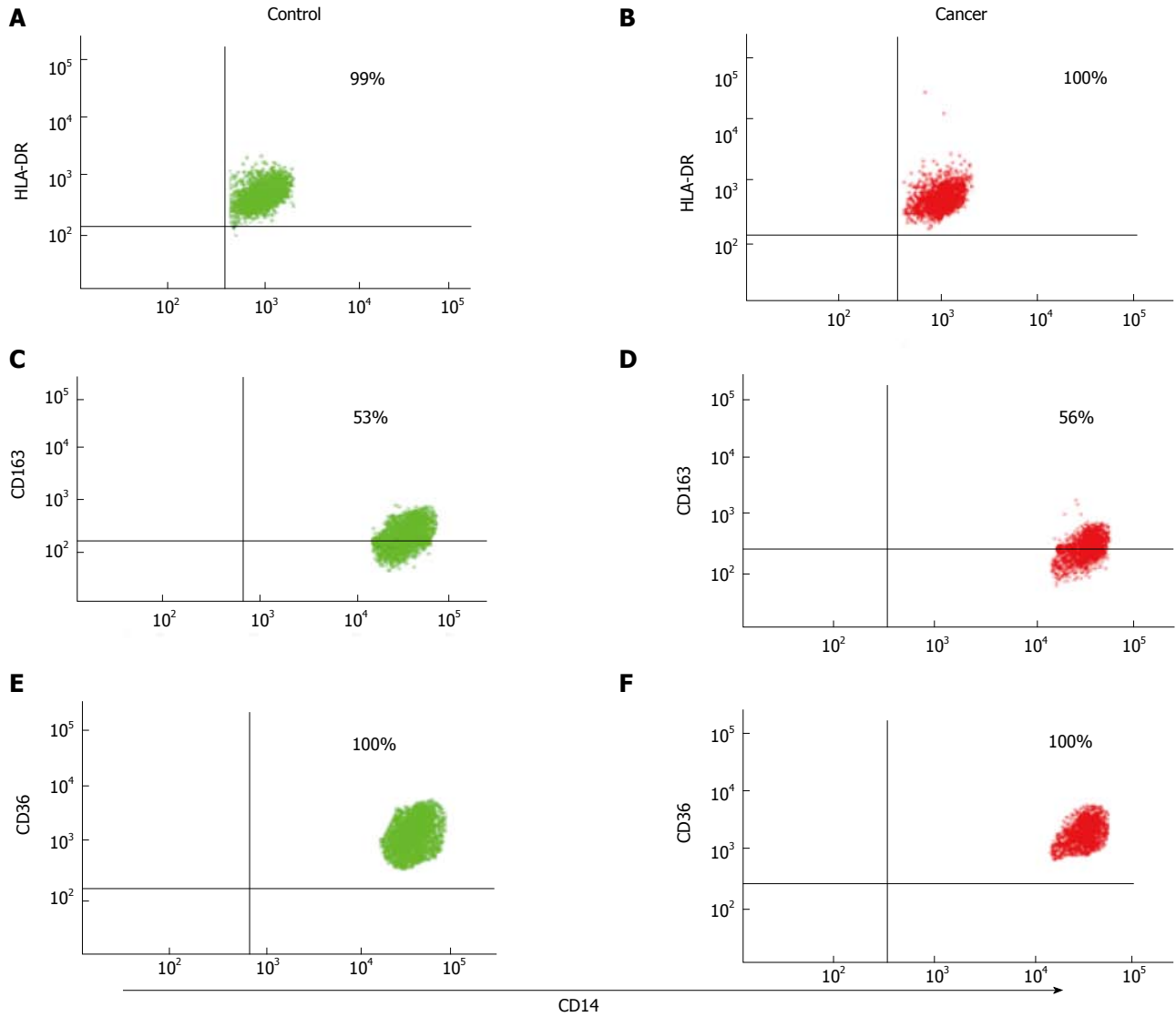
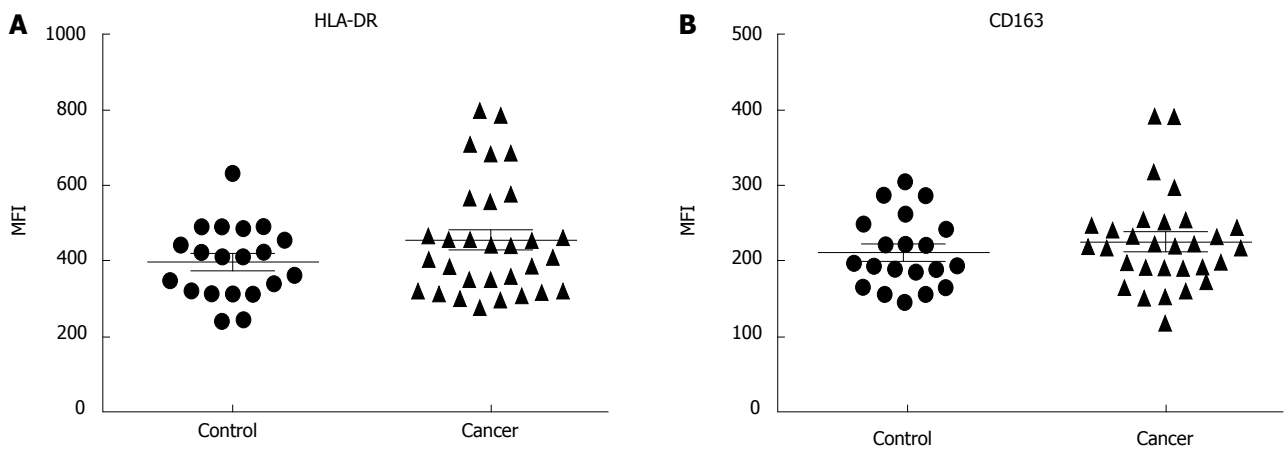


Figure 1 HLA-DR, CD163 and CD36 surface expression on (CD14+/CD16-) blood classical monocytes in patients with primary non-small cell lung cancer compared to controls. A-F: Representative flow cytometry dot plots from PBMC stained against CD14, CD45 and CD16 and then co-stained with HLA-DR, CD163 and CD36 on patients with non-small cell lung cancer (red colour) and non-cancer controls (green colour).



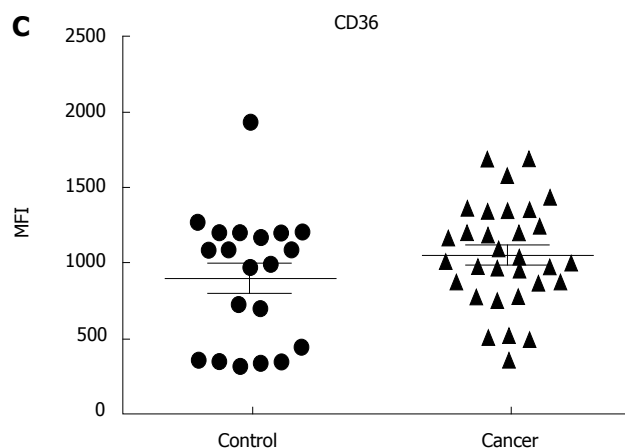


Figure 2 HLA-DR, CD163 and CD36 expression on (CD14+/CD16-) blood classical monocytes in patients with primary non-small cell lung cancer compared to controls. Summary graphs show the mean values of MFI \pm SEM of (A) HLA-DR, (B) CD163 and (C) CD36 markers from patients with non-small cell lung cancer (NSCLC) vs non-cancer controls.

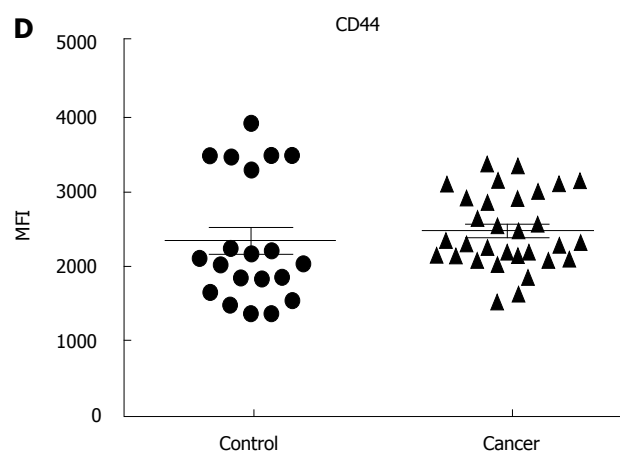
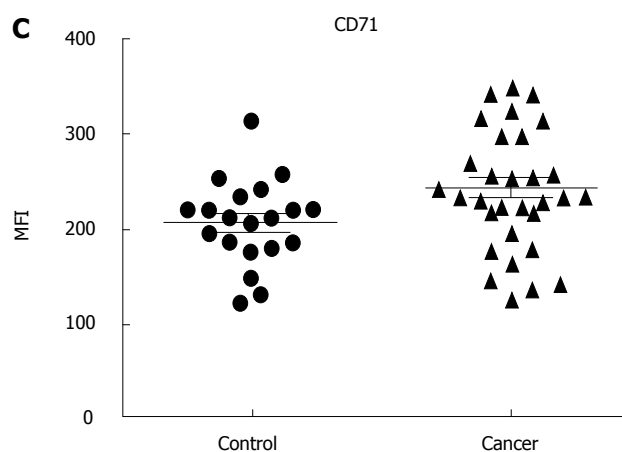
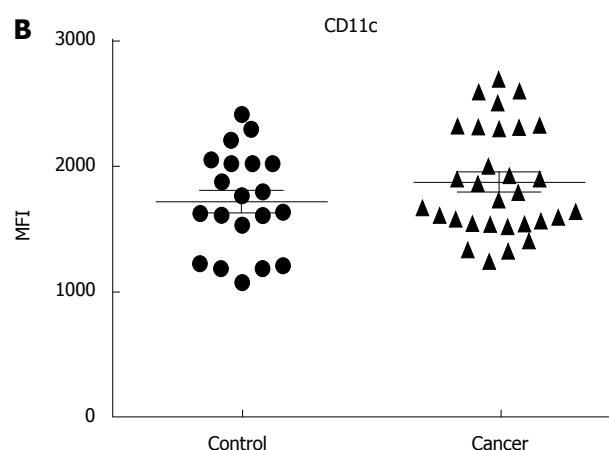
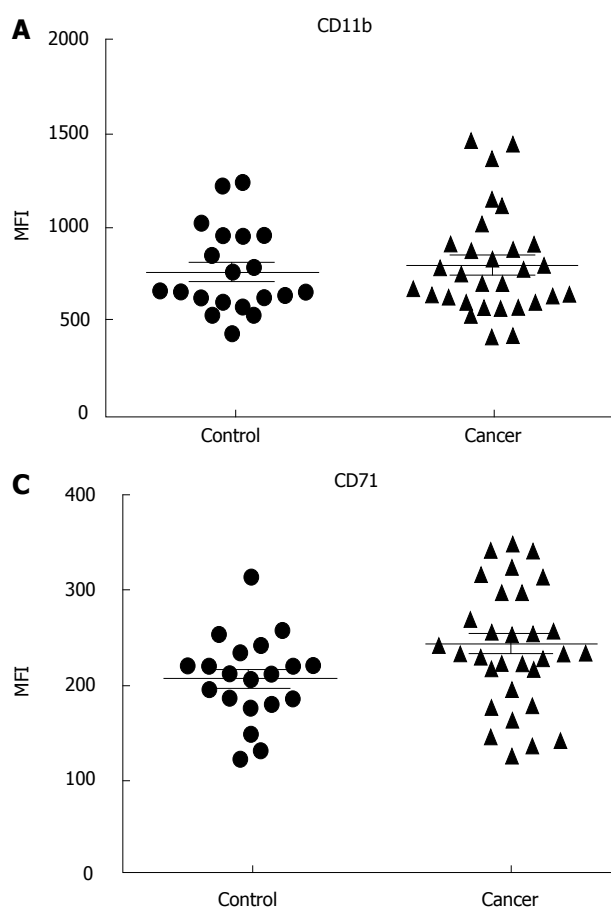


Figure 3 CD11b, CD11c, CD71 and CD44 expression on (CD14+/CD16-) blood classical monocytes in patients with primary non-small cell lung cancer compared to controls. Graphs show the mean values of MFI \pm SEM of (A) CD11b, (B) CD11c, (C) CD71 and (D) CD44 markers from patients with non-small cell lung cancer vs non-cancer controls.

were similar between patient groups. The %SE and MFI of the myeloid marker CD11b marker was not different between cancer and non-cancer control subjects: %SE ($P = 0.58$), MFI ($P = 0.61$). Also CD71 results indicated that there were no significant differences in the %SE ($P = 0.97$) and MFI ($P = 0.41$) of the transferrin receptor marker in patients with NSCLC compared to non-cancer controls.

CD11c results showed that there were no significant difference in the %SE ($P = 0.93$) and MFI ($P = 0.20$) of the CD11c in patients with NSCLC compared to non-cancer controls. In addition, CD44 results revealed that there were no significant differences in the %SE ($P = 0.50$) and MFI ($P = 0.53$) of the CD44 marker in NSCLC patients compared to non-cancer controls. The graphs (Figure

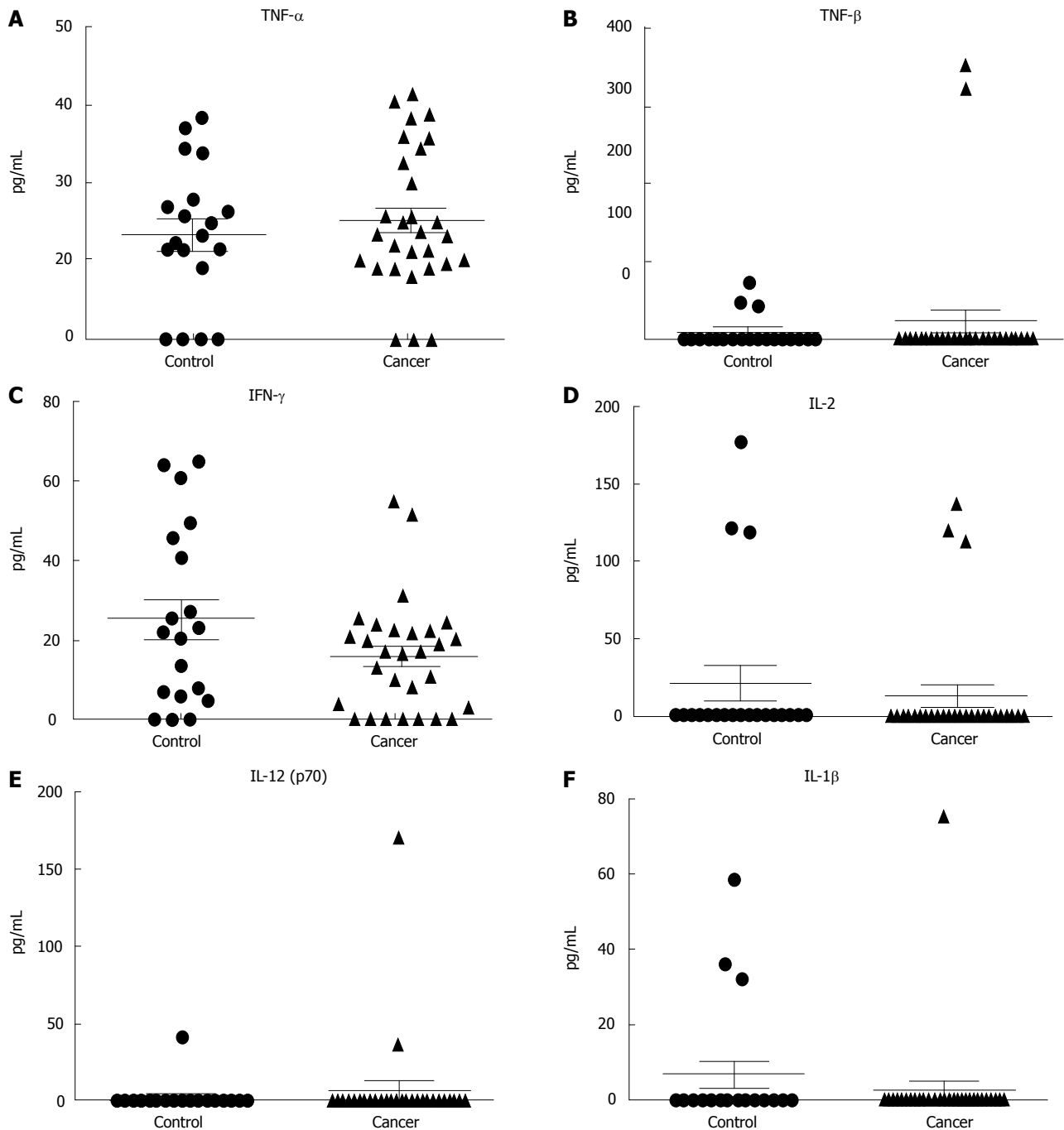


Figure 4 Th1 cytokine secretion profiles in plasma of patients with non-small cell lung cancer compared to controls. Whole plasma was analysed for (A) TNF- α , (B) TNF- β , (C) IFN- γ , (D) IL-2, (E) IL-12 (p70) and (F) IL-1 β by cytometric bead array technique using flow cytometry. Data was analysed using the FCAP Array™ v3.0.1 Software (BD Biosciences) and results are expressed as mean (pg/mL) \pm SEM. IL: Interleukin; TNF: Tumor necrosis factor; IFN: Interferon.

3) show MFI \pm SEM of all the markers (CD11b, CD71, CD11c and CD44) in classical monocytes from non-cancer controls and cancer patients (%SE results not shown).

No significant difference in plasma levels of Th1/Th2 in patients with primary lung cancer compared to non-cancer controls

Cytokine analysis revealed no significant difference in Th1/Th2 cytokines plasma levels in patients with primary lung cancer compared to non-cancer controls (Fig-

ures 4 and 5). Monocytes are known to produce several cytokines, chemokines including IL-1 β , IL-2, IL-4, IL-5, IL-8, IL-10, IL-12 (p70), TNF- α , TNF- β , and IFN- β . Cytokines were analysed in patients with NSCLC and non-cancer controls by CBA analysis. Cytokines that were detectable in patient plasma samples included Th1 cytokines TNF- α , TNF- β , IFN- β , IL-2, IL-12 (p70), IL-1 β and Th2 cytokines IL-4 and IL-5 showed no significant differences between patients with NSCLC compared to non-cancer controls.

