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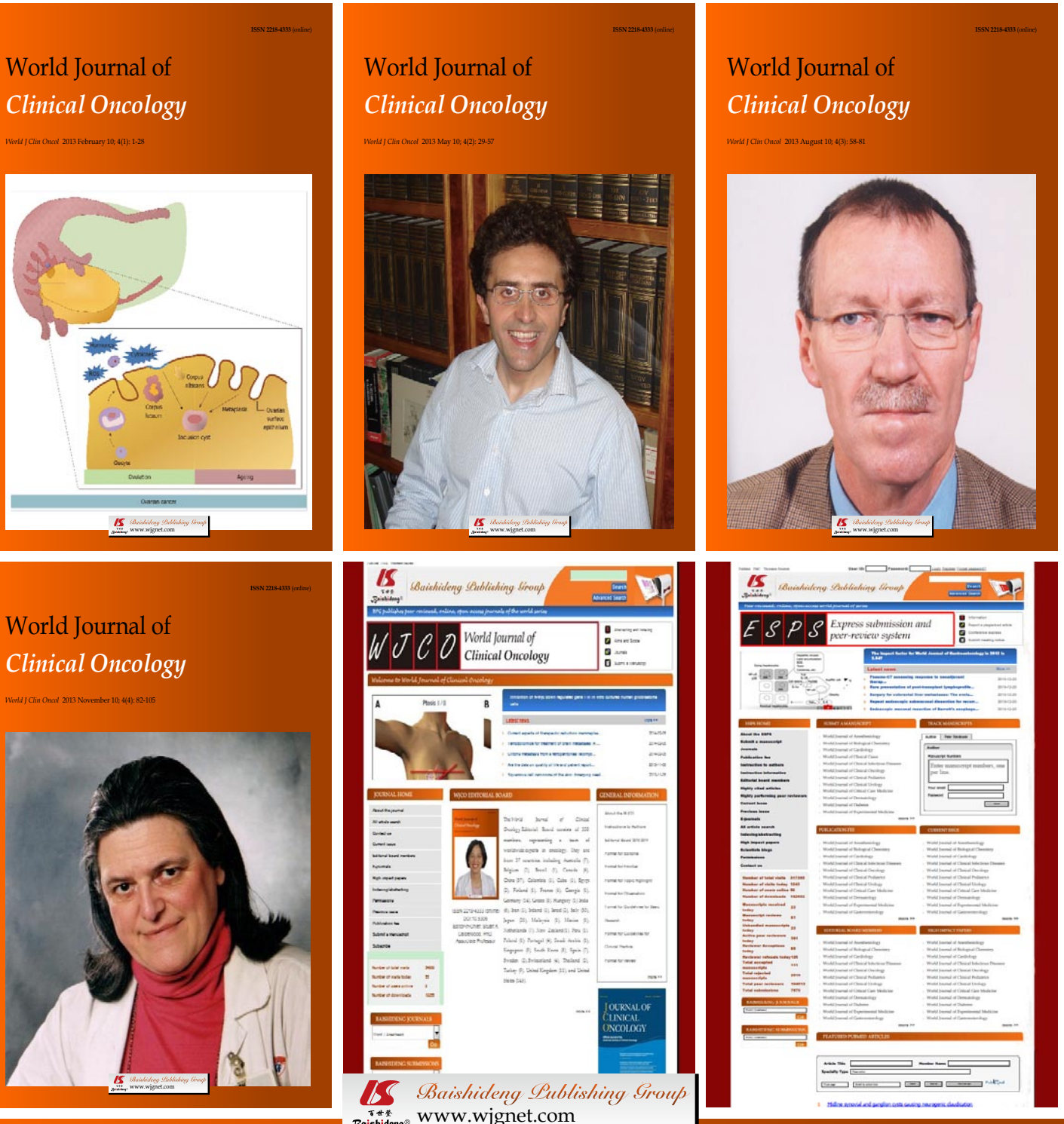

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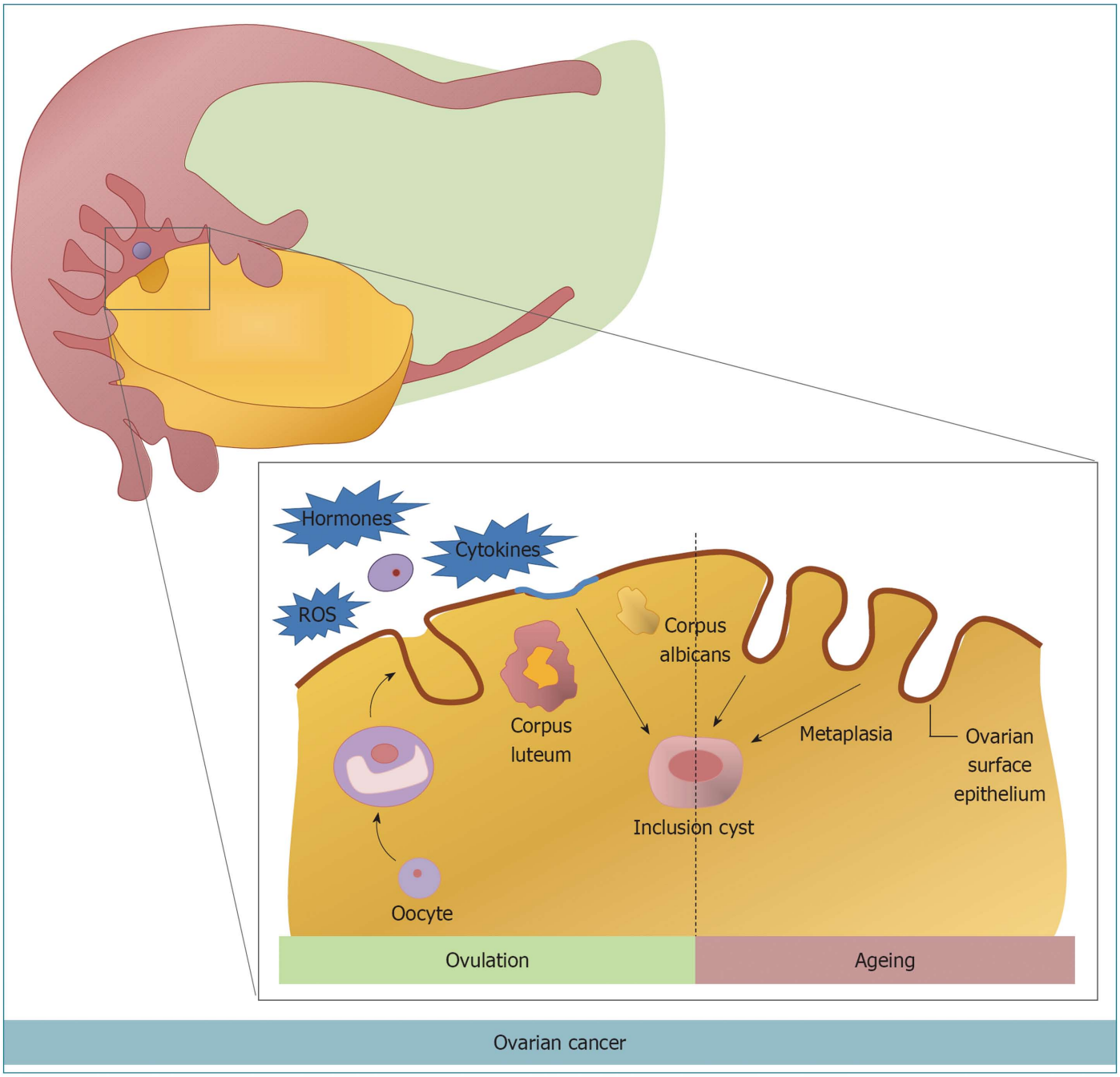

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EDITORIAL

Cathepsin D: Autoantibody profiling as a diagnostic marker for cancers

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Abstract

Current diagnostic assays for many cancers are antigen-based and rely on the detection of circulating proteins that are associated with a particular cancer. These assays depend on the expression, synthesis, and release of specific proteins by cells (*e.g.*, tumor cells) through either active secretion or shedding, or as a consequence of cell death (either necrosis or apoptosis). As such, these antigenic proteins must "escape" the primary site of disease, saturate the antigen-processing capacity of the individual's immune components, gain access to the circulation, and reach a sufficient steady-state concentration to be detected by enzyme- or radiolabel-based immunoassays. These events usually occur after the initial establishment of disease. Thus, and despite the fact that certain specific antigenic epitopes exhibit common recognition among patients with the same tumor types, the use of these antigen-based cancer assays has not been widely accepted in clinical practice, and many individual countries differ in the use of these potential diagnostic factors. Lately, an increasing number of studies demonstrated that procathepsin D secreted from cancer cells, acts as a mitogen on cancer cells and stimulates their pro-invasive and pro-metastatic properties. In this report, we focused on the possibility to use anti-procathepsin D autoantibodies as a diagnostic and/or predictive marker for cancers.

Key words: Cathepsin D; Procathepsin D; Autoantibodies; Diagnosis; Marker

Vetvicka V, Fusek M. Cathepsin D: Autoantibody profiling as a diagnostic marker for cancers. *World J Clin Oncol* 2013; 4(1): 1-3 Available from: URL: http://www.wjgnet.com/2218-4333/full/v4/i1/1.htm DOI: http://dx.doi.org/10.5306/wjco.v4.i1.1

INTRODUCTION

It is a well-established fact that early diagnosis significantly influences prognosis of diseases. If breast cancer is diagnosed and treated while it is still confined to the breast, the cure rate can approach 100%[1]. However, the five-year survival rates in breast cancer are very low in those patients diagnosed in later stages as compared to those diagnosed in early stages[2].

Currently, biomarkers in breast cancer lack reliability for screening. The only validated serum biomarkers for breast cancer, including carcinoembryonic antigen, cancer antigens (CA)27.29 and CA15.3, are used primarily to monitor advanced diseases and do not have sufficient clinical sensitivity for early detection[3,4]. Therefore, lack of a reliable, highly sensitive and specific screening diagnostic test is truly an unmet medical need for overwhelmingly prevalent breast cancer, resulting in a high mortality/morbidity in women in the United States and worldwide.

Cancer patients frequently develop autoantibodies. These autoantibodies (AAb) produced by the patient's own immune system upon exposure to tumor-associated antigens (TAA) or tumor-related molecules are emerging as promising biomarkers for the early detection of

cancers[5,6]. AAbs are specific, secreted in large quantities despite the presence of a relatively small amount of the corresponding antigen[2,3,7]. AAbs are present in the serum before the antigens can be detected and are secreted prior to the first clinical signs[7]. AAbs are also expected to have persistent concentrations and long half-lives ($t_{1/2}$ between 7 and 30 d) in blood due to limited proteolysis and clearance from the circulation, making sample handling much easier[7].

Although AAbs are proposed as early indicators of cancers, not all antigens are capable of eliciting adequate autoimmune response[3]. For instance, the sensitivity of detection of AAbs to a panel of 6 TAAs in breast cancer ranges from 20% to 73% (55%, 62% and 73% in grades 1, 2 and 3 primary invasive breast cancers, respectively; 20%, 62% and 41% in early, intermediate and high grade ductal carcinomas *in situ,* respectively)[1]. Clearly, these levels of sensitivities of AAbs to individual or panel of breast cancer TAAs are clearly not sufficient to build a reliable screening/diagnostic test[3,7]. To increase the predictive value of tumor-specific antibodies for use as immunodiagnostics, several groups have begun testing multiple antigens in parallel[3]. Therefore, it is necessary to identify and validate AAbs against a tumor specific antigen/s with a high sensitivity.

PROCATHEPSIN D

Numerous clinical studies reported an association between procathepsin D/cathepsin D levels and prognosis, incidence of metastasis, tumor aggressiveness and a degree of chemoresistance in a variety of solid tumor types[8]. In the last two decades, an increasing number of studies demonstrated that procathepsin D (pCD), secreted from cancer cells, acts as a mitogen on both cancer and stromal cells and stimulates their pro-invasive and pro-metastatic properties[9-13]. Studies dealing with pCD diagnostic and prognostic value in cancer are complicated by the fact that there are several forms of cathepsin D in a cell at the same time: pCD, intermediate enzymatically active cathepsin D and mature two-chain cathepsin D. It is highly probable that tumor-promoting function of secreted cathepsin D is specific for only zymogen form of it. On the other hand, most of anti-cathepsin D antibodies recognize all forms, making prognostic evaluation difficult to interpret.

Recently, a new possibility to use the current knowledge of procathepsin D-cancer association appeared using anti-pCD autoantibodies as a promising marker[14,15]. Research performed in our laboratory has demonstrated the presence of anti-pCD autoantibodies[16]. As these antibodies are specific to pCD and do not recognize mature CD[17], they represent a fine target for comparison of the pCD secretion with cancer progression. It is possible that the level of anti-pCD autoantibodies correlates with the stage of several solid tumors, thus offering development of a non-invasive screening test. We prepared an enzyme-linked immunosorbent assay for evaluation of the presence of anti-pCD antibodies using a specifically modified synthetic activation peptide as an antigen assay. Employing multiple antigen peptide, we were able to measure the level of anti-pCD autoantibodies in patient serum[5,18].

We hypothesize that the amount of the pCD in the patient's serum will change with the progress of the cancer disease, thus corresponding with the increased number of pCD-releasing cancer cells. This hypothesis configures well with our preliminary findings on breast cancer and clearly shows higher levels of antibodies in more advanced stages. These preliminary data define the high clinical potential of this assay.

Different approach was suggested by Luo *et al*[15]. This group separated proteins from a lung adenocarcinoma cell line and then immunoblotted them with serum samples from patients diagnosed with lung cancer. When compared with autoantibody profiles from three years prior to the appearance of cancer, several immunoreactive spots were found to be cathepsin D. Detailed studies showed that the majority of patients had a most intense reaction against pCD.

Although both studies are somewhat preliminary and used only a limited number of patients, these data strongly suggest that the search for correlation between pCD secretion and cancer development or cancer detection promises to find a clinically relevant possibility to diagnose and/or screen for cancers. In order to identify the optimal types of tumors and the best technique, it is first needed to analyze a larger number of samples.

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REVIEW

Development of animal models underlining mechanistic connections between prostate inflammation and cancer

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Abstract

The characterization of animal models has indicated that the genetic, dietary and environmental factors and hormonal imbalance may influence the risk to develop prostate inflammatory lesions and prostate cancer (PC) confirming human epidemiologic data. It is now established that the prostate inflammatory response typically results in major changes in the local microenvironment of epithelial cells of the prostate gland, including an intense stromal remodeling, activation of fibroblasts, infiltration of immune cells such as mast cells, macrophages and B and T lymphocytes and collagen deposition. The immune cells recruited at prostate inflammatory lesions and myofibroblasts may contribute to the release of numerous pro-inflammatory cytokines and chemokines that in turn can promote the oxidative stress, genomic instability and proliferation of epithelial cells. The accumulation of additional genetic and/or epigenetic alterations in prostatic stem/progenitor cells may subsequently culminate to their malignant transformation and PC initiation and progression and more particularly with advancing age. The potential mechanistic relationships between the molecular events associated with the persistent inflammatory response and prostate carcinogenesis have important implications for optimizing the current therapies against different prostatic disorders and PCs.

Key words: Animal models; Prostate inflammation; Tumor microenvironment; Stromal remodeling; Prostate cancer; Therapies

Mimeault M, Batra SK. Development of animal models underlining mechanistic connections between prostate inflammation and cancer. *World J Clin Oncol* 2013; 4(1): 4-13 Available from: URL: http://www.wjgnet.com/2218-4333/full/v4/i1/4.htm
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INTRODUCTION

Prostate cancer (PC) is the most common malignancy and the second leading cause of cancer-related deaths in men in the United States[1-5]. Significant improvement of screening tests had led to a more effective therapeutic intervention for patients diagnosed with localized PCs[1,2,5-9]. Although this advance, the progression of organ-confined PCs to the locally advanced or metastatic castration-resistant PCs (CRPCs), which are resistant to conventional treatments by anti-hormonal therapy, radiotherapy and first-line systemic docetaxel-based chemotherapies, typically culminates in the death of patients after about 12 to 19 mo[1-6,8,10]. The molecular events responsible for PC initiation and progression to metastatic CRPCs, treatment resistance and disease relapse remain poorly understood. Consequently, the establishment of deregulated gene products in PC cells and the changes in their local tumor microenvironment that play critical functions for the prostate carcinogenesis, metastases, treatment resis-

tance and disease recurrence is of major importance in developing novel molecular biomarkers and therapeutic targets.

PC is a complex, heterogeneous and multifactorial disease (Figure 1). In this regard, the epidemiologic data have indicated substantial geographic and racial disparity in the PC incidence and mortality, including a higher occurrence of aggressive PCs in black American men as compared to white American and Asian men[11,12]. This suggests that the genetic and environmental factors, including the diet and behavior, may influence the PC development[12,13]. Importantly, a growing body of evidence has also revealed that the accumulation of genetic and epigenetic alterations in prostate stem/progenitor cells and their differentiated progenies concomitant with the changes in their local microenvironment, including in reactive stromal cells, may occur during severe injury, inflammation, oxidative stress and aging of the prostate gland and lead to PC development (Figures 1 and 2)[6,7,14-18]. Moreover, it has been shown that PC stem/progenitor cells and their differentiated progenies can acquire more malignant phenotypes during epithelial-mesenchymal (EMT) program and PC progression to locally invasive and metastatic CRPCs[6,17]. In this matter, we review some investigations that have been carried out with animal models in last years to establish the genetic and environmental changes that may contribute to the development of human proinflammatory lesions and their potential relationship with the prostate carcinogenesis and progression.