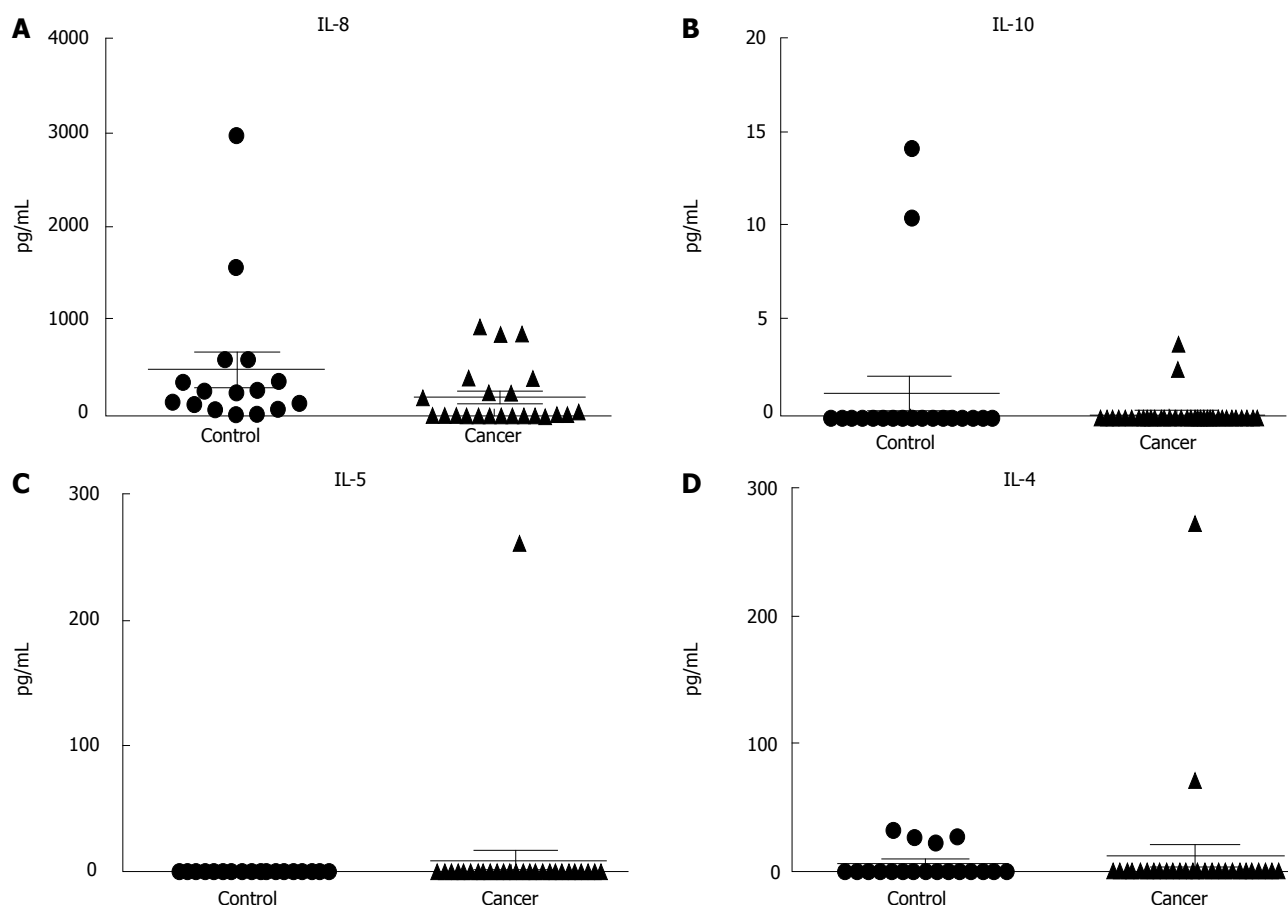


Figure 5 Th2 cytokine secretion profiles in plasma of patients with non-small cell lung cancer compared to controls. Whole plasma was analysed for (A) IL-8, (B) IL-10, (C) IL-5 and (D) IL-4 by cytometric bead array technique using flow cytometry. Data was analysed using the FCAP Array™ v3.0.1 Software (BD Biosciences) and results are expressed as mean (pg/mL) ± SEM.

DISCUSSION

The concept that the immune system has a protective role in tumour development is well established^[3]. Recent work has demonstrated that the immune system can both prevent tumour formation but potentially function to promote tumour initiation and progression^[4]. In particular, immune cells such as blood monocytes can function differently depending on the cancer types^[8]. While some studies indicated at monocyte function in cancer patients within normal range^[7,15], other studies have shown impairment in monocyte function^[6].

In this study we demonstrated that the phenotype of freshly blood classical monocytes from patients with NSCLC is not altered and does not show skewing from a pro-tumour (M1) to an anti-tumour (M2) phenotype. There were no significant differences in expression of M1 marker (HLA-DR), M2 markers (CD163 and CD36), CD11c and CD44 marker, myeloid marker CD11b and/or transferrin receptor CD71 in patients with NSCLC compared to non-cancer controls. In addition, there were no significant differences in the secretion of Th1/Th2 cytokines between NSCLC patients and non-cancer controls.

M1 phenotype was assessed here using HLA-DR. HLA-DR molecule plays a vital role in the immune re-

sponse by regulating the interaction between antigen-presenting cells including monocytes^[16,17]. It has been described as an M1 marker in the monocyte-macrophage system^[18]. Some studies have reported reduced HLA-DR expression on blood monocytes in human cancers^[5,19]. As well as examining M1 phenotype, M2 phenotype markers (CD163 and CD36) were used to investigate skewing from M1 to M2 markers on blood classical monocytes in patients with NSCLC. CD163 is a scavenger receptor that plays a major role in the anti-inflammatory response and has been identified as a M2 marker^[20,21]. CD36 is also expressed on monocytes and is involved mainly in phagocytosis^[21,22]. Sugai *et al.*^[23] studied the alteration of monocyte characteristics by examining the intracellular expression of IL-10 and IL-12 cytokines^[23]. They found that patients with advanced gastric cancer had different monocyte phenotypic characteristics compared to those with early stage cancer and non-cancer control subjects^[23]. In our study, there were no significant differences in expression of CD163 and CD36 between non-cancer controls and NSCLC patients. Also there was no apparent influence of tumour stage upon expression of these markers. These results when viewed in the context of previous studies raises questions regarding the impact of experimental design such as culturing and the use of molecules like

lipopolysaccharide (LPS) on monocyte polarisation and function.

CD11b and CD11c are myeloid cell markers that are expressed on monocytes and macrophages^[24]. CD11b has been shown to play a major role in many functions of myeloid cells including adhesion, migration, chemotaxis and phagocytosis^[24,26]. In this study we therefore investigated the effect of NSCLC on the monocyte expression of CD11b and CD11c. There were no significant differences in expression of CD11b and CD11c in NSCLC patients compared to non-cancer controls. These results are consistent with Mariotta *et al*^[7] study outcomes, which suggested NSCLC does not affect monocyte adherence and phagocytosis in lung cancer patients compared to healthy controls^[7].

Another marker that was examined was transferrin receptor (CD71). CD71 is known to be associated with rapidly proliferation cells such as cancer cells and plays a major role in cell growth and DNA synthesis, proliferation and cell survival^[27,28]. Increased CD71 expression has been demonstrated in cancer patients including lung cancer in lung tissue and BALF (bronchoalveolar lavage fluid) but not in blood serum^[28,29]. Dowlati *et al*^[28] investigated soluble CD71 in the serum of NSCLC patients. They verified no difference in the level of secreted CD71 in blood serum between NSCLC and control groups^[28]. Similar to this outcome we demonstrated that there were no significant differences in surface expression of CD71 on classical monocytes in NSCLC patients compared to non-cancer controls.

CD44 expression was also investigated in this study as it has been suggested a potential marker of tumour initiation in lung cancer. Elevated CD44 expression has been observed in the serum of cancer patients such as gastric and renal cancer^[30,31]. However, another study showed that NSCLC does not influence CD44 expression in the serum of NSCLC patients compared to benign lung disease^[15]. Similarly, this study revealed no significant difference in surface expression of CD44 on classical monocytes in NSCLC patients compared to non-cancer controls.

The presence of cytokines is essential for immunity initiation. Th1 cells have been found to play a major role in anti-tumour immunity and stimulation of cell-mediated responses. Pro-inflammatory cytokines such as TNF- α and IFN- γ are known to stimulate Th1 cells. In contrast, Th2 cells are known to act as the helper cells that influence B-cell development and produce anti-inflammatory cytokines such as IL-4 and IL-10^[32,33]. Analysis of Th1 and Th2 cytokines in the plasma revealed no differences in NSCLC patients in compared to non-cancer controls. Similarly, Gürsel *et al*^[34] also observed no differences in TNF- α concentration between pleural effusion and serum in patients with cancer^[34]. Although many studies have not looked at specific cytokine profiles in lung cancer, it has been shown that freshly prepared monocytes do not show any differences in pro-inflammatory and anti-inflammatory cytokine responses except IL-12 (p70)

in endometrial cancer patients compared to controls^[6].

Although the findings of this study are interesting, there are some limitations including an inability to compare results from subtypes within NSCLC grouping as all patients samples were lung adenocarcinoma and squamous cell lung carcinoma and no large cell lung carcinoma. Also the majority of samples were from patients with advanced stage disease, so we could not confidently address the question of whether the NSCLC influences monocyte function and polarisation changes with tumour progression. In future studies, examining monocyte polarisation and function in non-cancer controls *vs* lung cancer should be done on all monocytes subset by using freshly un-stimulated monocytes as well as cytokine treated monocytes at the same time to observe any variation that may occur. Also other lung cancer subtypes should be considered to inspect if they have any potential role in altering monocyte functions and phenotypes.

Our results demonstrate that freshly isolated peripheral blood monocytes from patients with NSCLC (lung adenocarcinoma and squamous cell lung carcinoma) do not show an altered phenotype and/or cytokines secretion. Therefore, these results suggest that peripheral classical monocytes are not altering into M2-like phenotype in bloodstream. More studies are needed to investigate the connections between monocyte-macrophage phenotype polarisation and tumour progression associated with lung cancer.

COMMENTS

Background

Lung cancer is a common cancer that has high incidence and death rates. Monocytes play an important role in the immune response against tumour cells including lung tumour cells. Diverse monocyte phenotypes were defined previously and they act differently to tumour cells.

Research frontiers

The role of monocyte phenotype in human lung cancer patients is conflicting and still need further investigations. More studies are needed to investigate the connections between monocyte-macrophage phenotype polarisation and tumour progression associated with lung cancer.

Innovations and breakthroughs

Previous studies highlighted the importance of monocyte phenotype and function in patient with cancer including lung cancer. This is the first study that has broadly analysed classical monocytes to give a better understanding of the lung cancer, monocyte phenotype and some functions.

Applications

This study suggests that there is no monocyte-specific systemic impairment in patients with lung cancer (in particular, lung adenocarcinoma and squamous cell lung carcinoma subtypes).

Terminology

Monocytes are immune cells that are known to play an important part of the innate immune response to cancer.

Peer review

This is an interesting study. Good design and analysis. Good writing and focus.

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Competing risks of death in younger and older postmenopausal breast cancer patients

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Abstract

AIM: To show a new paradigm of simultaneously testing whether breast cancer therapies impact other causes of death.

METHODS: MA.14 allocated 667 postmenopausal women to 5 years of tamoxifen 20 mg/daily \pm 2 years of octreotide 90 mg, given by depot intramuscular injections monthly. Event-free survival was the primary endpoint of MA.14; at median 7.9 years, the tamoxifen+octreotide and tamoxifen arms had similar event-free survival ($P = 0.62$). Overall survival was a secondary endpoint, and the two trial arms also had similar overall survival ($P = 0.86$). We used the median 9.8 years follow-up to examine by intention-to-treat, the multivariate time-to-breast cancer-specific (BrCa) and other cause (OC) mortality with log-normal survival analysis adjusted by treatment and stratification factors. We tested whether baseline factors including Insulin-like growth factor 1 (IGF1), IGF binding protein-3, C-peptide, body mass index, and 25-hydroxy vitamin D were associated with (1) all cause mortality, and if so; and (2) cause-specific mortality. We also fit step-wise forward cause-specific adjusted models.

RESULTS: The analyses were performed on 329 patients allocated tamoxifen and 329 allocated tamoxifen+octreotide. The median age of MA.14 patients was 60.1 years: 447 (82%) $<$ 70 years and 120 (18%) \geq 70 years. There were 170 deaths: 106 (62.3%) BrCa; 55 (32.4%) OC, of which 24 were other malignancies, 31 other causes of death; 9 (5.3%) patients with unknown cause of death were excluded from competing risk assessments. BrCa and OC deaths were not significantly different by treatment arm ($P = 0.40$): tamoxifen patients experienced 50 BrCa and 32 OC deaths, while tamoxifen + octreotide patients experienced 56 BrCa and 23 OC deaths. Proportionately more deaths ($P = 0.004$) were from BrCa for patients

< 70 years, where 70% of deaths were due to BrCa, compared to 54% for those ≥ 70 years of age. The proportion of deaths from OC increased with increasing body mass index (BMI) ($P = 0.02$). Higher pathologic T and N were associated with more BrCa deaths ($P < 0.0001$ and 0.002 , respectively). The cumulative hazard plot for BrCa and OC mortality indicated the concurrent accrual of both types of death throughout follow-up, that is the existence of competing risks of mortality. MA.14 therapy did not impact mortality ($P = 0.77$). Three baseline patient and tumor characteristics were differentially associated with cause of death: older patients experienced more OC ($P = 0.01$) mortality; patients with T1 tumors and hormone receptor positive tumors had less BrCa mortality (respectively, $P = 0.01$, $P = 0.06$). Additionally, step-wise cause-specific models indicated that patients with node negative disease experienced less BrCa mortality ($P = 0.002$); there was weak evidence that, lower C-peptide ($P = 0.08$) was associated with less BrCa mortality, while higher BMI ($P = 0.01$) was associated with worse OC mortality.

CONCLUSION: We demonstrate here a new paradigm of simultaneous testing of therapeutics directed at multiple diseases for which postmenopausal women are concurrently at risk. Octreotide LAR did not significantly impact breast cancer or other cause mortality, although different baseline factors influenced type of death.

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Key words: Breast cancer; Postmenopausal; Hormone receptor positive; Competing risks; Tamoxifen; Octreotide LAR

Core tip: With earlier detection and improved therapies, patients with early breast cancer simultaneously face multiple health risks; 54% of women ≥ 70 years at diagnosis died from other causes. Octreotide LAR, an early drug targeting the insulin pathway, might have affected both breast and other cause mortality. We demonstrated a method of jointly assessing the impact of therapy and baseline patient characteristics on multiple causes of death. Older patients with higher body mass index experienced more other cause mortality, while women with smaller hormone receptor positive tumours and less lymph node involvement were less likely to die from breast cancer.

Chapman JAW, Pritchard KI, Goss PE, Ingle JN, Muss HB, Dent SF, Vandenberg TA, Findlay B, Gelmon KA, Wilson CF, Shepherd LE, Pollak MN. Competing risks of death in younger and older postmenopausal breast cancer patients. *World J Clin Oncol* 2014; 5(5): 1088-1096 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i5/1088.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i5.1088>

INTRODUCTION

The focus of decades of breast cancer research has been

to reduce breast cancer mortality. In the United Kingdom in the early 1970s, 52% of women survived breast cancer for five years; five-year survival improved to 85% in 2005-2009^[1]. The US had corresponding improvements with about 75% of women diagnosed during 1975-1977 surviving 5 years, while 89% survived 5 years in 2005-2008^[2].

The early 1970s were characterized by women with larger primary breast cancer tumours with greater involvement of lymph nodes and higher risk of systemic disease at diagnosis. Systemic therapy was with less efficacious chemotherapy drugs and regimens; endocrine therapy, when administered, was tamoxifen. We lacked biologic therapy, particularly anti-human epidermal growth factor receptor 2 treatment, and there was an absence of tumour molecular assessments such as intrinsic subtyping and genetic profiling for familial cancer, which precluded our current increasing interest in targeted delivery of therapy. Radiotherapy regimens were more toxic causing more morbidity and therapeutic mortality.

Factors contributing to improvements in survival included: the routine use of screening mammography to detect invasive breast cancer earlier and increase detection of non-invasive breast cancer; use of systemic agents to prevent invasive breast cancer in high risk women; and improved management of invasive breast cancer.

Earlier detection of breast cancer has resulted in a decrease in the median age of diagnosis for invasive breast cancer: 65 years at the time Adjuvant! Online was developed^[3], to 61 years in the US in 2011^[4]. Recent studies have shown that more than fifty percent of women may now be expected to die from a cause other than breast cancer, with an increasing proportion of non-breast cancers occurring at older ages^[3,5,6].

Adjuvant! Online was established from the large prospectively and uniformly collected US Surveillance Epidemiology and Endpoints Results data and the Oxford overviews of breast cancer clinical trial meta-analyses^[3]. A patient at age 65 who was ER-positive, node negative with 1- to 2-cm tumour without adjuvant therapy would have a 10% chance of dying of breast cancer and 10% chance of dying of other causes in the next 10 years. The Adjuvant! Online website permits clinicians the opportunity to explore trade-offs in risk-benefits for different patient management options for different ages, taking into account the severity of presenting disease, and differing co-morbidity states^[4].

Concurrently, experience was reported from a smaller prospectively accrued single institution phase IV cohort at Women's College Hospital, where routine annual mammography starting at age 35 was employed in the 1960's, following early scientific reports of its effectiveness^[7,8]. This experience was from an era before both routine adjuvant radiotherapy and systemic therapy, and illustrated the effects of early detection^[7,8]. At a median 8.2 years follow-up, 20% of those older than 65% and 3% of those under 65 died of other causes ($P < 0.001$).

We previously reported the effects of competing risks in the NCIC Clinical Trials Group (CTG) MA.17 inter-

national phase III randomized placebo-controlled clinical trial of letrozole in post-menopausal hormone-receptor positive breast cancer in women who recently received 5 years of tamoxifen^[6]. MA.17 patients had a median age of 62 years. Non-breast cancer deaths accounted for 60% of the known causes of death for all patients, 72% of deaths for those 70 or older.

We face the prospect that older women may simultaneously face dual health risks, increasingly presenting with concomitant competing risks from which at least half of them are likely to die. Women have reaped survival benefits from decades of multi-modality transdisciplinary research that lead us to a new paradigm for early breast cancer management^[9]. In this context, it will be important to consider therapeutic regimens which may affect several disease outcomes.

We examine competing risks of mortality in NCIC CTG MA.14 which investigated whether octreotide LAR would affect insulin-like growth factors and subsequent clinical outcome in postmenopausal women with hormone-positive breast cancer^[10]. Octreotide was postulated to target the insulin-growth factor pathway so patients were assessed for insulin-growth factor 1 (IGF-1), insulin-growth factor binding protein 3 (IGFBP-3), and C-peptide, a byproduct created when the hormone insulin is produced. Octreotide significantly lowered IGF-1, IGFBP-3, and C-peptide ($P < 0.001$). High levels of C-peptide and body mass index (BMI) were associated with poor outcome, but octreotide did not significantly affect event-free survival ($P = 0.62$).