ANIMAL MODELS OF PROSTATE INFLAMMATION AND PC

The characterization of phenotypic features of different animal models of prostate inflammation and PCs has indicated that the genetic background of animal strains, dietary and environmental factors, such as carcinogenic substances, high-fat diet and cholesterol as well as advancing age and hormonal imbalance may influence the incidence and progression of inflammatory lesions and PCs as suggested by human epidemiologic data (Figure 1)[19-27]. For instance, it has been observed that 72% of Lewis rat developed spontaneous prostatitis with advancing age compared to only 27% for Wistar rats while the administration of 17β-estradiol or castration promoted the incidence and severity of non-bacterial prostatitis to 100% in old adult Wistar rats[20]. The up-regulation of the systemic endogenous estrogens, including serum 17β-estradiol, concomitant with a decrease in the serum testosterone level by overexpressing aromatase (AROM) in FVB/N mice has also been observed to induce a chronic inflammation at 48 wk of age[28]. The inflammatory responses in *AROM*$^{+/+}$ mice was also associated with an enhanced number of mast cells, macrophages, neutrophiles and T-lymphocytes and culminated to the formation of prostatic intraepithelial neoplasias (PINs) at 52 wk of age[28]. In this regard, a treatment of immortalized, non-transformed and androgen-responsive rat NRP-152 prostatic epithelial cell line with 17β-estradiol at concentrations 1-3 μmol/L for a period of 2-6 wk has also been observed to induce their capacity of forming colonies in soft agar and tumors in immunodeficient nude mice[29]. The oncogenic effect of 17β-estradiol on NRP-152 cells was accompanied by an increase of expression levels of estrogen receptor-α (ER-α) and PC stem cell-like markers (integrins $\alpha_2\beta_1$, CD44, CD133, ABCG2 and CXCR4) but a decrease of ER-β and androgen receptor (AR) expression levels[29]. Moreover, it has been reported that the androgen replacement therapy with 4-dihydrotestosterone (4-DHT) or testosterone may prevent the 17β-estradiol-induced inflammatory reaction and proliferative epithelial response in the rat prostate of castrated Noble rats in a dose-dependent manner[30]. Altogether, these data suggest that the hormone imbalance, including the decrease of serum testosterone level in men with advancing age, which may promote the development of different prostate disorders, inflammation and pre-neoplastic lesions could be attenuated by a treatment aiming to increase the androgen-to-17β-estradiol ratio in serum.

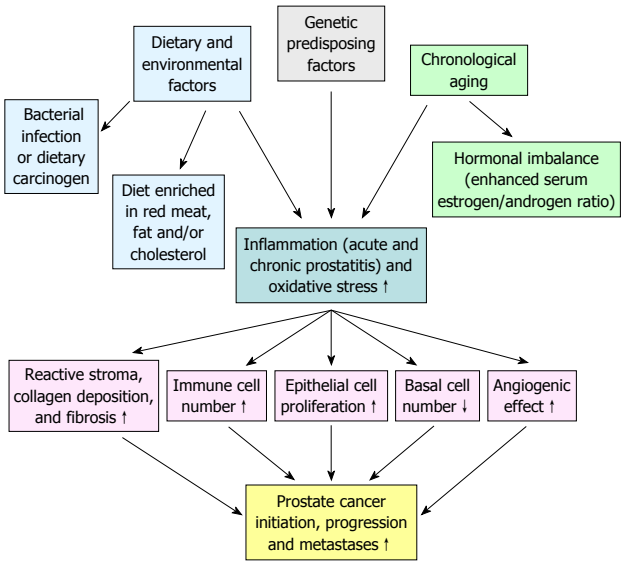

Figure 1 Potential risk factors predisposing to the development of prostate inflammation and their promoting effects on prostate cancer initiation and progression.

In addition, a non-bacterial mouse model of acute prostatitis has also been developed which consists to induce the inflammation in the anterior, dorsolateral and ventral prostate in prostate ovalbumin expressing transgenic mice (POEAT-3) or POEAT-3/Luc/tensin deleted on chromosome 10 (*PTEN*$^{-/+}$) mice by an adoptive transfer of ovalbumin-specific CD8$^+$ T cells[31]. The acute prostatitis in these mice was characterized by the leukocyte infiltration, enhanced levels of pro-inflammatory cytokines and chemokines, marked epithelial cell proliferation, activated stromal cells and increase of collagen deposition that was maintained for up to 80 d after the adoptive transfer of CD8$^+$ T cells[31]. Hence, future investigations by crossing these *POEAT-3* mice with transgenic mouse models of PCs should help to shed the light

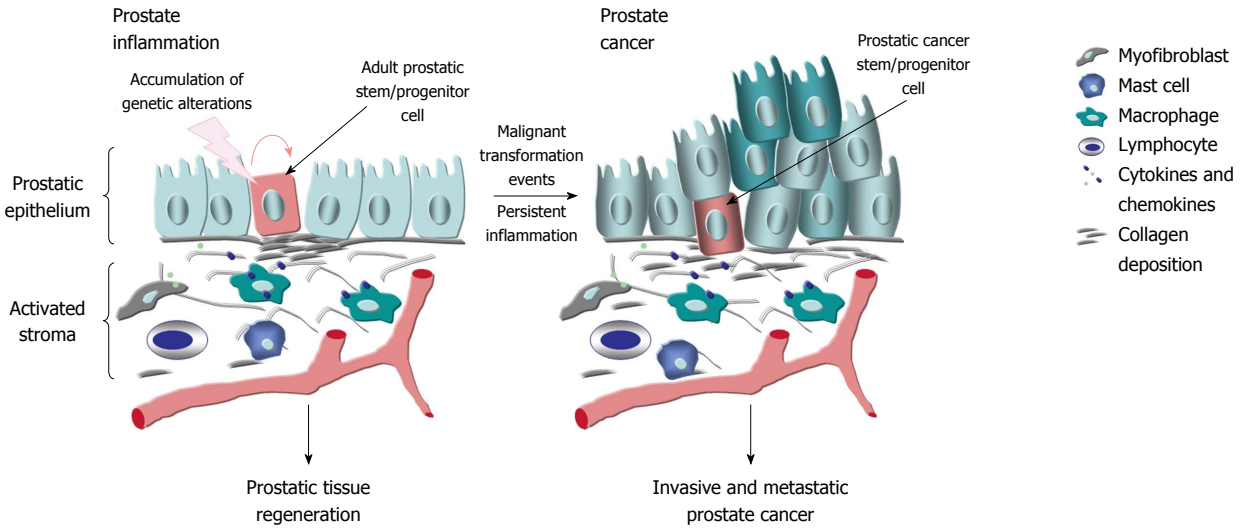

Figure 2 Potential model of the interconnections between the persistent activation of prostate inflammatory response and prostate cancer etiopathogenesis and progression. The inflammatory response which involves the changes in the reactive stroma, including the differentiation of fibroblasts into myofibroblasts and enhanced number of immune cells such mast cells, macrophages and T and B lymphocytes that can release different proinflammatory cytokines and chemokines is illustrated. The transient induction of inflammation may promote the repair of the damaged prostatic tissue under homeostatic conditions. The persistent activation of inflammatory response combined with the accumulation of genetic alterations in prostatic stem/progenitor cells and their progenies which can contribute to the induction of foci of proliferative glandular epithelium, angiogenesis and prostate cancer development and progression are also indicated.

on the molecular events involved in the prostatitis and their potential implications in PC development. In this regard, it has been reported that the induction of chronic bacterial prostatitis in C3H/HeOuJ mice by performing an infection with *Escherichia coli* (*E. coli*) bacteria led to intense inflammatory infiltrates in the stroma, genotoxic stress and focal atypical hyperplasia in prostatic epithelium[32]. These molecular events were associated with a loss of the expression levels of AR, glutathione-S-transferase, $p27^{Kip1}$ and PTEN tumor suppressor proteins as compared to control mice[32]. In the same way, the induction of bacterial prostatitis in C3H/HeOuJ mice by intraurethral inoculation of *E. coli* has also been associated with a marked decrease of the expression level of Nkx3.1 tumor suppressor protein in infected prostate lobes and development of chronic inflammatory response within 14 d postinoculation[33]. The down-regulation of Nkx3.1 also correlated with an increased expression of a proliferation marker, reduction of AR level and a marked increase in the basal cell marker p63[33]. Hence, the decrease expression of key tumor suppressor products, including $p27^{Kip1}$, PTEN and Nkx3.1 in these animal models of bacterial prostatitis that are frequently down-regulated during PC development provide a potential link between the persistence of prostate inflammation and carcinogenesis.

In addition, a treatment with a dietary charred meat carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) of humanized mice engineered for expressing cytochrome P450 1A enzyme (CYP1A) also induced prostate inflammation and development of atrophy of acini, low-grade PINs and high-grade PINs after 30-50 wk in the prostate gland of these rodents[22]. The high-grade PINs observed in these CYP1A humanized mice treated with PhIP expressed AR but exhibited a loss of expression levels of basal cell marker p63, PTEN and E-cadherin[22]. Importantly, it has also been observed that the administration of high-fat stress diet to PhIP-treated CYP1A humanized mice promoted prostate carcinogenesis and formation of carcinoma *in situ*[22]. In the same pathway, the treatment of male Fischer rats with mutagenic heterocyclic amine PhIP in the diet for 20 wk also induced inflammation, post-inflammatory proliferative or glandular atrophy and PIN lesions in the ventral prostate that culminated to invasive carcinomas after a subsequent treatment of rats with testosterone propionate[23,34]. Hence, these *in vivo* data suggest a potential relationship between the induction of a persistent prostate inflammatory response and PC development.

Transgenic mouse and rat models of prostate inflammation and PC

Different transgenic mouse and rat models of prostate inflammation and PC have also been generated by performing prostate-specific gene alterations using probasin (PB) promoter constructs[7,35-48]. It has been shown that the down-regulation of NKX3.1, PTEN, p53 and/or $p27^{Kip1}$ tumor suppressor proteins and up-regulation of different oncogenic growth factor receptors and intracellular signaling elements such as Myc and Akt, and altered metabolism in prostate epithelial cells may cooperate to their malignant transformation, PC development, metastases and treatment resistance[7,35-49]. For instance, it has been observed that the *PTEN* knockout transgenic mouse models mimic the continuum of genetic and histopathological changes that are frequently associated with human PC initiation, progression and metastases and development of androgen-independent (AI) PCs after castration[7,35-38,49]. More specifically, the characteriza-

tion of *PB-Cre4 PTEN*$^{-/-}$ transgenic mice with prostate-specific *PTEN* deletion has indicated that the enhanced pAkt in Sca^{+} prostatic stem/progenitor cells localized in the basal compartment of prostate led to their malignant transformation into PC stem/progenitor cells and culminated to the development of PINs that progressed to invasive PCs in 100% of transgenic mice at about 9-12 wk of the age[37,38]. The metastases or micrometastases have been detected in some *PB-Cre4 PTEN* null transgenic mice at 12-29 wk at lymph nodes and lungs as well as an intense bone remodeling activity[37]. Moreover, it has also been shown that the compound *PB-Cre4 PTEN*$^{-/-}$*;p53*$^{-/-}$, *PB-Cre4 PTEN*$^{-/-}$*;Smad4*$^{-/-}$ and *Nkx3.1*$^{CE2/+}$*;PTEN*$^{-/-}$*;Braf*$^{CA/+}$ transgenic mice developed the prostatic tumors that progressed more rapidly to invasive and metastatic states and which were invariable lethal as compared to *PTEN* knockout mice[39-41,49]. In the same way, the crossing of transgenic mice overexpressing constitutively activated human Akt-1 (MPAkt) and human MYC (Hi-Myc) in the prostate also resulted in MPAkt/Hi-Myc bigenic mice that exhibited an accelerated progression of PIN lesions to microinvasive tumors as compared to littermate control mice[50]. A stromal remodeling and infiltration of macrophages and B- and T-lymphocytes resembling to the inflammation observed in human prostate tumors were also seen in MPAkt/Hi-Myc mice in the early stage during the development of PINs and persisted along the PC progression[50].

Among other largely investigated animal models of PC, the prostate-specific expression of the simian virus 40 (SV40) large antigen (Tag) in prostate epithelial cells under the control of PB promoter has also led to transgenic adenocarcinoma of mouse prostate (TRAMP) mice that developed epithelial hyperplasia, high-grade PIN lesions at about 8 wk that progressed to differentiated adenocarcinomas at about 18 wk of age[42,43]. A loss of E-cadherin was also observed during the progression of primary tumors to a less differentiated state in TRAMP mice and associated with distant metastases to the lymph nodes or lungs in 100% of transgenic mice at 28 wk of age[42]. It has also been shown that the castration in TRAMP mice at 12 wk of age led to a reduction of prostatic tumor burden while the progression to poorly differentiated tumors and metastases were not delayed in castrated TRAMP mice relative to non-castrated TRAMP mice[44]. Importantly, the treatment of TRAMP mice with a diet enriched in fat and cholesterol [Western-type diet containing 21.2% fat and 0.2% cholesterol (wt/wt)] accelerated the incidence, burden and histological grade of the prostate tumors, angiogenesis and lung metastases as compared to control mice fed with a regular diet (chow diet containing 4.5% fat and 0.002% cholesterol (wt/wt))[51]. The expression of SV40 Tag driving by rat PB promoter using Sprague Dawley or Lewis strain of rats has also led to the generation of transgenic animals designated as transgenic rat adenocarcinoma of the prostate (TRAP)[45,46]. TRAP displayed atypical epithelial cell proliferation in prostate at about 4 wk and formed well-differentiated adenocarcinomas with 100% incidence before 15 wk of age, respectively[45,46]. In contrast to TRAMP mice, the castration in TRAP rats at 5 or 20 wk of age completely prevented or induced a complete involution of androgen-dependent prostate tumors in the most of transgenic rats[45,46]. Of therapeutic interest, since the prostate-specific antigen and prostate acid phosphatase (PAP) are expressed in rat prostate as observed in human prostate but not in mouse prostate, TRAP rats may constitute a good animal model to test novel vaccine strategies targeting these antigens[46,52]. For instance, it has been observed that the immunization of Lewis TRAP rats with a DNA vaccine encoding PAP triggered an autologous PAP-specific T-cell responses in transgenic rats[46]. Nevertheless, the major disadvantage of these SV40 Tag transgenic mouse and rat models of PC is that they frequently give arise to tumors that are characterized by a high level of neuroendocrine differentiation and which are observed only in a small subset of PC patients limiting thereby their clinical relevance.

Recent studies have also revealed that enhanced levels of transforming growth factor-β1 (TGF-β1) and TGF-β3, macrophage-inhibitory cytokine-1/growth differentiation factor-15 (MIC-1/GDF-15), interleukin-8 (IL-8)/CXCL8 and its receptors CXC chemokine receptors (CXCR1 and CXCR2), toll-like receptor 4, IL-17 and nuclear factor-κB (NF-κB) may play critical functions for the development of prostate inflammatory lesions, PC progression and metastases[53-60]. These inflammation-associated factors may contribute to the prostate stromal remodeling, fibrosis, host immune cell modulation and induction of the EMT process in PC cells and angiogenic switch under normoxic and hypoxic conditions, which in turn may promote the invasion and metastatic spread of PC cells at distant sites including bones (Figure 2)[55-57]. For instance, transgenic mice engineered for overexpressing epitope tagged TGF-β1 in prostate epithelial cells developed severe focal attenuation of epithelium, discontinuous basal lamina, intense fibrosis, collagenous micronodules in collapsed acini concurrent with inflammation in nerve ganglia and small vessels in an age-dependent manner as observed during human PC development[53,61]. It has also been shown that the graft of a tissue recombinant prepared of immortalized and non-tumorigenic benign prostatic hyperplasia (BPH)-1 human prostatic epithelial cells plus human prostatic cancer-associated fibroblasts (CAFs) expressing high elevated levels of both TGF-β1 and stromal cell-derived factor-1 (SDF-1)/CXCL12 under the renal capsule of severe combined immune deficient (SCID) mice resulted in the development of fibrous stroma and rapidly growing and poorly differentiated prostate tumors[54]. The tumorigenic effects of TGF-β1 appear to be mediated in part through the up-regulation of CXCR4 expression in BPH-1 cells, stimulation of CXCR4 by SDF-1 released by CAFs and activation of Akt in BPH-1 cells[54]. In addition, it has also been noted that the expression of dominant negative TGF-βRII construct in BPH-1 cells or a treatment of mice with an antibody (2G7)

directed against TGF-β ligand significantly suppressed the tumor volume and invasion in this tissue recombinant xenograft model of PC[54]. The blockade of TGF-β1 signaling pathway by using TGF-β1 latency-associated peptide or neutralizing antibody also inhibited the angiogenesis and tumor formation by LNCaP PC cells xenografted in nude mice[62]. Importantly, systemic delivery of oncolytic adenovirus targeting TGF-βRII or a treatment with a selective TGF-βRI kinase inhibitor, LY2109761 was also effective at suppressing the growth of MDA PCa-2b or PC-3 cells in the bone of SCID mice as compared to the untreated mice[63,64]. In the same way, the overexpressing of MIC-1, a divergent member of the TGF-β superfamily, in PC cells has also been associated with an enhanced rate of metastases at distance sites, including bones, treatment resistance and poor outcome of PC patients[18,65-71]. Moreover, a significant increase of MIC-1 expression, at both the mRNA and mature protein level, has been detected in prostatic hyperplasias and PINs formed in LBT Tag 12T-7s transgenic mouse model, a modified SV40 early region driven by the prostate-specific rat PB promoter, and associated with a stimulation of prostatic epithelial cell proliferation[35,72]. These data suggest that an enhanced expression of mature MIC-1/GDF-15 form in prostate epithelial cells may constitute an early transforming event during prostate carcinogenesis. Although the development of locally invasive PCs was observed in this transgenic 12T-7s mouse model, no metastasis at distant organs has however been detected in these mice[72]. It has also been reported that the crossing of TRAMP mice with syngeneic mice overexpressing MIC-1 in myeloid cells under control of the myeloid cell specific *c-fms* promoter (MIC-1^{fms}) produced syngeneic TRAMP$^{fmsmic-1}$ mice that exhibited smaller prostate tumor size but marked increase of metastases at distant sites as compared to wild-type TRAMP mice[73]. The number of lung tumor colonies formed by TC1-T5 PC cell line derived from TRAMP mice intravenously injected in MIC-1^{fms} mice was also superior to the number of lung colonies detected in control C57BL/6 mice[73]. Similarly, the overexpression of MIC-1 in human and AI PC3 cells was also effective at promoting their metastases at distant sites in an orthotopic tumor model in mice[74,75]. Hence, in considering the fact that MIC-1 is typically overexpressed in the majority of PCs and can contribute to the metastases of PC cells and to their resistance to current docetaxel-based chemotherapeutic treatments, which are the major causes of the death of CRPC patients, it will be of interest to develop novel transgenic mice with prostate-specific MIC-1 expression[18,65-71].