Nevertheless, the MA.14 trial illustrates the new paradigm we propose, that trials simultaneously target and assess more than one chronic disease. Octreotide's action could affect both breast cancer and insulin-related components of cardiovascular disease. Thus, its efficacy would be better assessed concurrently for multiple disease components of overall survival, rather than examined in totality to avoid confounding therapeutic benefits and trade-offs.

MATERIALS AND METHODS

Patient population

Between 1996 and 2000, NCIC CTG MA.14 (ClinicalTrials.gov Identifier: NCT00002864) enrolled 667 postmenopausal women with histologically proven adenocarcinoma of the breast who underwent lumpectomy or total mastectomy^[10]. Patients were to have no previous or concurrent malignancies except adequately treated carcinoma of the skin (basal cell), cervix, endometrium, colon, or thyroid, treated more than 5 years before study entry, and were presumed cured, and had no inter-current illness expected to reduce life expectancy to less than 5 years from the date of surgery. Baseline serum was assessed for IGF-1, IGFBP-3, and C-peptide for 646 patients (96.9%), and 25-hydroxy (OH) vitamin D was centrally assessed for 607 of the MA.14 patients (91%)^[10]. More detailed descriptions about patient eligibility and trial conduct are

available at ClinicalTrials.gov and in the main trial paper^[10].

Study design

Patients were randomly assigned to tamoxifen 20 mg orally once daily for 5 years or tamoxifen 20 mg orally once daily for 5 years plus octreotide long-acting release 90 mg intramuscularly monthly for 5 years by minimization (tamoxifen+octreotide). In July 2000, the duration of octreotide was reduced to 2 years because of a greater incidence of gallbladder toxicity in the octreotide arm of National Surgical Adjuvant Breast and Bowel Project (NSABP) B-29.

Stratification

Patients were stratified by adjuvant chemotherapy (none, concurrent, or sequential), nodal status (none, one to three, four or more, or unknown), and receptor status (ER and/or PgR positive, ER and PgR negative, or ER and PgR unknown).

End points

The primary end point of the trial was event-free survival (EFS); events included recurrence of disease, second malignancy, or death as a result of any cause. Overall survival (OS) was a secondary end point. The effects of insulin-like growth factor physiology on outcome were another secondary objective.

MA.14 trial experience

MA.14 investigations were by the intention-to-treat principle. Event-free survival was the primary endpoint of MA.14; at the final analysis at median 7.9 years, the tamoxifen+octreotide and tamoxifen arms had similar event-free survival ($P = 0.62$). Overall survival was a secondary endpoint, and the two trial arms also had similar overall survival ($P = 0.86$)^[10]. We used the median 9.8 years follow-up in intention-to-treat analyses here.

Primary objective of competing risks investigation

The primary objective of this investigation was to examine whether there was evidence of competing risks operative in the MA.14 trial, by way of whether there were significantly different factor effects indicated for different causes of death. The Lagakos method was used for this purpose^[11]. We assumed independent cause-specific risks for death with or from breast cancer (BrCa) and death from other causes (OC). Fuller details about the method used are reported elsewhere^[6].

We tested 3 hypotheses (H1, H2, and H3): (1) H1: A factor does not affect type or time to death, $\beta_{\text{BrCa}} = \beta_{\text{OC}} = 0$, tested with a likelihood ratio criterion ($-2\log R$) [$-\chi^2_{(2)}$], where β_{BrCa} and β_{OC} are the cause-specific effects of the factor. With rejection of H1, H2 was tested; (2) H2: A factor has the same effect for both types of death, $\beta_{\text{BrCa}} = \beta_{\text{OC}}$, tested with $-2\log R$ [$-\chi^2_{(1)}$]. With rejection of H2, a factor was differentially associated with type of death so

Table 1 Number of deaths by treatment arm and factor subgroups *n* (%)

Factor	Number of patients	Breast cancer	Other cause ¹	P-value ²
Treatment				
Tamoxifen	329 (50)	50	32	
Tamoxifen + Octreotide LAR	329 (50)	56	23	0.40
Age				
< 70	544 (83)	85	37	
≥ 70	114 (17)	21	18	0.004
BMI				
< 25	185 (28)	30	7	
25-30	221 (34)	29	20	
> 30	213 (32)	41	24	0.02
Tumour size				
T1	382 (58)	38	26	
≥ T2, unknown	276 (42)	68	29	< 0.0001
Nodal status				
N0	346 (53)	39	29	
N1, N2, Nx	312 (47)	67	26	0.002

¹Nine patients are excluded with unknown type of death; ²P-value is based on Pearson chi-square test.

was assessed separately for cause-specific mortality: β_{BrCa} (β_{OC}). When H2 is not rejected, there is a common effect, β , on all cause mortality, which was tested by H3^[11]; and (3) H3: A factor is not associated with time to all cause mortality (overall survival), $\beta = 0$, tested with $-2\log R [-\chi^2_{(1)}]$, which should produce a significant result due to rejection of H1.

Statistical analyses

Cumulative hazard plots for breast cancer and other cause mortality were used to examine the presence of substantive competing risks, that is overlapping time periods for different types of death. We then used the Lagakos method described above. Factor effects were examined in models that included all of the factors ("full-factor" models).

We examined baseline MA.14 factors: (1) treatment (tamoxifen *vs* tamoxifen + octreotide); (2) age (in years); (3) race (white, other); (4) ECOG performance status (0, other); (5) pathologic T (1, other); (6) pathologic N (0, other); (7) breast surgery (segmental mastectomy, other); (8) IGF-1 (continuous); (9) IGFBP-3 (continuous); (10) C-peptide (continuous); (11) weight (kg), (12) body mass index (BMI; continuous); (13) vitamin D (continuous), and baseline values of the stratification factors; (14) number of positive nodes (0, Nx, 1-3 +, ≥ 4 +); (15) hormone receptor status (ER-PR-, unknown, ER+ and/or PR +); and (16) adjuvant chemotherapy (no, yes).

With log-normal survival analysis, the natural logarithm of survival time, $Y = \ln(t)$, is a linear function, $Y = \alpha + \sum \beta_j z_j + \sigma W$, where σ is a scale parameter; W is $N(0,1)$; z_j , j th baseline factor; and β_j effect of j th factor.

A normal probability plot was utilized to examine the assumption of log-normal survival time. The final model fit was examined with standardized residual plots.

Treatment and baseline values of the three stratification factors had forced inclusion in the multivariable models to account for MA.14 design structure. Other factors were considered in stepwise forward regression analyses, including a factor with P value ≤ 0.05 by $-2\log R [-\chi^2_{(1)}]$. Patient experience was depicted with log-normal survivor plots, where factor effects were adjusted for the effects of treatment, and other significant factors. Breast cancer and other cause 5-year and 10-year survival were reported. Royston described the log-normal model as a pragmatic tool that provides a continuous estimate of prognosis^[12]. Of note, both Kaplan-Meier and Cox plots present discontinuous estimates of prognosis.

RESULTS

We used the MA.14 median 9.8 years follow-up for our analysis. The median age of MA.14 patients was 60.1 years: 547 (82%) < 70 years and 120 (18%) ≥ 70 years. MA.14 patients experienced 170 deaths: 106 (62.3%) BrCa; 55 (32.4%) OC, of which 24 were other malignancies, 31 other causes of death; 9 (5.3%) patients had unknown cause of death, so were excluded from competing risk assessments.

The analyses were performed on 329 patients allocated tamoxifen and 329 allocated tamoxifen+octreotide (Table 1). There were no significant differences in the number of BrCa and OC deaths by treatment arm ($P = 0.40$): tamoxifen patients experienced 50 BrCa and 32 OC deaths, while tamoxifen+octreotide patients experienced 56 BrCa and 23 OC deaths. Proportionately more deaths ($P = 0.004$) were from BrCa for patients < 70 years, where 70% of deaths were due to BrCa, compared to 54% for those ≥ 70 years of age. The proportion of deaths from OC increased with increasing BMI ($P = 0.02$). Meanwhile, higher pathologic T and N were associated with more BrCa death ($P < 0.0001$ and 0.002, respectively).

The cumulative hazard plot for BrCa and OC mortality (Figure 1) indicates the concurrent accrual of both types of death throughout follow-up, or the existence of competing risks of mortality. Meanwhile, the normal probability plot (Figure 2) supports the assumption of a log normal model up to about 10 years follow-up. We utilized log-normal survival analysis for the competing risks assessments, providing survival experience up to 10 years.

Table 2 indicates the competing risks results. MA.14 therapy was not associated with mortality ($P = 0.77$). Baseline factors associated with mortality were age ($P = 0.02$), tumour size ($P = 0.01$), nodal status ($P = 0.002$), number of positive nodes ($P < 0.0001$), and hormone receptor status ($P = 0.08$). Age ($P = 0.03$), tumour size ($P = 0.04$), and hormone receptor status ($P = 0.04$) were differently associated with BrCa and OC so cause-specific multivariate analyses were used to investigate their effects. Nodal status ($P = 0.54$) and number of positive nodes ($P = 0.89$) did not show different associations with BrCa and OC, but were associated with all cause mortality ($P <$

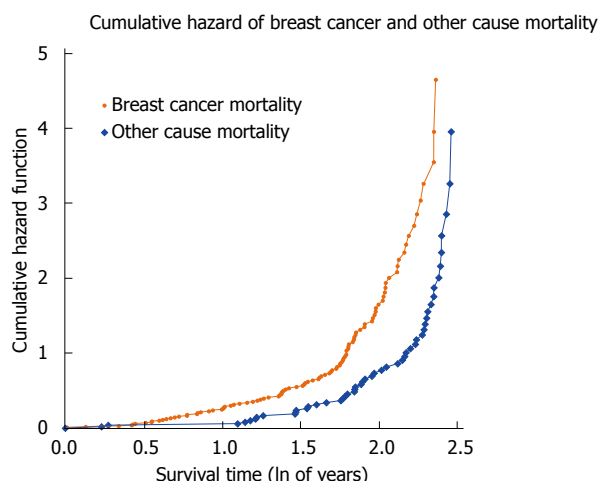


Figure 1 Cumulative hazard of breast cancer and other cause mortality by natural logarithm of survival time.

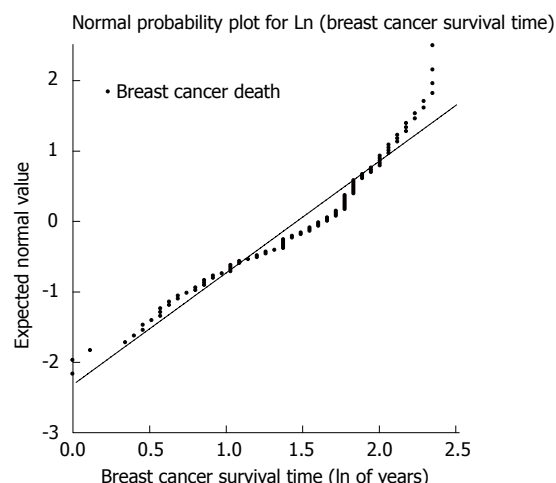


Figure 2 Normal probability plot for natural logarithm of breast cancer survival time.

Table 2 Competing risks assessment of factors associated with death

Factor	Association with death		Association by type of death	
	χ^2 ²¹	P-value	χ^2 ²²	P-value
Treatment (TAM, TAM + OCT)	0.52	0.77		
Age (yr)	7.90	0.02	4.73	0.03
Race (white, other)	1.85	0.40		
ECOG performance status (0, other)	0.56	0.75		
Tumour size (T1, \geq T2, unknown)	10.55	0.01	4.22	0.04
Nodal status (N0, N1, N2, Nx)	12.79	0.002	0.38	0.54 ³
Surgery (segmental mastectomy, other)	0.04	0.98		
IGF-1 (continuous)	1.17	0.56		
IGFBP-3 (continuous)	0.19	0.91		
C-peptide (continuous)	3.86	0.15		
Weight (kg)	0.27	0.88		
BMI (continuous)	1.38	0.50		
Vitamin D (continuous)	1.05	0.59		
Number of positive nodes (0, Nx, 1-3+, \geq 4+)	19.43	< 0.0001	0.02	0.89 ³
Hormone receptor status (ER-PR-, unknown, ER+ and/or PR+)	5.11	0.08	4.19	0.04
Adjuvant chemotherapy (no, yes)	2.39	0.30		

¹Hypothesis H1: Factor does not affect type or time to death. Test criterion was the likelihood ratio criterion that has an approximately chi-square distribution on 2 df ($-X^2$). Only factors with an indication of statistical significance were considered in H2; ²Hypothesis H2: Factor effect is the same for two causes of death. Test criterion was the likelihood ratio criterion that has an approximately chi-square distribution on 1 df ($-X^2$); ³Hypothesis H3: As H2 was not rejected, there is evidence of a common effect on both types of death, or on all cause mortality, which was tested by the third hypothesis, H3. H3 is that the factor has no effect on time of death. Test criterion was the likelihood ratio criterion that has an approximately chi-square distribution on 1 df ($-X^2$). H3 P-value for nodal status and for number of positive nodes was < 0.0001.

Table 3 Multivariate factor effects by type of death

Factor ¹	β (SE) ²	95%CI ²	P-value ³
Breast cancer survival ⁴			
Treatment (Tamoxifen, Tamoxifen + Octreotide LAR)	0.0008 (0.17)	[-0.33, 0.33]	1.00
Adjuvant chemotherapy (no, yes)	0.2553 (0.20)	[-0.14, 0.65]	0.20
Number of positive nodes (0, Nx, 1-3, \geq 4+)	-0.2524 (0.08)	[-0.41, -0.10]	0.002
Hormone receptor status (ER-PR-, unknown ER/PR, ER+ and/or PR+)	0.2821 (0.13)	[0.03, 0.54]	0.03
Tumour size (T1, \geq T2, unknown)	-0.6521 (0.18)	[-1.00, -0.30]	0.0003
Other Cause Survival			
Treatment (Tamoxifen, Tamoxifen + Octreotide LAR)	0.1236 (0.18)	[-0.23, 0.48]	0.49
Adjuvant Chemotherapy (no, yes)	-0.0703 (0.23)	[-0.52, 0.38]	0.76
Number of positive nodes (0, Nx, 1-3, \geq 4+)	-0.0723 (0.08)	[-0.23, 0.08]	0.38
Hormone receptor status (ER-PR-, unknown ER/PR, ER+ and/or PR+)	-0.3852 (0.26)	[-0.89, 0.12]	0.13
Age (years)	-0.0389 (0.01)	[-0.06, -0.02]	0.002
BMI (continuous)	-0.0395 (0.01)	[-0.06, -0.02]	0.01

¹Cause-specific survival model was built using $\ln(\text{survival time})$ having linear function of $\beta \times (\text{factor values})$. The model had forced inclusion of treatment and stratification factors (adjuvant chemotherapy, nodal status, and hormone receptor status) with step-wise forward inclusion of other baseline factors; ² β is effect of factor; SE is standard error; CI is confidence interval assuming β is approximately normally distributed; ³P-value is that for a Wald statistic; ⁴Higher values of continuous C-peptide were associated with worse BrCa ($P = 0.08$).

0.0001).

The step-wise multivariate models are provided for BrCa and OC in Table 3. Treatment did not significantly affect BrCa ($P = 1.0$) or OC ($P = 0.49$). The stratification factor adjuvant chemotherapy ($P = 0.20$) was not significantly associated with BrCa mortality; however, a

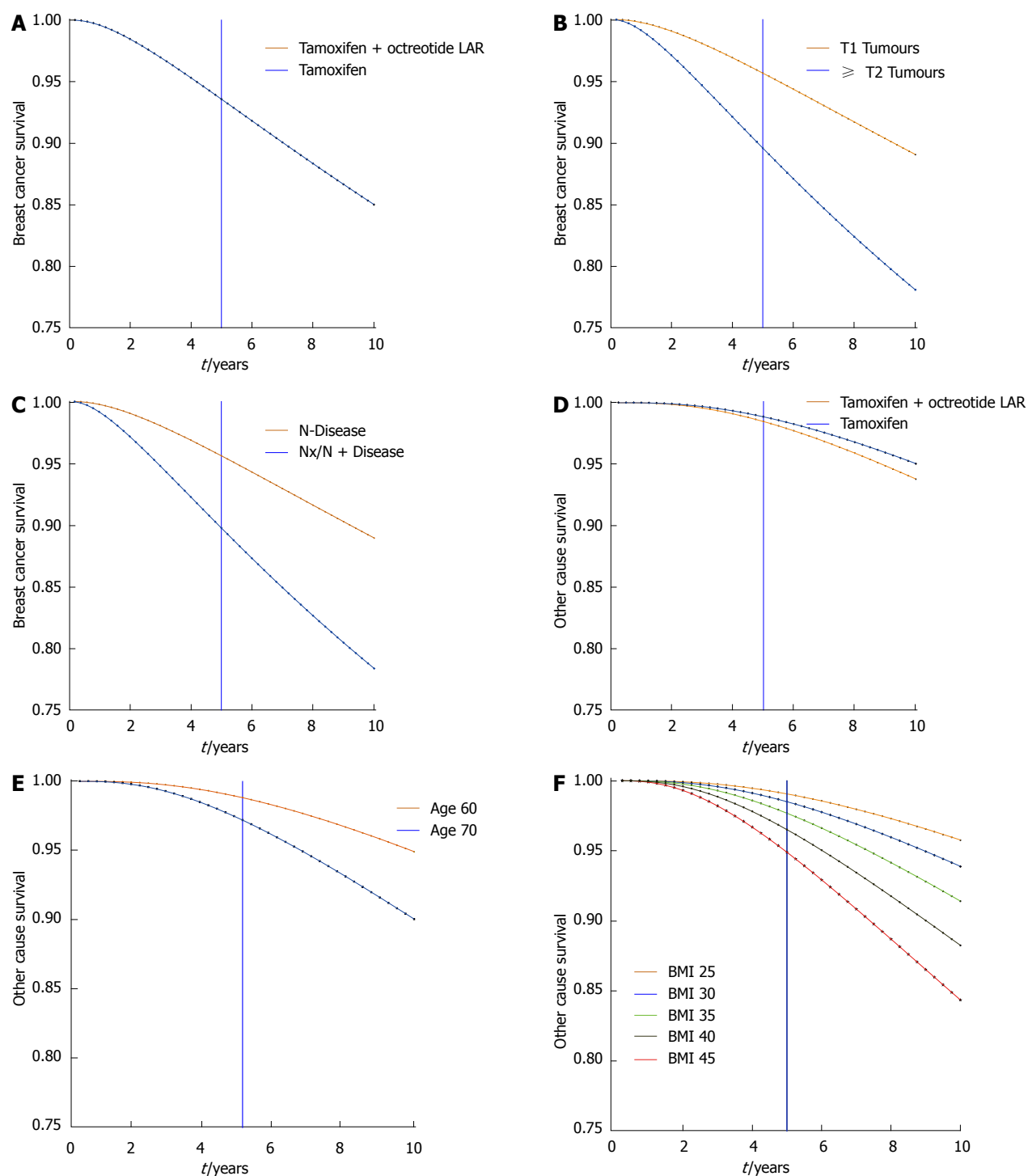


Figure 3 Breast cancer survival. A: Breast cancer survival by treatment: Experience for the two treatment arms, tamoxifen and tamoxifen + octreotide LAR overlay each other; B: Breast cancer survival by tumour size; C: Breast cancer survival by nodal status; D: Other cause mortality by treatment; E: Other cause mortality by age; F: Other cause mortality by body mass index.

lower number of positive lymph nodes ($P = 0.002$) and hormone receptor positivity ($P = 0.03$) were associated with less BrCa mortality. Larger tumours ($P = 0.0003$) led to more BrCa mortality. there was weak evidence that, lower C-peptide ($P = 0.08$) was associated with less BrCa mortality. None of the stratification factors impacted OC mortality. Older patients ($P = 0.002$) and higher BMI ($P = 0.01$) were associated with more OC mortality.