On the other hand, it has also been reported that the prostate-specific expression of a constitutively activated IκB kinase 2 (IKK2) form, which can stimulate the pro-inflammatory factor NF-κB, was insufficient for inducing prostate tumorigenesis in transgenic mice[76]. However, the crossing of *PTEN*$^{+/-}$ mice with *IKK2* mice generated compound *PTEN*$^{+/-}$*;IKK2*$^{+/+}$ transgenic mice that exhibited all prostate lobes enlarged at 8 mo and older, formation of cribriform structures, and increase in fiber in the fibroblastic stroma associated with inflammation as compared to littermate PTEN$^{+/-}$ mice which had only some hyperplasia and PINs[76,77]. The malignant transformation of prostate epithelial cells in *PTEN*$^{+/-}$*;IKK2*$^{+/+}$ transgenic mice was also associated with a persistent inflammation as revealed by the infiltration of granulocytes and macrophages and up-regulated expression levels of pro-inflammatory chemokines (CXCL5, CXCL15, CCL3, CXCL10, and CXCL2) and cytokines [tumor necrosis factor (TNF) and IL-1b] in the prostate epithelium and stroma[76]. Moreover, it has also been observed that the *Vav3*$^{+/+}$ transgenic mice generated by overexpressing a constitutive active form of guanine nucleotide exchange factors for Rho family GTPases, Vav3 under the control of *ARR2-PB* promoter in the prostatic epithelium exhibited a marked activation of AR, NF-κB and phosphatidylinositol 3-kinase-Akt signaling elements[78]. These molecular events led to the development of nonbacterial chronic prostatitis in the prostate gland which was associated with the infiltration of monocytes, lymphocytes, and plasma cells as well as the formation of PIN lesions and invasive PCs at the age as early as 3 mo[78]. In addition, it has also been reported that fibroblast growth factor-8b (FGF-8b)$^{+/+}$ transgenic mice overexpressing FGF-8b in the prostate epithelium exhibited activated stroma containing increased proportion of fibroblastic cells, collagen deposition, and aggregates of inflammatory cells, including T cells, B cells and macrophages and intensive neoangiogenesis[79]. The intensive stromal changes and inflammation in *FGF-8b*$^{+/+}$ transgenic mice preceded the development of PIN lesions that culminated to the tumor formation with phenotypical features of adenocarcinoma and sarcoma[79]. These data suggest that the overexpression and secretion of FGF-8b by prostate epithelial cells can promote prostate carcinogenesis in part *via* the stromal activation and induction of an inflammatory response. On the other hand, several investigations have also revealed the major contribution of the activation of AR in the stromal cells and recruitment of bone marrow (BM)-derived cells in the induction of inflammatory response and PC progression.

Functions of AR in the modulation of the development of prostatic inflammatory lesions and PC

The sustained activation of AR expressed by epithelial and adjacent stromal fibromuscular cells in the prostate gland, which plays critical functions in the modulation of stromal-epithelial interactions for the maintaining of normal prostate homeostasis, also can promote the development of prostatic inflammatory lesions, including inflammation-associated BPH and PCs[80-84]. For instance, it has been observed using an *in vitro* cell co-culture system that immortalized and non-tumorigenic BPH-1 human prostate epithelial cells significantly increased the migration of THP-1 macrophages which, in turn, induced the EMT marker expression, such as N-cadherin, snail and TGF-β2, in BPH-1 cells as well as their sphere-forming ability[80]. Moreover, the exogenous expression of AR in BPH-1-AR also promoted the THP-1 cell migration and

enhanced EMT marker expression and sphere-forming capacity of BPH-1-AR cells relative to BPH-1-vector cells used as control[80]. Conversely, the BPH-1/THP-1 co-culture in the presence of an anti-TGF-β2 antibody or silencing of AR function in BHP-1-AR cells using AR degradation enhancer, ASC-J9, has also been observed to decrease the THP-1 macrophage migration and suppress the induction of EMT marker expression in BPH-1 cells[80]. Moreover, the results from *in vivo* tissue recombination studies have indicated that the combination of BPH-1 cells with human PC-associated fibroblasts from PC surgical specimens generated large tumors while no tumor was formed by BPH-1 cells in the presence of normal prostatic fibroblasts[84]. The data from investigations performed with tissue recombinants composed of mouse or rat urogenital sinus mesenchyme expressing ARs and ERs and BPH-1 cells showing undetectable levels of ARs and ERs grown under the kidney capsule of male athymic nude mice have also revealed that a treatment with 17β-estradiol plus testosterone induced only invasive PC development in the presence of functional mesenchymal AR[80-83]. It has also been noted that the tumore derived from rat UGM plus BPH-1 cells metastasized to lymph nodes, liver and lungs[81]. Additionally, the selective AR knockout in fibroblasts and smooth muscle cells in the *dARKO/PTEN*$^{+/-}$ mouse model of PC has also been observed to inhibit the prostate epithelial cell proliferation concomitant with a decrease of the development of low- and high-grade PIN lesions and low-grade PIN progression as compared to wild-type *AR/Pten*$^{+/-}$ mice[85]. The AR deletion in fibromuscular cells of *dARKO/PTEN*$^{+/-}$ mice was also accompanied by a reduction of the extracellular matrix remodelling, collagen deposition and number of infiltrating immune cells, including T cells, B cells and macrophages and neovasculature formation in the stromal compartment of prostate gland[85]. Moreover, it has also been shown that the AR activation by 4-DHT in prostate stromal cells isolated from *Pten*$^{+/-}$ mouse prostates up-regulated the expression of pro-inflammatory cytokines and chemokines such as macrophage inflammatory protein-1α (MIP-1α), MIP-1β, MIP-2 and IL-10[85]. These pro-inflammatory factors, in turn, induced an immune cell recruitment and inflammatory response that promoted the PIN development in *Pten*$^{+/-}$ mouse prostate[85]. Of therapeutic interest, the down-regulation of AR in stromal fibromuscular cells and prostate epithelial cells with the AR degradation enhancer, ASC-J9, has also been observed to be effective at reducing the stromal remodeling and PIN development and progression in *Pten*$^{+/-}$ mice[85]. Hence, together these data supports the benefit to target AR in stromal and epithelial cells of the prostate to suppress the inflammatory response and prevent PC development.

Implications of BM-derived adult stem/progenitor cells in the development of prostatic inflammatory lesions and PCs

Several investigations have revealed that circulating BM-derived adult stem/progenitor cells, including hematopoietic stem/progenitor cells, mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs) and/or myeloid cells may be recruited at prostatic inflammatory lesions and contribute to the PC development and metastases[86-93]. In fact, although BM-derived adult stem/progenitor cells appear to play only minimal roles in prostate epithelial regeneration after severe prostate inflammation and glandular disruption *via* cell fusion with prostate epithelial cells or transdifferentiation into prostate epithelial-like cells, they can induce immunosuppressive and angiogenic effects that promote prostate carcinogenesis[86-93]. More specifically, the release of soluble pro-inflammatory chemokines and cytokines acting as chemoattractant factors, such as SDF-1 (CXCL12), chemokine (C-C motif) ligand 5 (CCL5, RANTES) and monocyte chemotactic protein-1 (CCL2/MCP-1) by prostate epithelial cells, activated stromal cells and/or immune cells may recruit BM-derived adult stem/progenitor cells expressing their cognate receptors at injured prostate site and PC[88-90]. More specifically, it has been observed that PC-derived stromal cells, which express fibroblast activation protein-α, CD90-, CD73- and CD105 but undetectable levels of CD14-, CD20-, CD34-, CD45- and human leukocyte antigen DR exhibited morphological and phenotypic features comparable to BM-derived MSCs[94]. It has also been noted that PC-derived stromal cells represented about 0.01%-1.1% of the total cells present in core biopsies from primary human PC specimens[94]. Moreover, the stimulation of murine RM-1 PC cells by inflammatory cytokines, such as interferon-γ and tumor necrosis factor-α has been shown to be accompanied by the production of platelet-derived growth factor-BB that in turn promoted the proliferation of MSCs *in vivo* and *in vitro*[88]. The exogenous and endogenous MSCs recruited into the tumor microenvironment was also able to promote the tumor growth of RM-1 cells subcutaneously implanted in mice[88]. These data suggest the potential implication of the recruitment of MSCs in prostate inflammatory microenvironment induced *via* the proinflammatory mediators in the induction of immunosuppressive effects that can allow PC cells to escape the immune surveillance and favor PC development.

In addition, it has been observed that CXCR4^{+}/sca-1^{+}, vascular endothelial growth factor receptor-2 (VEGFR-2^{+})/CD34^{+} and VEGFR-2^{+}/CD117^{+} BM-derived cell subpopulations were increased in the peripheral blood of SCID mice bearing PC cell xenografts and contributed to the tumor growth by promoting neoangiogenesis[91-93]. It has also been noticed that a treatment of tumor-bearing mice for 5 d with doxorubicin or daunorubicin was effective at reducing the tumor vascularization at least in part by inhibiting the recruitment of BM-derived cells at tumor *via* the inhibition of hypoxia-inducible factor-α[92]. Moreover, the data from BM transplantation/reconstitution and genetic lineage-tracing experiments have also revealed that BM-derived myelomonocytic cells can transform into lymphatic endothelial cells and integrated

into PC-associated lymphatic vessels in the TRAMP-C1 cell transplantation model and thereby contribute to lymphangiogenesis[93].

Of therapeutic interest, it has also been shown that parental or genetically-engineered MSCs and EPCs, which show an innate tropism for damaged epithelial tissues, including PCs may be exploited as vehicles for targeted-delivery of anti-inflammatory, cytotoxic and/or anti-angiogenic agents at injured prostatic sites. For instance, it has been observed that MSCs engineered for expressing secreted frizzled related protein-2 suppressed the tumor growth and increased apoptosis and necrosis within tumors formed by C4-2B human CRPC cells orthotopically implanted into the prostates of castrated host SCID mice[89].

CONCLUSION

The generation of different animal models of PC has led to a better understanding of the roles of specific genetic and environmental factors that may contribute to trigger molecular events occurring in the prostate epithelium and stroma during the pathogenesis of prostate inflammatory lesions and PCs. Future investigations to develop novel compound transgenic mouse and rat models of PC and metastases with prostate-specific gene alterations relevant to the molecular events that frequently occur during PC etiology and progression, metastases at distant sites including bones and treatment resistance is of great interest. More particularly, additional studies are necessary to further establish the molecular mechanisms by which the dietary factors, prostate inflammatory process and chronological aging of prostatic stem/progenitor cells and their differentiated progenies may lead to the damages to epithelium and reactive stroma, and thereby promote prostate carcinogenesis. It will be important to establish the potential promoting effect of crossing POEAT-3 mice or transgenic mice engineered for overexpressing TGF-β1 or MIC-1 in prostate epithelial cells with *PB-Cre4 PTEN*$^{-/-}$ or compound *PB-Cre4 PTEN*$^{-/-}$;*p53*$^{-/-}$ transgenic mice on inflammatory response and PC etiology, progression and metastases. The determination of anti-carcinogenic effects induced by different anti-inflammatory drugs, antioxidants and immune-based vaccines, alone or in combination with current anti-hormonal and chemotherapeutic treatments on transgenic mouse and rat models of PC is also important to develop novel effective combination therapies for treating PC patients diagnosed at early and late stages of the disease.

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REVIEW

Ovarian cancer and DNA repair: DNA ligase IV as a potential key

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Abstract

Ovarian cancer (OC) is the sixth most common cancer and the seventh cause of death from cancer in women. The etiology and the ovarian carcinogenesis still need clarification although ovulation may be determinant due to its carcinogenic role in ovarian surface epithelium. The link between ovarian carcinogenesis and DNA repair is well established and it became clear that alterations in DNA damage response may affect the risk to develop OC. Polymorphisms are variations in the DNA sequence that exist in normal individuals of a population and are capable to change, among other mechanisms, the balance between DNA damage and cellular response. Consequently, genetic variability of the host has a great role in the development, progression and consequent prognosis of the oncologic patient as well as in treatment response. Standard treatment for OC patients is based on cytoreductive surgery, followed by chemotherapy with a platinum agent and a taxane. Although 80% of the patients respond to the first-line therapy, the development of resistance is common although the mechanisms underlying therapy failure remain mostly unknown. Because of their role in oncology, enzymes involved in the DNA repair pathways, like DNA Ligase IV (LIG4), became attractive study targets. It has been reported that variations in LIG4 activity can lead to a hyper-sensitivity to DNA damage, deregulation of repair and apoptosis mechanisms, affecting the susceptibility to cancer development and therapy response. To overcome resistance mechanisms, several investigations have been made and the strategy to target crucial molecular pathways, such as DNA repair, became one of the important areas in clinical oncology. This review aims to elucidate the link between DNA repair and OC, namely which concerns the role of LIG4 enzyme, and how genetic polymorphisms in *LIG4* gene can modulate the activity of the enzyme and affect the ovarian carcinogenesis and treatment response. Moreover, we try to understand how LIG4 inhibition can be a potential contributor for the development of new cancer treatment strategies.

Key words: Ovarian Cancer; DNA Repair; DNA ligase IV; Polymorphisms; Susceptibility; Treatment response

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INTRODUCTION

Ovarian cancer (OC) is the third most common gynecological cancer among women worldwide, with an estimate 225 000 new cases and 140 000 deaths due to this disease each year[1]. In Europe, OC is the fifth most incident cancer in women but the main cause of death among the gynecological tumors[2].

OC presents itself as a high heterogeneous disease, which may develop from three different cell types: epithelial cells, sex cord-stromal cells or germ cells. In spite of the fact that the ovarian epithelial layer only represents a small percentage of all ovarian cell types, epithelial ovarian carcinoma (EOC) comprises 85% to 90% of all malignant ovarian tumors in adult women[3-5].

One of the most interesting aspects about EOC is the fact that while the transformation processes occur, the ovarian epithelium becomes more differentiated with the capability to undergo metaplasia into Müllerian epithelium. This aberrant transformation occurs in the majority of EOC and allows its histological classification into four main sub-types: serous, mucinous, endometrioid and clear cell tumors according to their histological and secretory resemblance with fallopian tube, endometrium, endocervix or vagina, respectively[6-8].

EOC incidence is age related and is a characteristic of postmenopausal women. The majority of cases happen in women after age 40, with a median age of diagnosis of 63 years[4,9]. By the lack of adequate experimental models to the study of this neoplasm, the etiology and the ovarian carcinogenesis still need clarification although some reproductive and hormonal events can be determinant. In this way, there have been made some possible hypothesis, which based on epidemiological and biological observations, pretend to explain susceptibility to OC[8] (Table 1). However, one of the more important risk factors established to OC is family history. About 5% to 10% of all OC are attributed to inherited mutations in high penetrance genes associated with hereditary breast and OC, as in *BRCA1* (3%-6%) and *BRCA2* (1%-3%), and with Lynch Syndrome, generally attributed to *MLH1* and *MSH2* (1%-2%) gene mutations[10].

EOCs are themselves a heterogeneous group of tumors. Shih *et al*[11] and Kurman *et al*[12] proposed an EOC sub-classification according to its clinic behavior, tumor progression, morphologic and genetic features. In this way, EOCs are divided in two main groups named Type I (25% of all cases), considered as lower grade, and Type II (75% of all cases), considered as higher grade. Type I tumors include all major sub-types but exhibit low-grade nuclear and architectural features, slow growth and can be linked to well-defined benign ovarian precursor lesions. These tumors are characterized by a relative genetic stability, although mutations in *KRAS*, *BRAF*, *PTEN* and *β-Catenin* genes are common, and frequently associated with a worse therapy response. On the other hand, Type II ovarian tumors are infrequently associated with benign or borderline ovarian precursor lesions, arising in an aggressive and spontaneous manner, being usually sensitive to chemotherapy. They are comprised almost exclusively of high-grade serous carcinomas (90%) but also include two less common subtypes (mixed epithelial and undifferentiated carcinomas) and those associated with *BRCA1* and *BRCA2* hereditary tumors. Type II ovarian tumors are characterized by genetic instability being usual mutations in *TP53* gene (50%-80%) and also amplification and overexpression of *HER2/neu* (10%-20%) and *AKT2* (12%-18%) oncogenes[11,12]. This sub-classification of EOC can be the result of two divergent pathways in ovarian carcinogenesis although more studies need to be done to confirm this suggestion[13].

As mentioned above, OC is considered the most lethal gynecological cancer[2]. This high mortality is due, essentially, to late diagnosis since, in 75% of OC cases, it is only made in an advanced disease stage, when the tumor is no longer confined to ovary[14]. In spite of diagnosis and prevention strategies improvement, some barriers to early OC detection exist and are due to its low incidence, hidden location of ovaries, not existence of well defined pre-invasive lesions and for being usually asymptomatic[15].