Adjusted 5 and 10 year survival is presented in Table 4 and depicted graphically for BrCa by treatment (Figure 3A), tumour size (Figure 3B), and lymph node involvement (Figure 3C) and for OC by treatment (Figure 3D), age (Figure 3E), and BMI (Figure 3F). In both the Table and plots survival is adjusted (as appropriate) for the effects of treatment, (other) stratification factors, and other significant factors.

Table 4 Survival at 5 and 10 yr

Factor	5 yr ¹	10 yr ¹
Breast cancer survival		
Treatment		
Tamoxifen	0.94	0.85
Tamoxifen + Octreotide LAR	0.94	0.85
Tumour size		
T1	0.96	0.89
≥ T2, unknown	0.90	0.78
Nodal status		
N0	0.96	0.89
N1, N2, Nx	0.90	0.78
Other cause survival		
Treatment		
Tamoxifen	0.98	0.94
Tamoxifen + Octreotide LAR	0.99	0.95
Age		
60	0.99	0.95
70	0.97	0.90
BMI		
25	0.99	0.96
30	0.98	0.94
35	0.98	0.91
40	0.97	0.88
45	0.95	0.84

¹Survival for is adjusted for the effects of other factors: treatment, stratification and other significant factors.

After the log-normal model was fit, each patient's observed survival time was compared to her expected survival time, based on patient and tumour characteristics. *i.e.*, for patient *i*, with survival time *t_i* [*s_i*, *y_i* = *ln(t_i)*], [*y_i* - (*α* + Σ *β_jz_{ij}*)]/*σ*] would be expected to have approximately a standard normal distribution with mean 0 and standard deviation 1. A plot of all the patient standardized residuals examines whether departures from the multivariate BrCa (Figure 4) and OC (Figure 5) models appear to be ascribable as random error. The residuals indicate that the *y_i* (*ln* of survival times) are consistently smaller than they are expected to be; there are explanatory factors for both BrCa and OC that are not included in this investigation.

DISCUSSION

The addition of octreotide therapy to tamoxifen in MA.14 was an early strategy to test the impact of targeting the insulin-like growth factor pathway for early postmenopausal breast cancer patients. In the main trial analyses, octreotide was not found to impact either the primary endpoint of EFS or OS potentially because the administration of octreotide was reduced to 2 years (from the originally planned 5 years) due to excess gallbladder toxicity^[10]. The trial was designed with the expectation that the 5 year EFS rate for patients allocated tamoxifen would be 73%. For patients treated with tamoxifen alone, the 5-year BrCa was 94%, while the 5-year OC experience was 98%. The MA.14 trial was under-powered to detect a significant octreotide effect.

However, the main trial indicated a possible role for the insulin-like growth factor pathway with significant

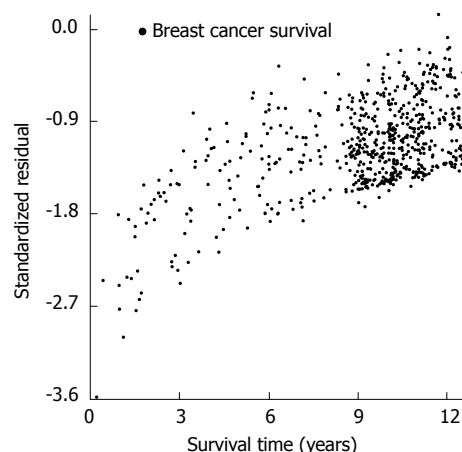


Figure 4 Breast cancer standardized residuals vs time: The residuals indicate that the natural logarithm of survival times are consistently smaller than they are expected to be; there are explanatory factors for breast cancer-specific that are not included in this investigation.

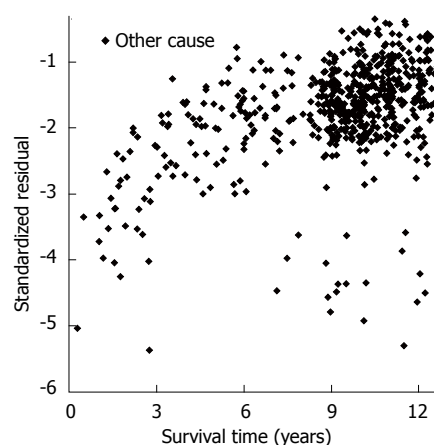


Figure 5 Other cause standardized residuals vs time: The residuals indicate that the natural logarithm of survival times are consistently smaller than they are expected to be; there are explanatory factors for other cause that are not included in this investigation.

treatment associated changes in the mandatory correlative biomarkers of IGF-1, IGFBP-3, and C-peptide as well as in BMI. Further, higher C-peptide levels, or higher BMI, were significantly associated (*P* < 0.001) with worse EFS. As EFS involved both breast cancer relapse and all cause mortality, the questions we addressed here were whether the patients simultaneously had substantive risks of both BrCa and OC mortality, and if so, whether there were differential effects of factors on cause-specific mortality.

We found that the early postmenopausal patients, 91% of whom had hormone-receptor positive tumours, faced the risk of both BrCa and OC mortality. The prognostic breast cancer factors of larger tumour size (*P* = 0.0003), higher number of positive lymph nodes (*P* = 0.002), and a tumour that was hormone receptor negative (*P* = 0.03) were associated with more BrCa mortality. Meanwhile, older age (*P* = 0.002) and higher BMI were associated with more OC mortality. The increase in OC mortality with older age replicates what was found with

the NCIC CTG MA.17 trial with extended adjuvant endocrine therapy where patients were randomized to 5 years of letrozole or placebo following 5 years of tamoxifen^[6].

Adjusted survival estimates were provided for BrCa by treatment, tumour size, lymph node status, and for OC by age and BMI. Graphical presentations were to 10 years, along with a table of specific 5- and 10-year survival rates.

This study represents a secondary use of a breast cancer trial database. We examined whether prognostic risk factors similarly impacted both BrCa and OC, and found that there was differential association of baseline factors and type of death. Age and higher BMI were associated with OC mortality, while T status, nodal involvement and hormone receptor status were associated with BrCa mortality. Adjuvant chemotherapy was neither mandated nor prohibited; 33% of patients received adjuvant chemotherapy (Pritchard *et al*^[10]). However, in this trial, age was not associated with BrCa death, but OC mortality.

There is a limitation in ability to delineate comorbidity with MA.14 data. Eligibility criteria were that patients have no previous or concurrent other malignancy except for carcinoma of the skin, cervix, endometrium, colon, or thyroid adequately treated 5 or more years before study entry, and had no inter-current illness expected to reduce life expectancy to less than 5 years after surgery. The number of non-breast cancer deaths was too few to refine investigations since with a median 9.8 years follow-up, 24 patients experienced other malignancies and 31 other causes of death; the two classifications were combined. Prospective collection of death type in larger trial populations, over longer follow-up would be needed to improve disease-specific interpretation.

The MA.14 trial with octreotide was an early attempt at targeting the insulin pathway for adjuvant postmenopausal breast cancer patients. octreotide therapy did not impact either BrCa ($P = 1.00$) or OC ($P = 0.49$) mortality. However, the methodology demonstrated here may be of interest in eventually assessing the effects of competing risks and the insulin pathway on both BrCa and OC mortality in the much larger metformin placebo-controlled trial, NCIC CTG MA.32.

Alternatively, other joint assessment of chronic diseases is conceptually possible in trials where therapies may be postulated to have dual modes of action. NCIC CTG MA.27 originally had a factorial design with administration of celecoxib that was postulated to also be an anti-breast cancer agent until excess cardiac toxicity led to the closing of the celecoxib arms^[13]. The dual modalities for MA.27 would have been breast cancer and possibly musculoskeletal or arthralgic sequelae^[14] which could also be targets of celecoxib therapy. The prevention trial, NCIC CTG MAP.3, likewise had to close its celecoxib arms so is not a good venue for this joint testing^[15]. We await the results of the REACT trial which was able to accrue its full sample size to celecoxib, and postulate that it will be a good candidate for this modality of joint test-

ing^[16].

In summary, the progress for earlier detection and improved management of breast cancer means that women with early breast cancer will increasingly face joint mortality risks of other chronic diseases. This situation raises the opportunity for a new paradigm of simultaneously targeting several chronic diseases, or at least assessing the joint risks of mortality, for a better understanding of predisposition of patients to mortality other than breast cancer. MA.14 was an early trial of the insulin pathway targeted therapy, octreotide, which conceptually may have affected both BrCa and OC mortality, so was used here to demonstrate analyses that might be considered for the new assessment framework. The current work is offered as a proof of principle to demonstrate the relevance of collecting detailed cause-specific mortality data and factors which may be associated with non-breast cancer death.

COMMENTS

Background

Improvements in detection and management of breast cancer lead to the prospect that many postmenopausal women will now not die from the disease as they face joint mortality risks of other chronic diseases. Investigators need to determine baseline characteristics differentially associated with breast cancer vs other causes of death for more effective overall patient care. This paper proposes a method that might provide a basis for such discernment. The method is demonstrated for a phase III clinical trial which tested an early target of the insulin pathway that could have impacted both breast cancer and other cause mortality.

Research frontiers

Successful development of therapies which would simultaneously target several chronic diseases would improve whole population survival. Additionally, the management of elderly breast cancer patients is currently an important research area given the general increase in proportion of older people worldwide.

Innovations and breakthroughs

The usual process of inferring different factors influencing different causes of death involves qualitative observations. The competing risks method demonstrated here has quantitative formal tests. Older age and higher BMI increased the prospect that a patient would die of other causes than breast cancer, as did patients having smaller, hormone-receptor positive tumours and less lymph node involvement. The authors also reported quantitative patient survival over time by type of death.

Applications

This work could be extended to existing uniformly collected databases where there was careful collection of cause of death to better delineate factors affecting a broader range of competing risks of death. The more routine collection of multiple causes of death would assist future work.

Terminology

Log-normal survival analysis examines the linear effects of factors on the natural logarithm of survival time to provide smooth continuous functions and plots of factor effects.

Peer review

Well-prepared article offered new insights in breast cancer research and in evaluation of treatment, particularly in postmenopausal women.

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Risk factors and natural history of breast cancer in younger Chinese women

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Abstract

AIM: To investigate the age differences in the risk factors, clinicopathological characteristics and patterns of treatment of female breast cancer patients.

METHODS: Seven thousand one hundred and fifty-two women with primary breast cancer from the Hong Kong Breast Cancer Registry were recruited after receiving patients' consent, they were asked to complete standardized questionnaires which captured their sociodemographic characteristics and risk factors associated with breast cancer development. Among them, clinicopathological data and patterns of treatment were further collected from medical records of 5523 patients with invasive breast cancers. Patients were divided into two groups according to the age at diagnosis: younger (< 40 years old) vs older patients (\geq 40 years old) for subsequent analyses.

RESULTS: Analysis on the sociodemographic characteristics and exposure to risk factors were performed on 7152 women with primary breast cancer and the results revealed that younger patients were more likely to have unhealthy lifestyles; these include a lack of exercise (85.4% vs 73.2%, $P < 0.001$), having high stress in life (46.1% vs 35.5%, $P < 0.001$), having dairy/meat-rich diets (20.2% vs 12.9%, $P < 0.001$),

having alcohol drinking habit (7.7% *vs* 5.2%, $P = 0.002$). Younger patients were also more likely to have hormone-related risk factors including nulliparity (43.3% *vs* 17.8%, $P < 0.001$) and an early age at menarche (20.7% *vs* 13.2%, $P < 0.001$). Analyses on clinicopathological characteristics and patterns of treatment were performed on 5523 women diagnosed with invasive breast cancer. The invasive tumours in younger patients showed more aggressive pathological features such as having a higher percentage of grade 3 histology (45.7% *vs* 36.5%, $P < 0.001$), having a higher proportion of tumours with lymphovascular invasion (39.6% *vs* 33.2%, $P = 0.003$), and having multifocal disease (15.7% *vs* 10.3%, $P < 0.001$); they received different patterns of treatment than their older counterparts.

CONCLUSION: Younger patients in Hong Kong are more likely to encounter risk factors associated with breast cancer development and have more aggressive tumours than their older counterparts.

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Key words: Hong Kong Breast Cancer Registry; Breast cancer; Younger Chinese women; Sociodemographic characteristics; Risk factors; Clinicopathological characteristics; Breast cancer treatment

Core tip: We conducted this study to investigate the age differences in the risk factors associated with breast cancer development of female breast cancer patients in Hong Kong. Further, among patients with invasive cancers, we compared the clinicopathological characteristics and the treatments received between these two groups of patients. Younger patients in Hong Kong were found to be more likely to encounter risk factors associated with breast cancer development and have more aggressive tumours than their older counterparts. Based on the current findings, we will conduct further research to evaluate the impact of age at diagnosis on the outcomes of the disease.

Yeo W, Lee HM, Chan A, Chan EYY, Chan MCM, Chan KW, Chan SWW, Cheung FY, Cheung PSY, Choi PHK, Chor JSY, Foo WWL, Kwan WH, Law SCK, Li LPK, Tsang JWH, Tung Y, Wong LLS, Wong TT, Yau CC, Yau TK, Zee BCY. Risk factors and natural history of breast cancer in younger Chinese women. *World J Clin Oncol* 2014; 5(5): 1097-1106 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i5/1097.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i5.1097>

INTRODUCTION

Breast cancer has been the most frequent cancer affecting women in Hong Kong since 1993. Female breast cancer cases diagnosed in Hong Kong has more than doubled from 1152 in 1993 to 3014 in 2010. In 2010, breast cancer comprised of 24.1% of all cancers among

women and was the third leading cause of cancer death in women^[1]. On average, about 8 women in Hong Kong were diagnosed with breast cancer every day. The median age at diagnosis among breast cancer patients was 53, compared to that of age of 61 in the United States^[2], 61 in Australia^[3] and 53 in Singapore^[4].

Breast cancer is an age-related disease and increasing age is the single most important risk factor after female gender^[5]. In Hong Kong, the age-specific incidence rates for female with invasive breast cancer increased drastically after the age of 40. However, 8.9% of the cases in Hong Kong were diagnosed before the age of 40 in 2010^[1], which was higher than that in Australia (aged below 40) (5.6%)^[6] and the United Kingdom (aged below 40) (4.2%)^[7], while the reported figure for United States patients diagnosed younger before age 45 was 11.4%^[8].

It has been reported that risk factors for early onset breast cancer differ from those for postmenopausal breast cancer. Risk factors identified for premenopausal breast cancer include positive family history, high energy (caloric) intake, sedentary lifestyle, early age at menarche, heavy alcohol consumption and a high intake of red meat, while for postmenopausal breast cancer, high body mass index is identified as a risk factor. On the other hand, intense physical activity has been associated with a decreased breast cancer risk in premenopausal women^[9].

Although breast cancer in young patients is relatively uncommon, it represents a substantial clinical problem. Extensive researches have been carried out to study the differences in the pathological features of the tumours between younger and older patients. When compared to older patients, it has been suggested that invasive breast cancer in young women has a higher proportion with more aggressive features; these include presence of lymphovascular invasion, Grade 3 histology, extensive intraductal component, presence of necrosis, over-expression of the human epidermal growth factor receptor-2 (HER-2) oncogene and absence of estrogen receptor. Irrespective of the pathological differences, younger patients were also found to have poorer prognosis, with different treatment outcome and survival pattern, when compared with their older counterparts^[10]. Younger patients were more likely to recur both locoregionally and distantly^[11]. Survival rates were also found to be relatively lower for younger women when adjusted for the histologic subtypes and stages^[9].

Although the differences in the sociodemographic characteristics, risk factors associated with breast cancer development, clinicopathological characteristics and patterns of treatment among breast cancer women in different ages have been studied extensively worldwide, only two studies were conducted to study the differences in the clinicopathological characteristics and patterns of treatment between the younger and older breast cancer patients in Hong Kong^[12,13]. These latter two studies have reported that younger patients were more likely to be diagnosed at more advanced cancer stage, had higher pathological grade, more nodal involvement and presence of lymphovascular permeation; at the same time, a

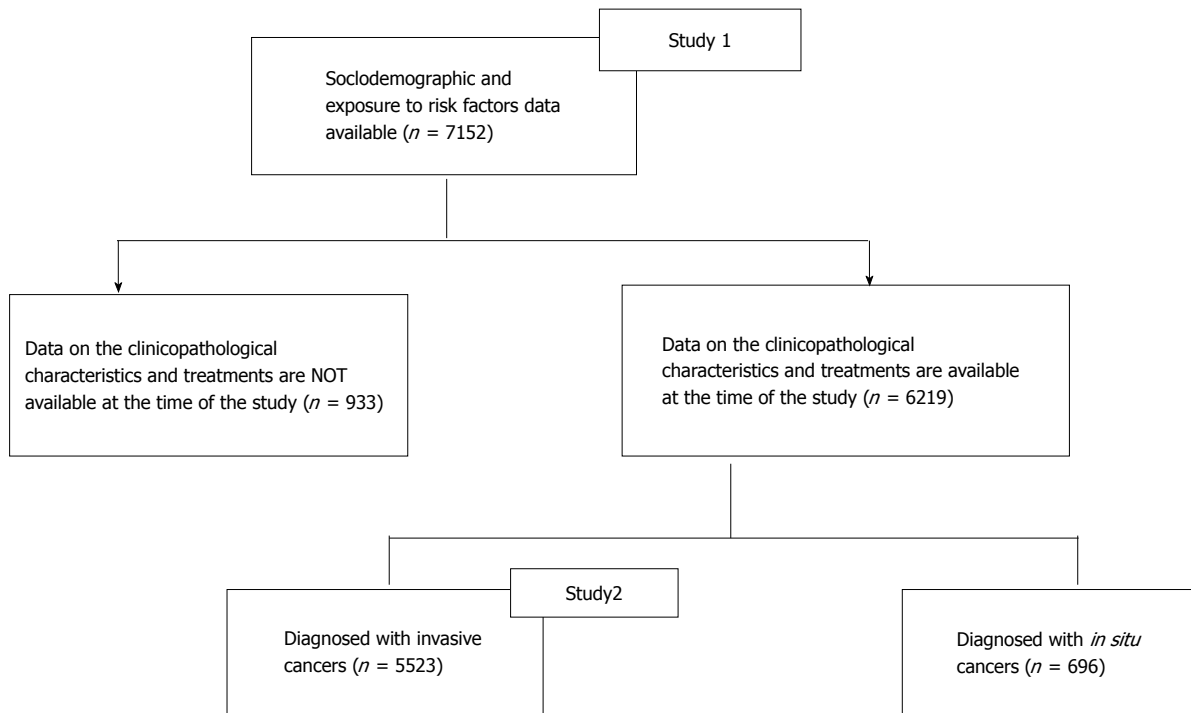


Figure 1 Patients included in the two studies. Study 1: Sociodemographic characteristic and exposure to risk factors analysis; Study 2: Clinicopathological and patterns of treatment analysis.

higher proportion of them underwent breast conservation surgery and reconstructive surgery. However, neither has focused on the differences in the sociodemographic characteristics and the risk factors associated with breast cancer development^[12,13]. Further, these studies recruited patients at a single hospital only, and as such the data might not reflect the overall clinical pattern of breast cancer in Hong Kong.

Thus, using the data from Hong Kong Breast Cancer Registry, we conducted this study to investigate the differences in the sociodemographic characteristics and risk factors associated with breast cancer development between younger female breast cancer patients and their older counterparts in Hong Kong. Further, among patients with invasive cancers, we compared the clinicopathological characteristics and the treatments received between these two groups of patients.

MATERIALS AND METHODS

Seven thousand one hundred and fifty-two consecutive Chinese women with primary breast cancer, recruited during the period of February 2008 to April 2012, from the Hong Kong Breast Cancer Registry (HKBCR) were studied. The HKBCR recruited breast cancer patients, regardless of the year of their diagnosis, from 35 centres including both private clinics/hospitals and public hospitals in Hong Kong. Among these women, 4320 (60.4%) were recruited from public hospitals. Ethics approvals for collecting patients' data for the HKBCR were obtained from the respective institutional review boards

of each participating hospital. The overall study consists of 2 parts: Study 1 and Study 2 (Figure 1). All registrants participated in Study 1 upon voluntary written consent; they were asked to complete standardized questionnaires which captured their sociodemographic characteristics data and the risk factors associated with breast cancer development. Among these 7152 patients, 5523 patients with confirmed invasive breast cancers participated in Study 2. The clinicopathological data, including method of first detection, clinical presentation for self-detected cancers, cancer stage, tumour histological type, grade, tumour size, presence of lymphovascular invasion, nodal status, breast cancer biological markers including estrogen receptor (ER), progesterone receptor (PR) and the HER-2 receptor statuses, and the treatments received were further collected for these 5523 patients from their medical records. Patients were arbitrarily divided into two groups according to age at diagnosis: younger (< 40 years old) *vs* older patients (\geq 40 years old) for subsequent analyses.

Statistical analysis

The Chi-square test was used to evaluate differences in categorical variables between the different age groups. T-test was used to evaluate the differences in the continuous variable between the two groups of patients. Median test, a non-parametric test, was used where assumptions for parametric tests were not met. A *P*-value < 0.05 was considered to be statistically significant. All statistical analyses were performed using SPSS software version 19.0 (SPSS Inc., Chicago, IL, United States).

Table 1 Sociodemographics and breast screening habits of the total patient cohort (n = 7152)

	< 40 yr (n = 1008) n (%)		≥ 40 yr (n = 6144) n (%)		P value
Age					
< 20	1	(0)			--
20-29	84	(1.2)			
30-39	923	(12.9)			
40-49			2 978	(41.6)	
50-59			2 102	(29.4)	
60-69			698	(9.8)	
70-79			268	(3.7)	
80+			98	(1.4)	
Education level					
No schooling	4	(0.4)	344	(5.7)	$\chi^2 = 473.730$, ^b P < 0.001
Primary	47	(4.7)	1 544	(25.5)	
Secondary	488	(49.3)	2 986	(49.4)	
Matriculation	133	(13.4)	425	(7)	
Undergraduate/postgraduate	318	(32.1)	748	(12.4)	
Occupation					
Professional/Clerical	608	(63)	1 845	(31)	$\chi^2 = 412.754$, ^b P < 0.001
Non-clerical/Labor	126	(13.1)	1 298	(21.8)	
Self-employed	45	(4.7)	190	(3.2)	
Housewife	170	(17.6)	2 043	(34.3)	
Retired/Unemployed	16	(1.7)	585	(9.8)	
Monthly household income					
< HK\$10000	77	(10.4)	837	(22.5)	$\chi^2 = 88.503$, ^b P < 0.001
HK\$10000-29999	290	(39.1)	1 591	(42.8)	
≥ HK\$30000	375	(50.5)	1 287	(34.6)	
Breast screening habits					
Breast self examination					
Never	332	(33.8)	2 359	(39.7)	$\chi^2 = 13.136$, ^b P = 0.001
Occasional	428	(43.6)	2 295	(38.6)	
Monthly	222	(22.6)	1 288	(21.7)	
Clinical breast examination					
Never	380	(38.5)	2 625	(44.1)	$\chi^2 = 10.934$, ^b P = 0.004
Occasional	132	(13.4)	718	(12.1)	
Regular ¹	476	(48.2)	2 611	(43.9)	
Mammogram					
Never	--	--	3 929	(66.1)	--
Occasional	--	--	554	(9.3)	
Regular ¹	--	--	1 463	(24.6)	
Ultrasound					
Never	--	--	4 029	(70.3)	--
Occasional	--	--	520	(9.1)	
Regular ¹	--	--	1 179	(20.6)	

¹Regular means having breast examinations annually, or once every 2-3 years.

RESULTS

Sociodemographic characteristics

Table 1 summarizes the sociodemographic characteristics of the patient cohort. Although population-based breast screening is not conducted in Hong Kong, Table 1 also provides information on patients' self-initiated breast screening habits. The age at diagnosis ranged from 18.8 to 101.4 years. Majority (71.0%) of the women was diagnosed in the age group of 40-59 years old, with a mean age \pm SD of 50.1 ± 10.4 years and a median age of 48.8 years. Around half (40.5%) of the patients were premenopausal women. 1008 (14.1%) of the patients were categorized as younger patients while 6144 (85.9%) were categorized as older patients. Younger patients were found to be more educated (education level at matriculation or above) (45.5% *vs* 19.4%, $P < 0.001$). As expected,

more younger patients were engaged in an occupation before cancer diagnosis (80.8% *vs* 56.0%, $P < 0.001$) and thus had higher monthly household income (\geq US\$3,750: 50.5% *vs* 34.6%, $P < 0.001$) than their older counterparts. In addition, younger patients were also found to have higher rates of breast self-examination (66.2% *vs* 60.3%, $P = 0.001$) and clinical breast examination (61.6% *vs* 56.0%, $P = 0.004$).

Risk factors associated with breast cancer development

The associated risk factors within the patient cohort are summarized in Table 2. The proportions having positive family history was significantly higher in younger patients (17.7% *vs* 14.5%, $P = 0.010$). A significantly higher proportion of younger patients did not have enough exercise (< 3 h/wk) (85.4% *vs* 73.2%, $P < 0.001$). Compared to their older counterparts, they also had a significantly

Table 2 Associated risk factors for breast cancer development in the total patient population (*n* = 7152)

	< 40 yr (<i>n</i> = 1008) <i>n</i> (%)		≥ 40 yr (<i>n</i> = 6144) <i>n</i> (%)		<i>P</i> value
Family history of breast cancer					
Yes	178	(17.7)	891	(14.5)	$\chi^2 = 6.788$, ^a <i>P</i> = 0.010
No	830	(82.3)	5 253	(85.5)	
Lifestyle-related risk factors					
Lack of exercise (< 3h/wk)					
Yes	861	(85.4)	4 497	(73.2)	$\chi^2 = 68.848$, ^b <i>P</i> < 0.001
No	147	(14.6)	1 647	(26.8)	
High level of stress (> 50% of time)					
Yes	465	(46.1)	2 179	(35.5)	$\chi^2 = 42.272$, ^a <i>P</i> < 0.001
No	543	(53.9)	3 965	(64.5)	
Being overweight/obese (BMI ≥ 23.0)					
Yes	241	(23.9)	2 344	(38.2)	$\chi^2 = 76.104$, ^b <i>P</i> < 0.001
No	767	(76.1)	3 800	(61.8)	
Diet rich in meat/dairy products					
Yes	204	(20.2)	791	(12.9)	$\chi^2 = 39.205$, ^b <i>P</i> < 0.001
No	804	(79.8)	5 353	(87.1)	
Frequently night shifts					
Yes	81	(11.4)	285	(9.3)	$\chi^2 = 2.958$, <i>P</i> = 0.091
No	632	(88.6)	2 796	(90.7)	
Alcohol drinking habit					
Yes	77	(7.7)	316	(5.2)	$\chi^2 = 10.008$, ^b <i>P</i> = 0.002
No	925	(92.3)	5 747	(94.8)	
Hormone-related risk factors					
Breastfeeding					
Yes	226	(46.3)	1 933	(40.9)	$\chi^2 = 5.249$, ^a <i>P</i> = 0.023
No	262	(53.7)	2 788	(59.1)	
Nulliparity					
Yes	381	(43.3)	1 042	(17.8)	$\chi^2 = 297.601$, ^b <i>P</i> < 0.001
No	499	(56.7)	4 805	(82.2)	
First live birth age after 35					
Yes	21	(4.3)	238	(5.1)	$\chi^2 = 0.630$, <i>P</i> = 0.514
No	469	(95.7)	4 420	(94.9)	
Early menarche (< 12 yr old)					
Yes	197	(20.7)	759	(13.2)	$\chi^2 = 37.243$, ^b <i>P</i> < 0.001
No	755	(79.3)	4 982	(86.8)	
Use of hormone replacement therapy					
Yes	5	(8.1)	376	(11.7)	$\chi^2 = 0.778$, <i>P</i> = 0.546
No	57	(91.9)	2 841	(88.3)	

higher proportion of having high level of stress (> 50% of time) (46.1% *vs* 35.5%, *P* < 0.001), having diets rich in meat/dairy products (20.2% *vs* 12.9%, *P* < 0.001), and having alcohol drinking habit (7.7% *vs* 5.2%, *P* = 0.002). In addition, a significantly higher proportion of younger patients had an early age at menarche (< 12 years old) (20.7% *vs* 13.2%, *P* < 0.001) and were nulliparous (43.3% *vs* 17.8%, *P* < 0.001). On the other hand, a significantly higher proportion of younger patients who bore children had breastfed their children (46.3% *vs* 40.9%, *P* = 0.023). Overweight or obesity was more common in older patients (23.9% *vs* 38.2%, *P* < 0.001).

Clinicopathological data of patients with invasive cancers

Table 3 summarizes the differences in the clinicopathological characteristics of the invasive tumours between the two groups of patients. Most (90.5%) of the patients self-detected their cancers but a higher proportion of younger patients self-detected their cancers than the older patients (94.1% *vs* 90.3%, *P* = 0.001). Except for a higher proportion of younger patients having nipple discharge

as the presenting symptom (5.1% *vs* 3.0%, *P* = 0.012), younger and older patients did not show any differences in their presentation symptoms and the duration of symptoms prior to the first medical consultation. A significantly higher proportion of younger patients were diagnosed with early stage cancer (Stage I - II B) (88.2% *vs* 83.0%, *P* = 0.001). Although younger patients tended to have smaller tumour sizes (median tumour size: 1.90 cm *vs* 2.00 cm, *P* = 0.065) and negative axillary nodal status (59.4% *vs* 61.9%, *P* = 0.075), these tumours usually exhibited more aggressive features. Younger patients had a higher percentage of grade 3 histology (45.7% *vs* 36.5%, *P* < 0.001) and a higher proportion of tumours with lymphovascular invasion (39.6% *vs* 33.2%, *P* = 0.003). A higher proportion of younger patients had presence of multifocal disease (15.7% *vs* 10.3%, *P* < 0.001). On the other hand, the two groups of patients did not show any significant differences in hormone receptor status (ER and PR), as well as the HER2 status. The proportions of patients having triple negative biological subtype (ER-PR-HER2-) were also not significantly different between the two groups of patients.

Table 3 Differences in the pathological characteristics of patients with invasive breast tumours (*n* = 5523)

	< 40 yr (<i>n</i> = 706) <i>n</i> (%)	≥ 40 yr (<i>n</i> = 4 817) <i>n</i> (%)	<i>P</i> value
Method of detection			
Self-detected	589 (94.1)	3962 (90.3)	$\chi^2 = 9.513$, ^b <i>P</i> = 0.001
Screen-detected ¹	37 (5.9)	427 (9.7)	
Presenting symptoms (for self-detected cancers)			
Lump	553 (93.9)	3729 (94.1)	$\chi^2 = 0.049$, <i>P</i> = 0.780
Pain	29 (4.9)	173 (4.4)	$\chi^2 = 0.375$, <i>P</i> = 0.521
Nipple discharge	30 (5.1)	118 (3)	$\chi^2 = 7.291$, ^a <i>P</i> = 0.012
Nipple retraction	5 (0.8)	75 (1.9)	$\chi^2 = 3.237$, <i>P</i> = 0.090
Skin change	3 (0.5)	37 (0.9)	$\chi^2 = 1.061$, <i>P</i> = 0.475
Axillary node	5 (0.8)	23 (0.6)	NA ²
Asymmetry	2 (0.3)	11 (0.3)	NA ²
Duration of onset of symptoms (for self-detected cancers)			
< 1 mo	93 (39.9)	536 (39.6)	$\chi^2 = 0.466$, <i>P</i> = 0.926
1-3 mo	90 (38.6)	515 (38.1)	
4-12 mo	33 (14.2)	212 (15.7)	
> 12 mo	17 (7.3)	89 (6.6)	
Cancer stage			
Early stage (Stage I - II B)	603 (88.2)	3900(83)	$\chi^2 = 11.490$, ^b <i>P</i> = 0.001
Advanced stage (Stage III A -IV)	81 (11.8)	797(17)	
Tumour size ⁴			
Mean ± SD (cm)	2.15 ± 1.30	2.24 ± 1.49	<i>T</i> = -1.514, <i>P</i> = 0.130
Median (cm) ³	1.9	2	<i>P</i> = 0.065
IQR (cm)	1.40-2.70	1.40-2.80	--
≤ 2.0	361 (55.4)	2283 (51.5)	$\chi^2 = 3.407$, <i>P</i> = 0.071
> 2.0	291 (44.6)	2150 (48.5)	
Bloom and Richardson grade ⁴			
1	80 (12.9)	806 (19.2)	$\chi^2 = 24.682$, ^b <i>P</i> < 0.001
2	258 (41.5)	1865 (44.3)	
3	284 (45.7)	1537 (36.5)	
Lymphovascular invasion ⁴	229 (39.6)	1336 (33.2)	$\chi^2 = 8.972$, ^b <i>P</i> = 0.003
Disease ⁴			
Multifocality	104 (15.7)	463 (10.3)	$\chi^2 = 17.472$, ^b <i>P</i> < 0.001
Multicentricity	21 (3.2)	120 (2.7)	$\chi^2 = 0.597$, <i>P</i> = 0.442
Estrogen receptor + ve (ER+) ⁴	481 (75)	3389 (75.6)	$\chi^2 = 0.100$, <i>P</i> = 0.768
Progesterone receptor +ve (PR+) ⁴	400 (62.7)	2836 (63.5)	$\chi^2 = 0.162$, <i>P</i> = 0.693
Human Epidermal Growth Factor Receptor 2 +ve (HER2+) ⁴	151 (28.5)	970 (26.8)	$\chi^2 = 0.728$, <i>P</i> = 0.402
Triple negative subtype ⁴	74 (14.0)	450 (12.5)	$\chi^2 = 1.000$, <i>P</i> = 0.327
Nodal status (no. of positive nodes) ⁴			
0	409 (61.9)	2680 (59.4)	$\chi^2 = 6.908$, <i>P</i> = 0.075
1-3	184 (27.8)	1198 (26.6)	
4-9	44 (6.7)	420 (9.3)	
10+	24 (3.6)	212 (4.7)	

¹Screen-detected includes monthly self-exam, clinical breast exam (routine checkups), mammography or ultrasound screening; ²Chi-square test was not available due to the small number of patients; ³Mann-Whitney U test was used; ⁴227 patients underwent neoadjuvant therapy and did not have pathology reports, thus had missing values for these parameters: tumour size, Bloom and Richardson grade, presence of lymphovascular invasion, multifocality, multicentricity, endocrine receptor status, human epidermal growth factor receptor 2 status, and nodal status.

Treatments for patients with invasive cancers

Table 4 summarizes the treatments received by the patients. Younger patients were significantly more likely to undergo breast conserving surgery (46.1% *vs* 34.3%, *P* < 0.001), reconstruction after mastectomy (35.7% *vs* 11.7%, *P* < 0.001), and sentinel node biopsy (32.2% *vs* 28.7%, *P* = 0.014). Younger patients were significantly more likely to receive radiotherapy (72.0% *vs* 66.2%, *P* = 0.002), however, such difference was not observed after adjusted for the type of surgery they received. A higher proportion of younger patients received chemotherapy; specifically, statistical significance were observed in stages I and II B patients (Stage I: 59.2% *vs* 39.7%, *P* < 0.001; Stage II B: 100.0% *vs* 91.1%, *P* < 0.001). However, younger patients were less likely to receive endocrine therapy (68.7%

vs 74.2%, *P* = 0.003).