Despite the high degree of phenotypic and genotypic variability between the sub-types of EOC, all patients are treated identically upon diagnosis[13]. Standard treatment for OC patients is based on cytoreductive surgery, followed by chemotherapy with a platinum agent (carboplatin or cisplatin) and a taxane (paclitaxel or docetaxel). Although 80% of the patients respond to the first-line therapy, the development of resistance is common and several patients eventually recur with a 5-year survival rate only around 45%[16-18].

Over the past several decades, great advances have been made in surgical techniques and chemotherapy regimens used to treat OC. However, despite the best achievements between clinic and research, these strategies have not yet been shown to have an impact on overall mortality from advanced-stage disease, which 5-year survival rate has improved only 8% in the last 30 years and remain mostly unknown the mechanisms underlying therapy failure[16,18].

Efforts to improve long-term results of first-line therapy through addition of a third cytotoxic agent have not been successful. An improvement in the understanding of OC biology has led to the identification of molecular targets and biological agents that interfere with DNA repair, growth factors, membrane-bound receptors and tumor-associated angiogenesis[18-20]. Emerging data regarding inhibition of vascular endothelial growth factor (VEGF)-mediated angiogenesis and inhibition of poly[adenosine diphosphate (ADP)-ribose] polymerase (PARP)-mediated DNA repair are promising[18,21,22].

OC AND DNA REPAIR

The traditional view of OC asserts that the majority of OC share a common origin within ovarian surface epithe-

Table 1 Hypothesis to epithelial ovarian cancer development

Hypothesis	Biological mechanism proposed	Epidemiological evidence
Incessant ovulation[24]	Repetitive ovulation and quickly cellular proliferation in post-ovulation repair creates a propitious environment to carcinogenesis initiation by genetic alteration accumulation as well as inclusion cysts development Ovulation inhibition results in gonadotropin and oxidative stress levels reduction, deceleration of ovarian follicle depletion and to a diminished inclusion cysts development in ovarian epithelium	Events that suppress ovulation such as pregnancy, lactation and oral contraceptive use are protective factors
Gonadotropins[91]	Excessive stimulation of ovarian epithelium by FSH and LH conducts to downstream genes activation as well as to stimulation of hormonal production by the ovary (as estrogen) in order to enhance cellular proliferation and consequently to malignant transformation and angiogenesis The formation of a protective progestagenic hormonal milieu can stimulate apoptosis in genetically damaged ovarian epithelial cells, preventing tumor development	Oral contraceptive use and pregnancy are protective. Hyper-gonadotropic conditions are common in infertile, in polycystic ovarian syndrome and in post-menopausal women
Hormonal stimulation[92]	High androgen levels are harmful while an increase in progesterone levels is benefic	Protective effect due to multiparity and oral contraceptive use. Harmful effect is associated with higher androgen levels as in polycystic ovarian syndrome women
Inflammation[25]	Ovulation is accomplished by an inflammatory response: redox potential alteration, cellular infiltration, cytokine release that can introduce DNA damage in epithelial cells involved in ovary rupture/repair	Inflammatory gynecological diseases, as endometriosis, can enhance EOC risk. Non-steroid anti-inflammatory drugs can be a protective factor

FSH: Follicle-stimulating hormone; LH: Luteinizing hormone; EOC: Epithelial ovarian cancer.

lium (OSE). OSE is a monolayer of uncommitted mesothelial cells that cover the exterior surface of the ovary[7]. During the monthly ovulation, the OSE is enzymatically degraded in order to allow the follicular rupture and oocyte release, creating a breach that must be posteriorly repaired (Figure 1)[7,23,24].

Over the course of a woman's reproductive life, this process of damage and repair is repeated multiple times and may result in a stepwise accumulation of genomic alterations, as postulated by the incessant ovulation hypothesis (Table 1)[24]. In addition to physical trauma, OSE cells are subjected to ovulation-associated inflammatory cytokines, reactive oxygen species (ROS), and hormones (and its reactive metabolites) that are capable to damage DNA and conduct to a hormonal metabolism imbalance[3,19,23,25].

Ovarian epithelial inclusion cysts may develop as an ovulation result or due to ageing, becoming entrapped within the stroma (Figure 1). Once inside the ovary, epithelial cells lining the inclusion cysts are exposed to an environment of aberrant autocrine/paracrine stimulation by growth factors including hormones, phospholipids and VEGF[7,19,26]. If the epithelial cells harbor unrepaired DNA damage, they may be prime targets for neoplastic transformation[13].

As the link between DNA damage and ovarian carcinogenesis becomes stronger, it will become more important to completely understand the role of DNA damage response (DDR) proteins in OC prevention. Because defects in DNA repair genes involved in double-strand breaks (DSBs) repair, such as *BRCA1* and *BRCA2*, are implicated in familiar OC, overall DNA repair capacity may have an effect on the risk of sporadic OC as well[27].

With the human genome sequencing, the identification of genetic variations and the understanding of how common variations can affect normal cellular processes has become possible[28]. Genetic polymorphisms are naturally occurring sequence variations, about 90% of which are single nucleotide polymorphism (SNP)[29]. SNPs are single base pair positions in genomic DNA at which different alleles exist in normal individuals in some population(s), wherein the least frequent allele has an abundance of 1% or greater[30]. SNPs occur every 100-300 bases along the human genome and several studies suggest that the risk to many complex diseases, like cancer, can be extensively affected by the individual's SNP profile. Presumably, it will be the combination between the SNP profile and environmental factors that contribute to sporadic cancer development[30-32]. Genetic polymorphisms in DNA repair genes seem to determine the overall DNA repair capacity, which in turn may affect the risk of OC[15].

DDR pathways induce cell cycle arrest in response to DNA damage, in order to maintain genomic stability. In this way, these mechanisms are known to act as tumor suppressors and proteins involved in repair pathways are considered as genome caretakers. DDR pathways are controlled by specific sets of genes and although they are considered as good players in the cancer prevention, they can act as bad players in the treatment response[27,33]. The recognition of DNA damage and the consequent repair mechanism are crucial to the sensibility or resistance of cancer cells to treatment. This means that cells with proper DDR pathways are capable to efficiently repair the damage caused by chemo or radiotherapy, being responsible for the development of resistance in tumor cells[34].

Pharmacogenetics and pharmacogenomics are emerging areas that are essential to the development of personalized medicine, ultimately leading to drug prescription based on patient's individual genetic and molecular pro-

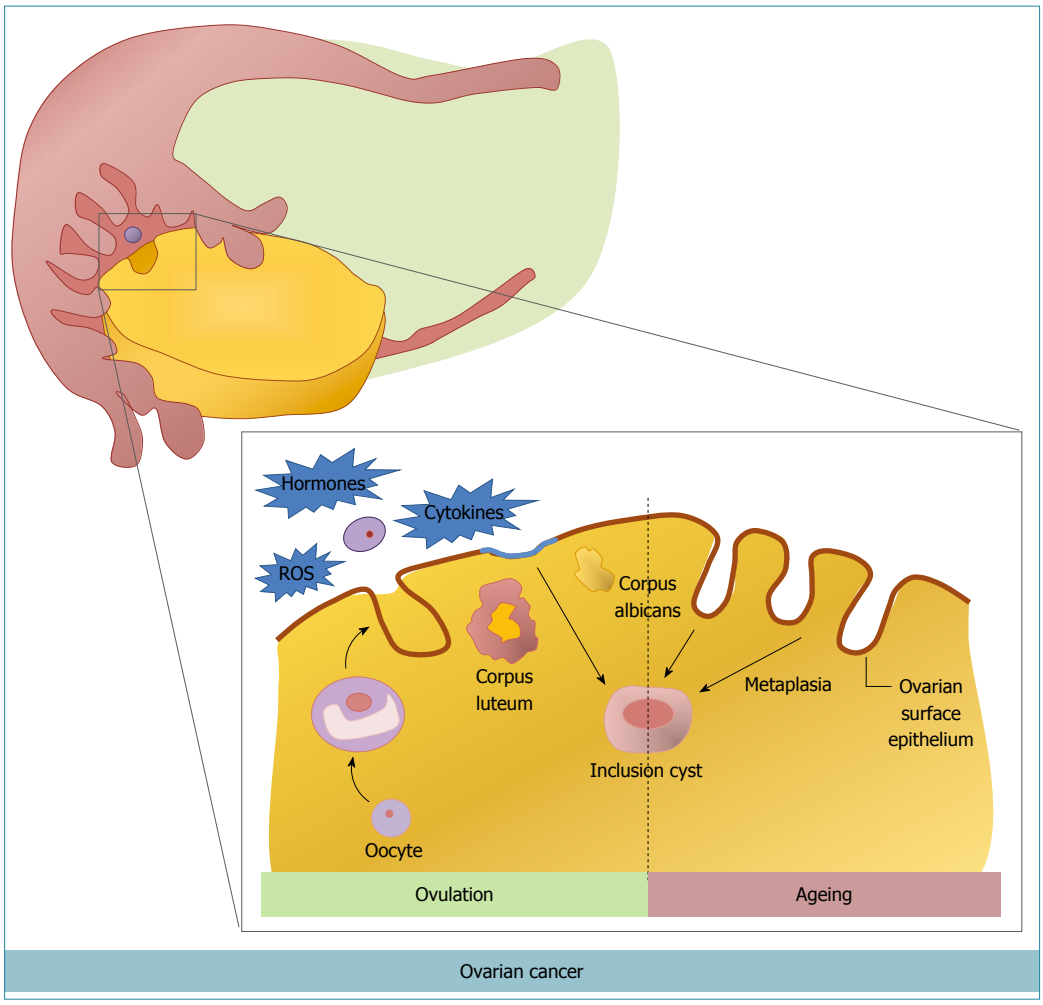

Figure 1 Link between ovulation/ageing and ovarian cancer development. Adapted from Levanon *et al*[3]. ROS: Reactive oxygen species.