DISCUSSION

This is the first comprehensive study to describe the differences in the sociodemographic characteristics, risk factors associated with breast cancer development, cancer characteristics and patterns of treatment among female breast cancer patients of different ages in Hong Kong. Around one-seventh (14.1%) of the patients were of younger age (aged < 40 years), which was higher than 8.7% recorded by the Hong Kong Cancer Registry^[1], reflecting a higher percentage of younger patients having participated in this voluntary study. This figure is also relatively higher than that reported from other areas of

Table 4 Treatments received by patients with invasive breast tumours (*n* = 5523)

	< 40 yr (<i>n</i> = 706) <i>n</i> (%)	≥ 40 yr (<i>n</i> = 4817) <i>n</i> (%)	<i>P</i> value
Surgery			
Breast conserving surgery	324 (46.1)	1638 (34.3)	$\chi^2 = 36.921$, ^b <i>P</i> < 0.001
Mastectomy	379 (53.9)	3135 (65.7)	
Reconstruction			
Yes	135 (35.7)	367 (11.7)	$\chi^2 = 158.189$, ^b <i>P</i> < 0.001
No	243 (64.3)	2762 (88.3)	
Nodal Surgery			
Sentinel node biopsy (SNB)	222 (32.2)	1351 (28.7)	$\chi^2 = 8.496$, ^a <i>P</i> = 0.014
Axillary dissection (AD)	336 (48.7)	2572 (54.6)	
SNB+AD	132 (19.1)	787 (16.7)	
Chemotherapy			
Stage I	151 (59.2)	633 (39.7)	$\chi^2 = 34.433$, ^b <i>P</i> < 0.001
Stage II A	210 (88.6)	1291 (83.8)	$\chi^2 = 3.572$, <i>P</i> = 0.067
Stage II B	97 (100)	631 (91.1)	$\chi^2 = 9.417$, ^b <i>P</i> < 0.001
Stage III	72 (98.6)	664 (93.7)	NA ¹
Stage IV	8 (100)	69 (89.6)	NA ¹
Radiotherapy (overall)			
All patients	496 (72)	3106 (66.2)	$\chi^2 = 9.189$, ^b <i>P</i> = 0.002
BCS patients	306 (96.2)	1522 (95.4)	$\chi^2 = 0.460$, <i>P</i> = 0.556
MTX patients	190 (51.6)	1564 (51.2)	$\chi^2 = 0.027$, <i>P</i> = 0.912
Irradiated regions (overall)			
Breast ± regional lymph nodes	187 (60.1)	998 (46)	$\chi^2 = 21.859$, ^b <i>P</i> < 0.001
Chest wall ± regional lymph nodes	124 (39.9)	1173 (54)	
Endocrine therapy	477 (68.7)	3496 (74.2)	$\chi^2 = 9.206$, ^b <i>P</i> = 0.003
Targeted therapy	59 (8.6)	339 (7.2)	$\chi^2 = 1.682$, <i>P</i> = 0.211

¹ χ^2 -test was not available due to the small number of patients.

the world^[6-8].

This study revealed that among patients in Hong Kong, younger patients were more likely to be exposed to risk factors associated with breast cancer development. These included unhealthy lifestyles, such as a lack of exercise, having high stress in life, taking dairy/meat-rich diets and having alcohol drinking habit. They were also more likely to be associated with hormone-related risk factors such as nulliparity, not having breastfeeding experience and having an early age at menarche. Younger patients also had higher proportions with positive family history and these results were in line with those found in studies from other areas of the world^[9,14-23]. Combinations of these risk factors might have increased the risks of early onset breast cancer in Hong Kong. Differences in the clinicopathological characteristics and patterns of treatment were also found between younger and older patients.

This study uses the age of 40 as the cut-offs for younger patients. Although most studies categorized women age less than 35 in the “younger” age group, Zhou *et al.*^[10] found that women “35 to 40 years of age or younger” defined a group of patients in which age was an independent risk factor associated with high rates of recurrence. The age cut-off in this study is arbitrarily set at 40 years because it has been shown that in Hong Kong women, the risk of having breast cancer after the age of 40 years is significantly increased^[1].

Although no population-based breast cancer screening policy exists, younger females in Hong Kong are more aware of breast health than their older counter-

parts, which can be reflected by their regular self-initiated breast screening habits (both breast self-examination and clinical breast examination) and this might explain why younger patients were more likely to be diagnosed at earlier stages. Despite that, invasive tumours in younger patients showed more aggressive features with a higher proportion having higher grade tumours, presence of lymphovascular invasion, and tumours being multifocal; these are similar to previous report on local Chinese women which included a smaller number of patients^[13]. Between the younger and older patient population, there was no difference in the hormone receptor status, and these results were slightly different from those found in other reports from western and Asian populations^[18,24-33]. More frequently, younger women were found to have breast cancer with negative endocrine receptors than the older women^[18,19,24,25,28,30-32]. In China, a study conducted in 2012 revealed that younger patients were found to have larger tumours, higher metastatic lymph node rates and higher positivity rates for HER-2 overexpression than older patients, while older patients were less likely to be negative for estrogen and progesterone receptors^[34].

In the present study, due to the young age at diagnosis and the relatively smaller tumour size, younger patients were more likely to undergo breast conserving surgery. This is associated with a higher rate of breast radiotherapy. Previous reports have described a more aggressive therapeutic strategy with the use of chemotherapy for younger patients in an attempt to optimize the outcome^[11,29]. This study concurs with these reports and shows that younger patients with early stage breast

cancer had a significantly higher percentage receiving chemotherapy. For patients with more advanced stage disease (stages III and IV), the differences in the rate of using chemotherapy did not reach statistical significance. It has to be noted that chemotherapy can cause age-specific problems such as infertility, bone loss and changes in sexual function and physical appearance, which are of great concerns to younger patients. Thus, whilst aiming to improve the prognosis of these patients with various anticancer therapies, considerations have to be made on treatment-related long term morbidities^[35,36].

There have been conflicting reports on young age at diagnosis in breast cancer patients in relation to associated risk factors for breast cancer development, cancer characteristics and prognosis^[11,37]. Gene expression profile was suggested to be a powerful predictor of disease outcome in young patients with breast cancer, but age *per se* was not an independent prognostic factor^[38]. However, gene expression profile and outcomes of the disease were not studied in this study, and further research has to be carried out to evaluate the effects of age at diagnosis on the survival outcomes in this group of patients.

Although data from a large number of breast cancer patients are described in this study, several limitations do exist. One of the limitations is that breast cancer in Hong Kong is not a statutory notifiable disease. Although the Hong Kong Cancer Registry captures the incidence and mortality rates for all types of cancers every year, detailed information on breast cancer patients, such as the exposure of risk factors, clinicopathological data etc, were not fully captured. The HKBCR provides a more comprehensive data collection system capturing detailed information on breast cancer cases with patients' consent. Nevertheless, the respondents of HKBCR are usually a self-selected group, patients who agreed to participate in the registry are likely to be more health conscious when compared to the non-respondents. In addition, since the HKBCR collects registrants' data by asking them to fill out standardized questionnaires, it is likely that the proportions of older patients and those with difficulty in filling out questionnaires (such as those with advanced disease or recurrent disease) maybe under-represented in the HKBCR. As a result, data on patients who had worse prognosis may have been under-estimated. Another limitation is that since questionnaires were used, data on patients' stress and nutritional aspects before cancer diagnosis could not be objectively assessed and were only based on patients' subjective impression.

In conclusion, this study shows that in Hong Kong, there are differences in the sociodemographics characteristics and associated risk factors for breast cancer development in younger vs older breast cancer patients. Further, among those with invasive breast cancers, the clinicopathological characteristics and patterns of treatment between younger and older breast cancer patients vary. A higher proportion of younger women is more likely to be exposed to various breast cancer risk factors and since breast cancer has become the most common

cancer among Hong Kong women, efforts have to be made to educate women to undertake primary preventive measures against the development of breast cancer with respective to modifiable factors, for example, leading an active lifestyle and modifying diets. In addition, secondary preventive measures should be considered to enable early detection of breast cancer by increasing breast cancer awareness and conducting prompt breast assessments as clinically indicated. Younger patients were found to have more aggressive tumours than their older counterparts and further research will be conducted to evaluate the impact of age at diagnosis on the outcome of diseases.

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COMMENTS

Background

Breast cancer has been the most frequent cancer affecting women in Hong Kong since 1993. It has been reported that risk factors for early onset breast cancer differ from those for postmenopausal breast cancer. To date, only two studies were conducted to address the differences in the clinicopathological characteristics and patterns of treatment between the younger and older breast cancer patients in Hong Kong, and the findings have been limited by small sample size.

Research frontiers

Using the data from Hong Kong Breast Cancer Registry, the authors conducted this study to investigate the differences in the sociodemographic characteristics and risk factors associated with breast cancer development between younger female breast cancer patients and their older counterparts in Hong Kong. Further, among patients with invasive cancers, the authors compared the clinicopathological characteristics and the treatments received between these two groups of patients.

Innovations and breakthroughs

Analysis on the sociodemographic characteristics and exposure to risk factors were performed on 7152 women with primary breast cancer. Younger female breast cancer patients in Hong Kong were found to be more likely to encounter risk factors associated with breast cancer development and have more aggressive tumours than their older counterparts.

Applications

Based on the current findings, the authors decided to conduct further research to evaluate the impact of age at diagnosis on the outcomes of the disease.

Peer review

An excellent review of the current status of breast cancer in Hong Kong. The data are used correctly, the methodology is sound and conclusions adequate. The paper represents significant contribution to this field.

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Lymphoepithelioma-like carcinoma of the breast presenting as breast abscess

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Abstract

Lymphoepithelioma-like carcinoma (LELC) is a rare type of neoplasm in which only twenty cases have been reported in the breast. This type of tumor can be difficult to distinguish from other breast tumors particularly medullary carcinoma and lymphoma in the breast. We present a case of LELC of the breast presenting as an abscess along with a review of the literature. This is the 21st reported case of LELC of the breast and the first case to present as an abscess. Her clinical picture could have been mistaken for other infectious or inflammatory diseases. Given the potential for favorable outcome, early detection and general knowledge of this neoplasm are essential to expedite treatment for this rare tumor type.

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Key words: Lymphoepithelioma-like carcinoma of the

breast; Breast cancer; Breast abscess

Core tip: We present a case of lymphoepithelioma-like carcinoma (LELC) of the breast, which is a rare tumor type that can be difficult to diagnose. This particular case is the first case of LELC of the breast presenting as an abscess with radiologic and histologic studies as well as literature review of this rare tumor.

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INTRODUCTION

Lymphoepithelioma-like carcinoma (LELC) is an undifferentiated carcinoma composed of malignant epithelial cells with a lymphocytic background, which was first described in the nasopharynx by Regaud, Reverchon and Schminke. These cells have been described in other sites including the stomach, salivary gland, lung, thymus, skin and cervix^[1]. LELC in the breast was first described by Kumar *et al*^[2]. LELC of the breast is a rare disease with only 20 reported cases described in the literature^[2-15]. Distinguishing LELC from medullary carcinoma and certain types of lymphoma has been a diagnostic challenge^[3,14,15]. Making this distinction has profound impact on therapy and overall prognosis.

In this paper, we present a case of LELC of the breast in a 64-year-old female with an unusual presentation and clinical course.

CASE REPORT

A 64-year-old African American woman presented with a painful palpable lump in her left breast for four weeks.

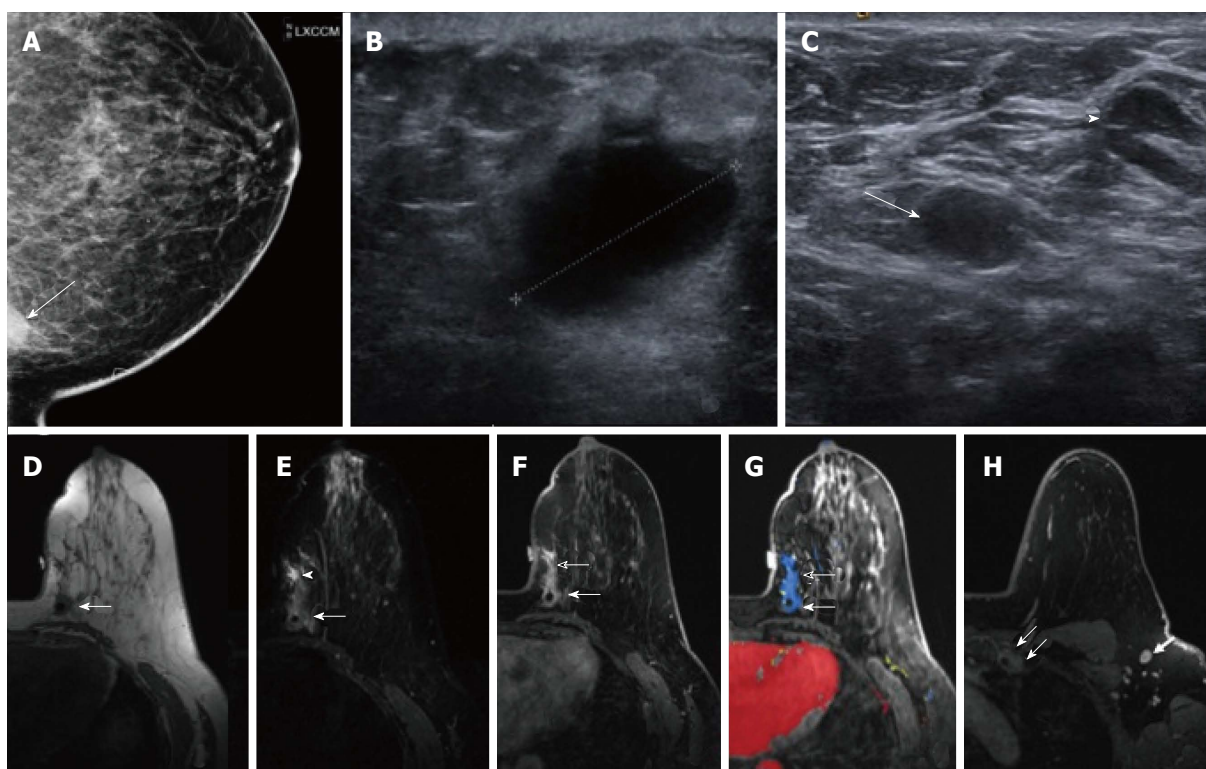


Figure 1 Imaging findings. A: Medially exaggerated cranio-caudal mammographic view of the left breast demonstrates the anterior aspect of the palpable mass that is a partially circumscribed and partially indistinct mass at 9 o'clock, posterior left breast (arrow); B: Ultrasound of the palpable lump demonstrates a 2.1 cm indistinct irregular hypoechoic solid mass with no posterior shadowing (caliper); C: Ultrasound of the left axilla demonstrates multiple oval and rounded lymph nodes measuring up to 1.4 cm. One oval 1.1 cm lymph node is diffusely hypoechoic (arrow) and is targeted for ultrasound guided Fine Needle Aspiration. The majority of the remaining lymph nodes demonstrate a thickened cortex (arrow head); D-H: Breast MRI: axial images of the left breast shown in multiple series as below; D: On pre-contrast T1W non-fat suppressed imaging the palpable mass is a 2 cm irregular mass with a central artifact from clip (arrow); E: On pre-contrast T2W fat suppressed imaging the mass is isointense to slightly hyperintense relative to the breast parenchyma (arrow). Similar signal is noted anterior to the mass with a higher intensity area more anteriorly (arrowhead); F: On first post-contrast fat suppressed T1W imaging the 2 cm irregular mass with a clip (arrow) is enhancing. Additional non-mass enhancement is noted anterior to the mass, measuring approximately 3 cm in the anterior-posterior plane (open arrow); G: Computerized color mapping demonstrates mostly persistent kinetic pattern of the mass and non-mass enhancement (area in blue, arrows); H: First post-contrast fat suppressed T1W imaging confirms the presence of multiple abnormal level 1 (arrow) and level 2 (not shown) axillary lymph nodes and abnormal left internal mammary chain adenopathy (double arrows).

She was initially seen by her primary care physician who prescribed antibiotics without any improvement. She subsequently underwent a mammogram, which demonstrated the anterior aspect of an ill-defined density located in the most posterior medial aspect of the left breast best seen on exaggerated cranio-caudal view (Figure 1A). This mass showed no associated focal architectural distortion or cluster of microcalcification. Her prior annual mammograms were all negative 4 years in a row. Ultrasound revealed an irregular markedly hypoechoic mass with indistinct margins at the 9 o'clock position of the left breast measuring approximately 2.1 cm in maximal diameter (Figure 1B). On color Doppler, no internal vascularity was detected. There were multiple enlarged left axillary lymph nodes detected on both mammogram and ultrasound (Figure 1C). The mass was classified as highly suggestive of malignancy according to the American College of Radiology Breast Imaging Reporting and Data System (ACR BI-RADS: 5). The patient subsequently underwent ultrasound-guided core needle biopsy of the left breast mass and a fine needle aspiration of the two dominant left axillary lymph nodes. Her initial biopsy specimen was

described as an abscess-like necrotic tissue but there were atypical cells that were highly suspicious for necrotic malignancy. The fine needle aspirations of the two enlarging axillary lymph nodes were negative for malignancy. This suspicious mass demonstrated low T1 and high T2 signal intensity on the pre-contrast imaging on a subsequent breast MRI (magnetic resonance imaging) with the susceptibility artifact from a biopsy clip (Figure 1D-F). After intravenous administration of gadolinium (Omniscan, GE), the mass showed irregular margins and enhancement, initially rapid and subsequently persisted (type III curve) (Figure 1F-H). Additional linear non mass-like enhancement with similar kinetic pattern was noted anterior to the mass, near the palpable marker, which could represent both tumor extension or post biopsy change. Her right breast showed no suspicious finding. Multiple abnormal enlarged and enhancing level 1 and level 2 left axillary lymph nodes and left inter-pectoral lymph nodes were detected, measuring up to 1.4 cm. There was also a 3.0 cm enhancing soft tissue mass with irregular margins in the left parasternal region, suggestive of left internal mammary adenopathy.

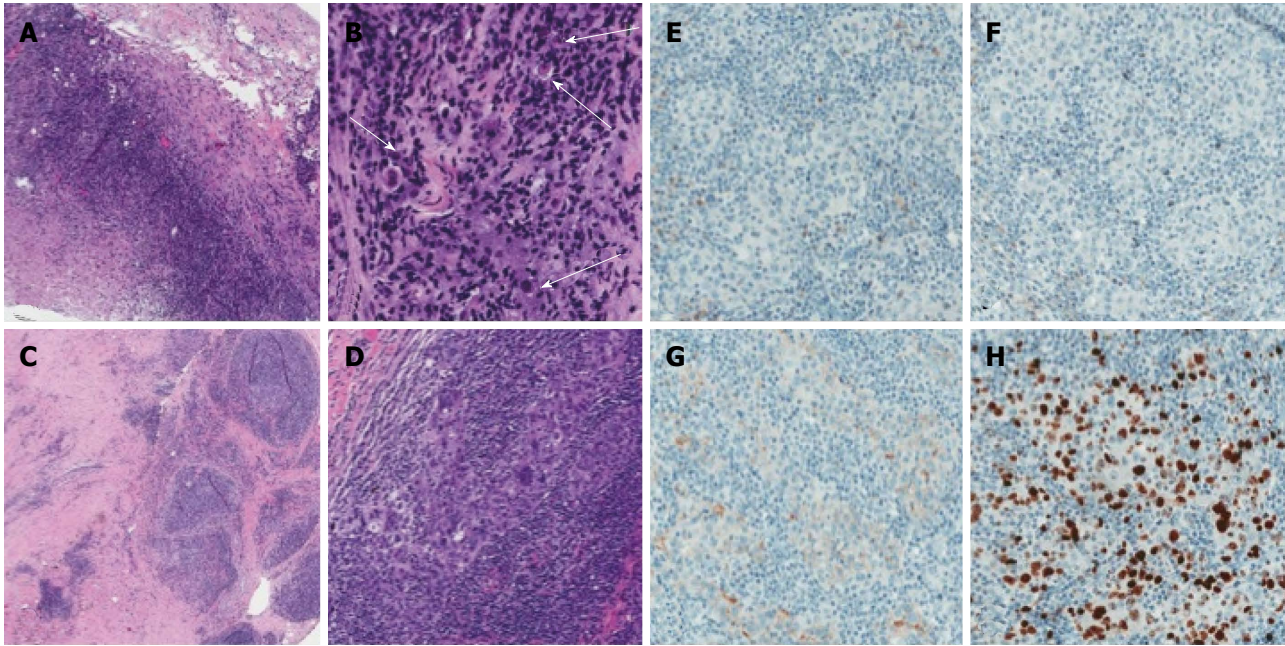


Figure 2 Pathology findings. A, B: Breast, core needle biopsy: large area of necrosis with chronic inflammation and fibrosis at periphery of lesion, 4X magnification (A); 16X magnification of A showing rare, atypical cells (arrows, B); C, D: Breast, surgical excision: poorly circumscribed lesion adjacent to previous biopsy site, 10X magnification (C); high grade carcinoma with marked pleomorphism and syncytial growth somewhat obscured by marked intra- and peri-tumoral inflammatory infiltrate (D) characteristic of LELC, 10X magnification; E-H: Immunophenotype: ER negative (E), PR negative (F), Her-2/neu 1+ (G), and Ki-67 27% (H). 10X magnification. ER: Estrogen-receptor; PR: Progesterone-receptor.

Subsequently, the patient underwent left lumpectomy with axillary node dissection. Positron emission tomograph scan showed no evidence of distant metastases. Her final pathology was consistent with stage IIA (pT1c, N1a, M0) lymphoepithelioma-like carcinoma (Figure 2A-D) with three out of twenty-three lymph nodes involved along with a small focus of extracapsular extension. Additional immunohistochemistry staining (Figure 2E-H) demonstrated that the tumor was negative for both estrogen-receptor and progesterone-receptor (ER 0%, PR 0%) and there was no overexpression of human epidermal growth factor receptor 2 (1+). The staining for proliferative index or Ki67 was 27%. The patient received adjuvant chemotherapy with dose dense doxorubicin and cyclophosphamide followed by weekly paclitaxel and a course of adjuvant radiation therapy.

Her clinical course was complicated by persistent inflammation and serosanguinous drainage from the surgical site. She was given antibiotics on multiple occasions for presumed cellulitis with minimal improvement. Follow-up punch skin biopsy and imaging done of the area showed no evidence of recurrence. These skin changes at the surgical site were thought to be secondary to post-surgical changes and radiation treatments. At her 3-year follow-up, the patient was doing well with no evidence of recurrent disease.

DISCUSSION

Lymphoepithelioma-like carcinoma of the breast is a rare tumor type characterized by epithelial neoplastic cells

with a background of lymphocytic cells. Only twenty cases of this type of breast neoplasm have been described in the literature. The reported cases are summarized in Table 1. Making the diagnosis of LELC can pose a diagnostic challenge due to its morphologic similarities with medullary carcinoma and certain types of lymphoma on pathologic examination^[5,14,15]. Making the distinction between LELC-B and other histologically similar tumors has significant impact on therapy and prognosis. Medullary carcinoma of the breast as described by Rapin and Ridolfi includes the following features: syncytial growth pattern > 75%, complete circumscription, diffuse mononuclear stromal infiltrate, moderate to marked nuclear pleomorphism and absence of microglandular features^[16,17]. LELC of the breast has similar features, but specifically obscures the neoplastic cells^[13]. Though poorly differentiated carcinomas, both medullary carcinomas and LELC-B consistently express cytokeratin markers^[12,18]. In our case, the neoplastic cells expressed CK7 and CK5/6, which differentiate these entities from a large cell lymphoma, which may also be on the histologic differential. Additionally, the extensive lymphoplasmacytic infiltrate associated with the epithelial cells may raise concern for a lymphoepithelial lesion, indicating a small cell lymphoma. Special studies for kappa and lambda expression by the B lymphocytes and plasma cells associated with medullary carcinoma and LELC-B show polyclonal cell population with expression of both kappa and lambda light chains. In situ hybridization for kappa and lambda light chains was performed in our case, showing a kappa-to-lambda ratio of approximately 2:1, ruling out a

Table 1 Summary of reported cases of lymphoepithelioma-like carcinoma of the breast

Case	Ref.	Year	Age	Presenting problem	Size (cm)	Lymph node (Y/N)	Surgery	Chemotherapy (Y/N) (agent listed if known)	Radiation therapy (Y/N)	Outcome (mo) ¹	ER status	PR status	Her2 status	EBV
1	Kumar <i>et al</i> ^[2]	1994	65		2	N	Mastectomy ALND	NR	NR	7	+	+	NR	NR
2	Cristina <i>et al</i> ^[3]	2000	54	Mass	1.5	N	Quadrantectomy ALND	Y	N	6	+	-	-	-
3	Dadmanesh <i>et al</i> ^[4]	2001	43	NR	1.9	Y	Quadrantectomy	N	N	60	-	-	-	NR
4	Dadmanesh <i>et al</i> ^[4]	2001	53	NR	2	N	NR	N	N	72	-	-	-	NR
5	Dadmanesh <i>et al</i> ^[4]	2001	49	NR	1	N	Quadrantectomy	N	N	2	-	-	-	NR
6	Dadmanesh <i>et al</i> ^[4]	2001	52	NR	2.7	N	Quadrantectomy	N	N	36	+	-	-	NR
7	Dadmanesh <i>et al</i> ^[4]	2001	64	NR	2	N	Mastectomy	N	N	60	-	-	-	NR
8	Dadmanesh <i>et al</i> ^[4]	2001	69	NR	2.3	N	Mastectomy	N	Y	48	-	-	-	NR
9	Naidoo <i>et al</i> ^[10]	2001	50	Mass	2.5	Y	Wide local excision ALND	N	N	3	NR	NR	NR	-
10	Pestereli <i>et al</i> ^[13]	2002	56	Mass	1.9	Y	Modified radical mastectomy ALND	Y	N	12	+	+	-	-
11	Ilvan <i>et al</i> ^[6]	2004	59	Mass	3.5	N	Wide local excision ALND	Y (Tamoxifen)	Y	52	+	+	NR	-
12	Ilvan <i>et al</i> ^[6]	2004	67	Mass	1.1	N	Quadrantectomy ALND	N	Y	46	+	+	NR	-
13	Sanati <i>et al</i> ^[15]	2004	62	Mass	3	NR	NR	NR	NR	36	+	-	-	-
14	Kurose <i>et al</i> ^[9]	2005	47	Mass	2.8		Total mastectomy ALND	Y (CEF)	N	12	+	+	+	-
15	Saleh <i>et al</i> ^[14]	2005	51	Mass	2	Y	Tamoxifen Lumpectomy ALND	N	N	NR	-	-	NR	-
16	Kulka <i>et al</i> ^[8]	2008	42	Mass	2.5	N	Lumpectomy	N	Y	NR	+	-	-	-
17	O'Sullivan-Meija <i>et al</i> ^[12]	2009	55	Abnormal mammo	2	N	Mastectomy	Y (Trastuzumab)	Y	22	-	-	+	-
18	Jeong <i>et al</i> ^[7]	2010	37	Mass	2.2	N	Modified mastectomy	Y	N	23	-	-	-	-
19	Dinniwell <i>et al</i> ^[5]	2012	55	Mass, tenderness	4	N	Excisional biopsy	N	Y	36	-	-	-	-
20	Nio <i>et al</i> ^[11]	2012	45	Mass	3	N	Quadrantectomy ALND	Y	Y	NR	-	-	-	NR
21	Present case	2012	64	Mass, tenderness	2	Y	Partial mastectomy ALND	Y (AC + paclitaxel)	Y	36	-	-	+	NR

¹Time after initial surgical procedure; ²Contralateral (right) LELC 3 years after initial diagnosis. ALND: Axillary lymph node dissection; NR: Not reported; CEF: Cyclophosphamide, epirubicin hydrochloride, 5FU; AC: Doxorubicin and cyclophosphamide; LELC: Lymphoepithelioma-like carcinoma; ER: Estrogen-receptor; PR: Progesterone-receptor; EBV: Epstein-Barr virus.

monotypic B cell population.

Although not tested in our patient, Epstein-Barr virus (EBV) and human papilloma virus (HPV) have been cited for their possible association with LELC of the breast. EBV has been linked to Burkitt's lymphoma and nasopharyngeal carcinoma. To date, EBV has not been shown to be associated with LELC of the breast; however, LELC in other anatomic sites namely salivary glands, sinonasal tract, stomach, thymus and lungs have been associated with EBV positivity^[1,19]. In addition, HPV has been associated with two cases of LELC-B^[8,11]. Another case has been seen with sclerosing lymphocytic

lobulitis^[10].

The case presented here, to the best of our knowledge, is the first case of lymphoepithelioma-like carcinoma of the breast to present as an abscess. Many of the prior case reports of LELC of the breast focus on the distinguishing histopathologic features of the disease and how to distinguish it from morphologically similar entities. Our case illustrates an unusual clinical presentation of this rare tumor type. Our patient presented with a painful breast lump and tenderness that could have easily been mistaken for an infection or other inflammatory processes which would have lead down a completely dif-

ferent diagnostic path and ultimately a significant delay in appropriate treatment. The patient's tumor also appeared to have necrotic and abscess-like features on initial pathologic examination, which has not been described in other cases of LELC. Initial clinical presentations were reported in 13 of the 20 cases. Twelve patients (60%) presented with a palpable mass while only 1 patient had abnormal findings on mammography. Her clinical course was also complicated by recurrent cellulitis and inflammation of the involved site minimally responsive to antimicrobial therapies. These changes may have been secondary to surgery and radiation therapy but did not appear to be consistent with recurrence of her primary disease.

All reported cases of LELC of the breast (Table 1) were found in women, ranging in age from 37 to 69 years. The median age was 54 years at time of presentation. The tumor sizes ranged from 1 to 4 cm and lymph node involvement seen in 25% of the patients including our patient. ER and PR status were evaluated in all but one patient (19 patients). ER was positive in nine patients (47%) and PR was positive in five patients (26%). Her-2 receptor status was reported in fifteen patients and was found to be overexpressed in three patients (20%).

Our patient regularly had annual mammography. Her last screening mammogram was performed six months prior to the detection of the palpable mass which showed no suspicious findings. However, the mass was probably not included in the field of view because of its very posterior location in the medial breast. This type of breast neoplasm appears to have overall favorable prognosis but early detection is imperative for proper treatment. After review of the literature, management of our patient went in line with most of the prior LELC-B cases. Seventeen of the reported cases mentioned some type of surgical intervention including mastectomy (complete or partial) in ten patients (59%) and axillary node dissections in nine patients (53%). Seven patients (34%) received some type of adjuvant chemotherapy and radiation therapy. Our patient underwent partial mastectomy and axillary node dissection followed by adjuvant chemotherapy along with radiation therapy. Disease-free outcome duration ranged from 2 to 72 mo in the cases reviewed while our patient was doing well without evidence of recurrent 36 mo after primary surgery.

In conclusion, this case and other cases of LELC-B demonstrate the diagnostic challenges for this particular type of tumor mainly due to its morphologic similarities with other neoplasms and rarity of the tumor. This is the first reported case of LELC of the breast presented with abscess-like clinical and pathologic features. Early recognition and appropriate diagnostic workup is the key for optimal management for this particular type of tumor.

COMMENTS

Case characteristics

A 64-year-old African American woman presented with a painful palpable lump.

Clinical diagnosis

Palpable mass of left anterior breast.

Differential diagnosis

Breast cancer, breast cellulitis/abscess.

Imaging diagnosis

Ultrasound showed an irregular markedly hypoechoic mass with indistinct margins at the 9 o'clock position of the left breast measuring approximately 2.1 cm in maximal diameter.

Pathological diagnosis

Her final pathology was consistent with stage IIA (pT1c, N1a, M0) lymphoepithelioma-like carcinoma. Additional immunohistochemistry staining demonstrated that the tumor was negative for both estrogen and progesterone receptors (ER 0%, PR 0%) and there was no overexpression of human epidermal growth factor receptor 2 (1+). The staining for proliferative index or Ki67 was 27%.

Treatment

The patient received adjuvant chemotherapy with doxorubicin and cyclophosphamide followed by weekly paclitaxel and a course of adjuvant radiation therapy.

Related reports

Lymphoepithelioma-like carcinoma (LELC) of the breast is a rare tumor type and the case presented is the first case of LELC of the breast presenting as a breast abscess.

Term explanation

LELC is an undifferentiated carcinoma composed of malignant epithelial cells with a lymphocytic background.

Experience and lessons

This case report emphasizes the importance of having knowledge of rare tumor types, particularly those in which early recognition can have profound impact on treatment outcome.

Peer review

It is an interesting and rare case of overlapping features where the diagnosis hangs between abscess and a tumour. The diagnosis is critical for appropriate management.

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Adult pulmonary blastoma: Report of an unusual malignant lung tumor

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Abstract

Pulmonary blastoma is an uncommon lung malignancy, usually presenting itself as a large chest mass causing pain, hemoptysis, cough and dyspnea; however, it is asymptomatic in up to 40% of patients. We present the case and suggestive images of a 37-year-old non-smoking lady with a monophasic pulmonary blastoma located in the lower lobe of the left lung who underwent a left posterolateral thoracotomy with lower lobectomy, hilar and mediastinal node dissection, followed by chemo and radiation therapy. After 36 mo, there is no disease progression and the patient is in good health, clinically stable and without significant chest pain.

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Key words: Pulmonary blastoma; Pleuropulmonary blas-

toma; Fetal adenocarcinoma; Lobectomy; Thoracotomy

Core tip: Pulmonary blastoma is a rare condition and diagnosis may be difficult to obtain. It is important to underline that the appropriate management of this unusual type of lesion can be achieved only by a multidisciplinary specialized team, able to plan the correct integrated strategy based on aggressive surgery and chemo-radiation therapy.

Magistrelli P, D'Ambra L, Berti S, Bonfante P, Francone E, Vigani A, Falco E. Adult pulmonary blastoma: Report of an unusual malignant lung tumor. *World J Clin Oncol* 2014; 5(5): 1113-1116 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i5/1113.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i5.1113>

INTRODUCTION

Pulmonary blastoma is an unusual lung malignancy, constituting about 0.5% of all lung tumors^[1-4]. Its histology resembles lung fetal tissue and can express both epithelial and mesenchymal features. Despite its assumed embryonal origin, the tumor predominantly affects adults^[5]. The literature reports a limited number of cases classified as adult type pulmonary blastoma or child pleuropulmonary blastoma^[5-8].

In adults, the neoplasm generally presents itself as a large and symptomatic mass causing cough, hemoptysis, fever and chest pain.

CASE REPORT

A 37-year-old non-smoking lady was admitted to the hospital with persistent chest pain, fever and hemoptysis. The chest X-ray showed a gross opacity in the left basal pulmonary field which was first suspected to be empy-

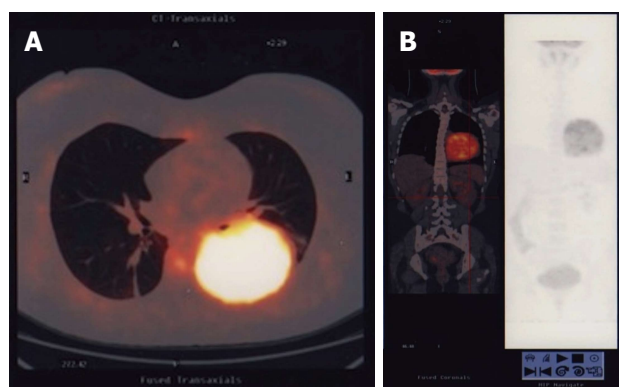


Figure 1 Computed tomography/positron emission tomography scan. A: CT-PET scan showing the large round mass in the left basal hemithorax; B: CT-PET scan showing a frontal vision of the mass inside the chest. CT-PET: Computed tomography/positron emission tomography.

ema complicating a pulmonary infection.

The computed tomography scan (CT scan) revealed the presence of a ten centimeter neoplasm located in the left basal hemithorax. Bronchoscopy showed neoplastic well vascularized vegetation occluding the left lower bronchus and histology through the biopsy permitted diagnosis of a monophasic pulmonary blastoma or the so called fetal adenocarcinoma. The immunohistochemistry obtained by fine needle aspiration biopsy of the lesion under CT scan guidance confirmed the rare lung tumor.

The stage was completed by a CT/positron emission tomography scan (CT/PET), showing an abnormal ^{18}F -FDG uptake limited to the round mass with an elevated SUV of 13, a specific feature of this type of blastoma^[1,9]. There was no evidence of other pathological localizations, confirming that the disease was enclosed in the left lung (Figure 1).

Pre-operative work-up showed no contraindications so the patient underwent surgery. We performed a left posterolateral thoracotomy with lower lobectomy and hilar and mediastinal node dissection (Figure 2).

The postoperative course was uneventful, the drains were removed three days after surgery and the patient was discharged three days later.

Definitive histology confirmed a pT3, pN2, M0 malignant monophasic pulmonary blastoma with only one metastatic mediastinal lymph node (Figure 3). The bronchial stump and the multiple specimens taken on the parietal and mediastinal pleura were negative.

A month later the patient was evaluated by our interdisciplinary team and was considered eligible for adjuvant chemo and radiation therapy.

Follow-up at 6, 12, 24 and 36 mo revealed no disease progression on PET/CT scan, with a serum decrease of oncological markers. The patient is now in good health, clinically stable and without significant chest pain.

DISCUSSION

Pulmonary blastoma is a rare neoplasm with distinctive

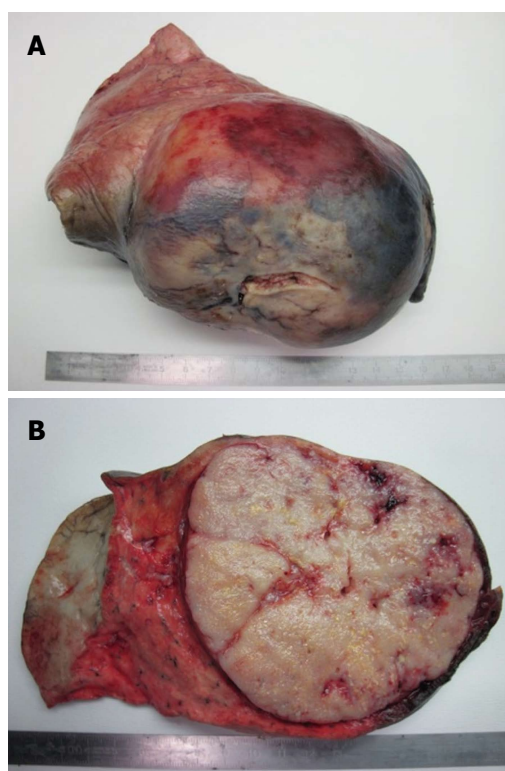


Figure 2 A left posterolateral thoracotomy with lower lobectomy. A: Macroscopic vision of the large tumor infiltrating the left lower lobe; B: The cutting surface of the large tumor inside the left lower lobe.

biological behavior^[4]. It was described for the first time in 1952 by Barnard as an “embryoma” and in 1961 was regularly classified by Spencer who established that the tumor arises from pulmonary blastema as tumors developed from fetal tissues^[5,10].

The neoplasm was divided by Koss *et al*^[11] into three groups: biphasic pulmonary blastoma, monophasic pulmonary blastoma with prevalent epithelial expression, and pleuropulmonary blastoma with mesenchymal expression^[2,5,10,11].

In adults, the tumor presents itself as a large chest mass causing pain, hemoptysis, cough and dyspnea; however, up to 40% of patients may be asymptomatic^[4]. Diagnosis may be difficult to obtain because of the unusual pleomorphic histology but it has to be suspected when there is cytological evidence of heterogeneity with epithelial and mesenchymal malignant cells^[12-14]. The radiological appearance consists of a well-delimited circle mass ranging in size from 1.5 to 13 cm in diameter.

Surgical excision is the treatment of choice^[4,15]. The prognosis reported in the literature is generally poor, with limited survival within 2-3 years from diagnosis. The difference in survival rate depends on mediastinal node involvement and over the last ten years a better prognosis has been shown with preoperative neoadjuvant chemotherapy^[14,16].

In a case of N2 disease, postoperative adjuvant radiation therapy and chemotherapy based on cisplatin and etoposide are considered the treatments of choice^[14,17,18].

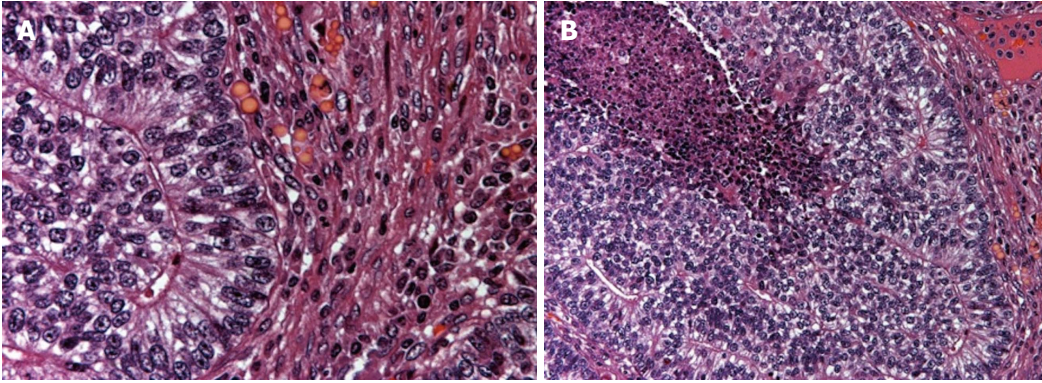


Figure 3 Monophasic pulmonary blastoma. A: Microscopic appearance of cytology in monophasic pulmonary blastoma; B: Monophasic pulmonary blastoma: Prevalence of epithelial on mesenchymal elements.

In conclusion, adult pulmonary blastoma is an unusual lung tumor presenting itself as a large invasive mass. Diagnosis, treatment and follow-up have to be planned by a multidisciplinary team. Integrated strategy based on aggressive surgery and chemo-radiation therapy may be the best choice for a successful treatment.

COMMENTS

Case characteristics

A 37-year-old non-smoking lady with persistent chest pain, fever and hemoptysis.

Clinical diagnosis

A ten centimeter neoplasm located in the left basal hemithorax.

Differential diagnosis

Empyema complicating a pulmonary infection.

Imaging diagnosis

A well-delimited circle mass ranging in size from 1.5 to 13 cm in diameter.

Pathological diagnosis

pT3, pN2, M0 monophasic pulmonary blastoma with one metastatic mediastinal lymph node.

Treatment

Left posterolateral thoracotomy with lower lobectomy and hilar and mediastinal node dissection followed by chemo and radiation therapy.

Related reports

The rare presentation of pulmonary blastoma can make obtaining a diagnosis difficult, so a meticulous evaluation by a multidisciplinary team is mandatory in suspected cases.

Experience and lessons

Pulmonary blastoma is a rare condition that has to be managed by a multidisciplinary team to plan the correct integrated strategy based on aggressive surgery and chemo-radiation therapy.

Peer review

This is a report of a rare case of a malignant lung tumor. The presentation is simple but adequate.

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Thyroid carcinoma showing thymus-like differentiation: Case presentation of a young man

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performed and interpreted the PET; Lombardi M interpreted the
histological data; Abeni C, Ogliosi C, Bertocchi P and Rota L
prepared the manuscript; Zaniboni A evaluated the draft and sug-
gested revisions; all authors read and approved the final manu-
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Diagnosis is complicated and requires careful histologi-
cal analysis (CD5- and P63-positive with presence of
Hassall's corpuscles); unfortunately there is no gold
standard treatment so, in this case, we administered a
sandwich of chemotherapy and radiotherapy.

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Key words: Carcinoma showing thymus-like differentiation;
thymic; CD5; Hassall's corpuscles; Thyroidectomy; Chemo-
therapy; Radiotherapy

Core tip: Carcinoma showing thymus-like differentiation
(CASTLE) is a very rare tumor and is very important to
differentiate it from others head and neck tumors be-
cause therapy and prognosis are different. Moreover, di-
agnosis is often complicated. Case reports on this topic,
reporting treatment modalities, are useful, because
there is no standard treatment for CASTLE.

Abeni C, Ogliosi C, Rota L, Bertocchi P, Huscher A, Savelli G,
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Abstract

Ectopic thymic tissue can be present in the thyroid gland and a carcinoma showing thymus-like differentiation (CASTLE) may arise from such tissue. We are reported the case of a 26-year-old man with CASTLE, with cervical subcutaneous nodules relapse, who showed a good response to treatment with surgery, chemotherapy and radiotherapy. The problematic aspect of this case was the diagnosis; only on review were we able to make a final diagnosis. CASTLE is a very rare neoplasm. It is important to differentiate this cancer from others tumors such as primary or metastatic squamous cell carcinoma of the head and neck or squamous cell thyroid carcinoma, because the therapy and prognosis are different.

INTRODUCTION

It is possible for ectopic thymic tissue to be present in the thyroid gland and a carcinoma showing thymus-like differentiation (CASTLE) may arise from such tissue. CASTLE is a rare type of cancer; first described by Miy-
auchi *et al*^[1] in 1981, it was not until 2004 that the World Health Organisation recognised it as an independent clinico-pathological entity and classified it as a type of

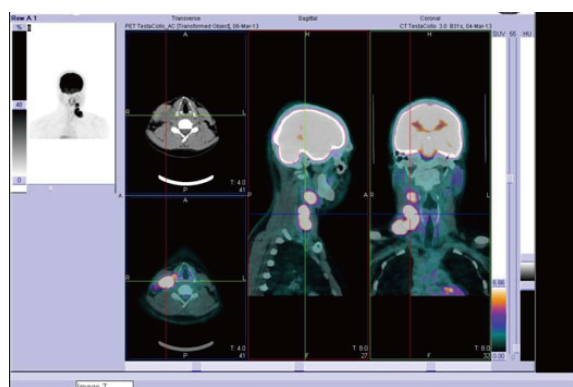


Figure 1 Positron emission tomography scan showing carcinoma showing thymus-like differentiation local disease recurrence.

thyroid tumor^[2]. We present the case of a young man with a diagnosis of locally advanced CASTLE.

CASE REPORT

A 26-year-old Caucasian male with no family history of neoplastic diseases and no comorbidities was examined by his general practitioner after developing minimal neck oedema and throat tightness; ultrasound of the neck was requested. The first step diagnostic procedures showed normal morphology and ultrasonography of the thyroid, with the exception of a suspicious nodule (about 3 cm of diameter) which was investigated cytologically with FNA and found to be positive for neoplastic cells (even if the diagnostic material was poor). The patient consequently underwent total thyroidectomy. The histological diagnosis was a poorly differentiated carcinoma of the thyroid, pT3N1b (6/6). Immuno-histochemistry (IHC): TTF1-positive (focal), thyroglobulin-positive (focal), CD56-positive (focal), NSE- and P63-positive. No adjuvant anti-neoplastic therapy was recommended. One month later, ultrasound examination of the neck revealed pathological changes at multiple right lateral cervical lymph nodes, confirmed by head and neck magnetic resonance. Positron emission tomography (PET) scanning did not show distant disease but detected neoplastic activity in bilateral cervical lymph nodes (Figure 1). Bilateral functional type lymphadenectomy of cervical lymph nodes was done, with 5/47 lymph nodes positive for metastases of poorly differentiated thyroid carcinoma and involvement of the right anterior margin of the sterno-mastoid muscle. About one month later, the patient came to our hospital for the first time. Physical examination showed multiple subcutaneous nodules near the surgical scar. This abnormal evolution of thyroid carcinoma prompted us to review the histological examinations. We found that the thyroid was characterised by intra-thyroid tumour growth including solid nests of epithelioid elements with high mitotic activity (14×10 HPF). There were also groups of squamoid cells similar to Hassall's corpuscles. The tumour had a lobulated profile and showed marked vascular invasion.

IHC analysis revealed: (1) P63: diffuse and strong nuclear positivity; (2) CD5: multifocal cytoplasmic positivity; (3) TTF1: nuclear positivity in the remaining follicular cells (both in follicles and in the collapsed regions within the tumour); (4) Thyroglobulin: positivity in the remaining follicular cells; and (5) Synaptophysin, calcitonin, chromogranin, CD56: negative; our revised diagnosis was CASTLE.

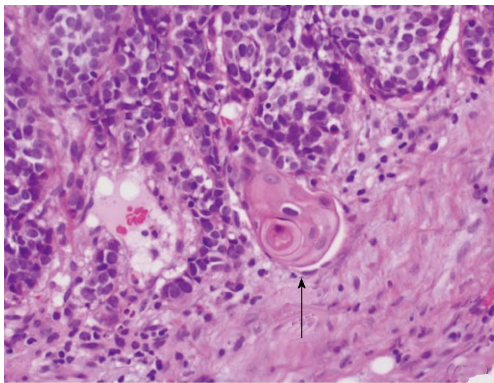
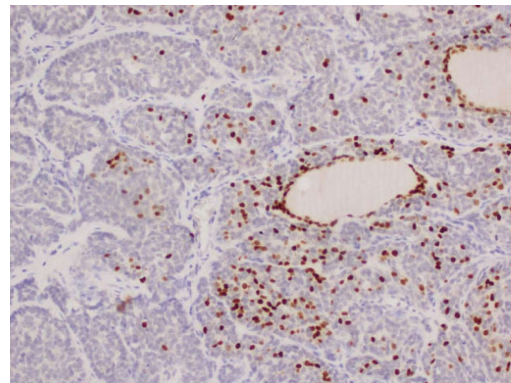
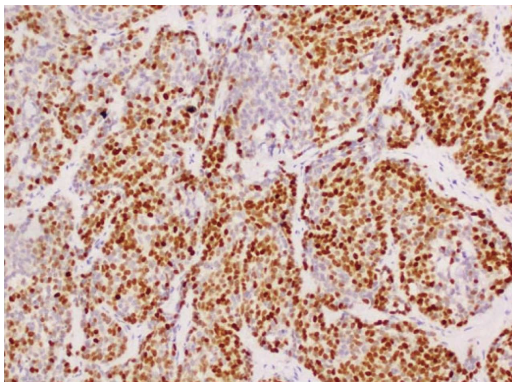
In view of the results of the histopathological review, a second local relapse within a few months, and a Computed Tomography (CT) scan negative for distant disease, we planned a therapeutic program which included chemo-radiotherapy: 2 cycles of chemotherapy, followed by radiotherapy, followed by 3 further cycles of chemotherapy using the same regimen. The chemotherapy administered was carboplatin AUC 6 and paclitaxel 225 mg/m² q21. Radiation was delivered by daily volumetric intensity-modulated arc therapy with cone-beam CT image-guidance. A parotid-sparing simultaneous integrated boost technique allowed the delivery of three different dose levels prescribed according to tumour burden: 66.0Gy in 33 fractions on the thyroid bed (site of macroscopic residual disease), 59.4Gy in 33 fractions on the right cervical nodes, levels II-V (site of positive extracapsular nodes) and a precautionary dose of 54.45Gy in 33 fractions on left cervical nodes, levels II-V and bilateral recurrent nodes showing excellent clinical response *i.e.*, disappearance of subcutaneous nodules. The most significant side-effects during the radiation treatment were: cervical skin erythema G2, desquamation in the thyroid bed, oropharyngeal mucositis G1 and sore throat; the chemotherapy was well-tolerated. At the end of the treatment the CT scan was negative and the first follow-up 3 mo later was also negative.

DISCUSSION

CASTLE is a very rare neoplasm which arises in the thyroid gland or the soft tissue of the neck. It is necessary to differentiate CASTLE from the others tumours such as primary or metastatic squamous cell carcinoma of the head and neck or squamous cell thyroid carcinoma, because the therapy and the prognosis are different^[3]. There are two theories of the histogenesis of this cancer, the first suggests that CASTLE arises from thymic nests near the thyroid gland which occur as a result of persistence of cervical thymic tissue during embryogenesis; the alternative theory proposes that it arises from remnants of the branchial pouches that differentiate along the thymic line^[3]. The expression of the IHC marker CD5 by CASTLE cells provides support for the latter theory^[4]. The diagnosis of this disease is complicated by its cyto-histological presentation. Microscopically it appears to be arranged in broad, smooth-bordered islands abutting a desmoplastic cellular stroma^[5]. The tumour cells show squamoid characteristics, having eosinophilic cytoplasm, oval nuclei and small distinct nucleoli. Within the lymphoid stroma Hassall's corpuscles may be seen at the

Table 1 Immunohistochemical markers in anaplastic thyroid gland/squamous cell carcinoma to be differentiated from carcinoma showing thymus-like differentiation

ICH	CD5	Calcitonin	P63	Synaptophysin	TTF-1	Thyroglobulin	Cromogranin	CD117
Anaplastic carcinoma	neg	Neg	Neg	Neg	Pos/neg	Neg	Neg	Neg/pos
CASTLE	Pos	Neg	Pos	Pos/neg	Neg	Neg	Neg	Focal pos
Squamous cell carcinoma	Neg	Neg	/	Neg	Neg	Neg	Neg	Neg/pos
Case report	Pos	Neg	Pos	Neg	Focal pos	Neg	Neg	/

**Figure 2 Hassall's corpuscle (arrow).****Figure 4 CD5 positive cells.****Figure 3 Diffuse nuclear p63-positive cells.**

periphery of the tumour; this may be an additional characteristic of this neoplasm^[6]. In this case the pathologist was alerted to the possibility of CASTLE by the presence of Hassall's corpuscles at the review of the histological findings. The IHC analysis showed a tumour that was strongly positive for pancytokeratin, CD5, P63, focal positive for CEA^[4-7], somewhat positive for chromogranin-A and synaptophysin^[8] and negative for TTF-1, thyroglobulin, chromogranin and calcitonin^[9] (Table 1). In this case CD5- and P63-positive IHC and the presence of Hassall's corpuscles were the two most important elements in the diagnosis (Figure 2, 3 and 4). In the literature this neoplasm is considered an indolent, slow-growing cancer even when regional lymph node metastasis is present^[7-10]. There is no gold standard treatment for this rare lesion although it appears that the first line treatment of choice is surgery with or without adjuvant radiotherapy^[3-11], we therefore suggested a treatment adapted to the needs of

our young patient. We used the type of chemotherapy that we normally give to patients with cancer of the thymus. In this case we administered a sandwich of chemotherapy and radiotherapy. The radiotherapy protocol was similar to that used for thymus tumours, but we reduced the dose intensity because the irradiated volumes were so big that the risk of toxicity for the patient was very high. Currently the patient is feeling well. If this patient had been given radiotherapy after the first surgery would he have relapsed? Unfortunately the literature does not provide evidence on this issue.

A further problem is determining the best radiological technique for staging and follow-up: there are currently no guidelines. In this case we did a total body CT-scan, PET-FDG and MRI of the neck for the staging, but used only a CT-scan for the first follow-up. Although our patient is younger than other cases reported in the literature, we were not able to find epidemiological, genetic or other explanations for his disease. This makes it more difficult to plan follow-up in order to prevent or achieve early diagnosis of other cancers or non-oncological diseases which may arise as ancillary pathological consequences of this rare tumour in this young patient.

COMMENTS

Case characteristics

A 26-year-old Caucasian male with a history of carcinoma showing thymus-like differentiation (CASTLE).

Clinical diagnosis

Neck edema and dysphagia.

Differential diagnosis

Metastatic squamous cell carcinoma of the head and neck or squamous cell thyroid carcinoma.

Laboratory diagnosis

Within normal limits.

Imaging diagnosis

The first step diagnostic was an ultrasonography of the thyroid that showed of a suspicious nodule, which was investigated cytologically and found to be positive for neoplastic cells.

Pathological diagnosis

The thyroid was characterised by intra-thyroid tumour growth including solid nests of epithelioid elements and also groups of squamoid cells similar to Hassall's corpuscles.

Treatment

The patient was treated with surgery followed by a sandwich of chemotherapy and radiotherapy.

Related reports

For this case there is no gold standard treatment so we were treated the patient as a patient with a thymic cancer.

Experiences and lessons

The importance of a multidisciplinary approach and the case's sharing could improve patient management.

Peer review

The manuscript is well written and reported diagnosis and treatment of a rare case of CASTLE.

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