file[30-32,35]. The aim of these areas is to establish a relationship between the genotype (*i.e.*, polymorphisms or mutations), gene expression profile and phenotype (both in drugs' pharmacokinetic and pharmacodynamics), interpreted as the variability between individuals concerning the toxicity, effectiveness and therapy outcome[35-39]. Polymorphisms in genes involved in DNA repair could result in variations in efficacy and accuracy of DNA repair enzymes and consequently significantly affect the toxicity, effectiveness and therapy outcome. By their role in therapy response, genetic polymorphisms in these genes can influence the patient's survival and be useful as prognostic and predictive markers in cancer[40-43].

Moreover, with the development of DDR protein inhibitors for cancer treatment, research on targeting molecular pathways, such as DNA repair, is becoming one of the most important areas in clinical oncology[43-48]. One of the enzymes involved in DDR is DNA Ligase IV (LIG4) enzyme, which is essential to catalyze the DNA phosphodiester bond formation, in the last step of one of the DNA repair mechanisms[49]. In this review, we explore the role of LIG4 in DDR, namely in OC carcinogenesis and treatment, as well as in the potential contribution to the development of new target therapies.

DDR

Along the cell cycle and during the lifetime of a cell, the genome is continuously exposed to a wide variety of agents and processes capable to damage the DNA[50]. Therefore, genetic stability is necessary and is maintained not only by precise replication mechanisms but also by accurate and redundant systems that detect and repair possible DNA lesions. Most of DNA injuries are transitory because after its recognition, a coordinated cellular response takes place in order to interrupt the cell cycle (allowing the repair) or to lead to cell death (if the damage is too serious), maintaining genomic stability[46,51].

There are several DNA repair mechanisms that use different enzymes to repair different kinds of damages[34]. One of the most deleterious DNA lesions is DNA DSBs. DSBs occur when the phosphodiester backbones of both strands are simultaneously broken and close enough to disrupt base pairing, whereas chromatin structure can not keep the ends juxtaposed[50,52]. The result is the release of two DNA ends that can get physically separated from each other, embarrassing the subsequent repair and providing an opportunity to inappropriate recombination[50]. These breaks can arise in all phases of the cell cycle from

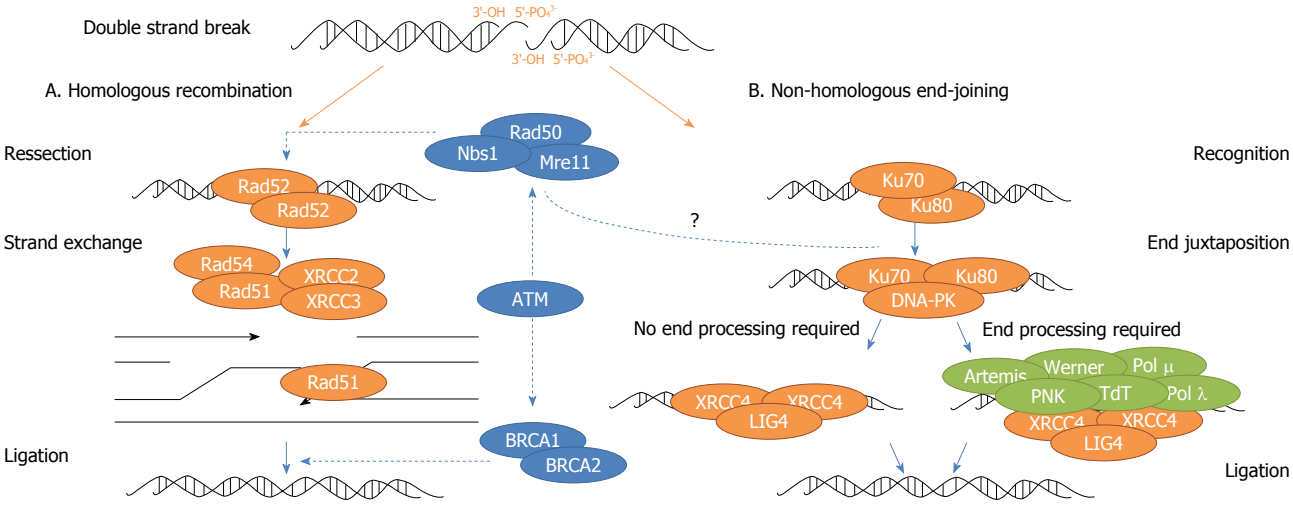

Figure 2 Simplified overview of homologous recombination and non-homologous end-joining. Homologous recombination (HR) pathway starts with break recognition and signaling by a complex containing NBS1, MRE11 and RAD50 (MRE11/RAD50/NBS1 - MRN complex). RAD51 and RAD52 catalyze and facilitate a strand exchange reaction. Assembly of RAD51 is facilitated by different RAD51 paralogs such XRCC2 and XRCC3. MRN complex also promotes activation of ATM, which in turn activates several DNA repair factors as BRCA1/2. HR finishes with DNA synthesis and final ligation. Non-homologous end-joining (NHEJ) pathway starts with the recruitment of Ku heterodimer (Ku70 and Ku80) to DNA ends. Once attached to double-strand breaks, Ku recruits and stimulates the DNA-PKcs, forming the DNA-PK holoenzyme. DNA-PK activates XRCC4-LIG4 complex, which links the broken complementary DNA ends together. If DNA ends are not ready to end joining, it is necessary a previous DNA end processing, which may involve numerous enzymes as Artemis, Werner, DNA Polimerases μ e λ, Polynucleotide kinase (PNK) and Terminal deoxynucleotidyl transferase (TdT), to conclude the NHEJ pathway. The role of MRN complex in NHEJ pathway it is still not clear.

a wide range of agents and processes: as result of normal cellular metabolism, by the action of ROS, or as result of physiological processes like V(D)J recombination, DNA replication or meiosis. Exogenous factors can include ionizing radiation as well as chemotherapeutic agents[52-54].

In eukaryotic cells, DSBs can be repaired by two main pathways: Homologous Recombination (HR) and Non-Homologous End-Joining (NHEJ)[34,50,52] (Figure 2).

In HR pathway, the break is repaired using the homologous chromosome or sister chromatid as template. This is considered an accurate repair pathway and it is thought that could be particularly important for DSB repair in S/G2 phases of the cell cycle, where replicated sequences are available to serve as repair templates[34,50,55]. In NHEJ pathway, the broken strands are crudely joined together at a site of micro-homology, frequently resulting in small alterations at the site of fusion, being often described as error-prone. Although been able to operate throughout cell cycle, NHEJ is the predominant pathway during G0, G1 and early S phases of the cell cycle[27,34,55-60].

NHEJ and HR proteins are highly conserved across all eukaryotes and ubiquitously expressed in multi-cellular organisms. HR appears to be the predominant DSB repair pathway in yeast although NHEJ is the main pathway in higher organisms such as mammals[50,52]. However, recent evidence suggests that these major repair pathways can cooperate and compete with each other at DSBs to promote efficient repair and genomic integrity[61].

LIGASE IV ENZYME AND ITS ROLE IN ONCOLOGY

DNA ligase enzymes are an evolutionary related protein

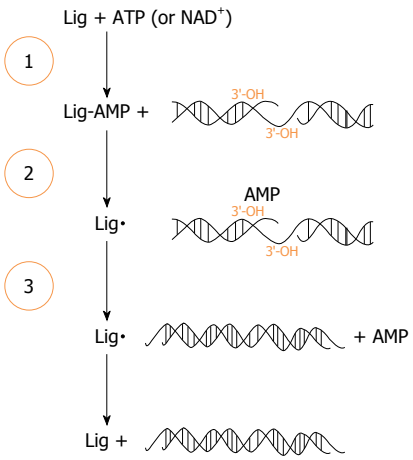

Figure 3 Enzymatic ligation of DNA by DNA ligase. The three-step reaction results in the sequential transfer of AMP (adenosine 5'-monophosphate) to an active-site lysine in Lig enzyme (step 1) then to DNA end (step 2), which results in the formation of a phosphodiester bond and consequently to a ligated DNA product (step 3). Lig: Ligase. Aapted from Ellenberger *et al*[49].

family, involved in innumerous cellular processes such as DNA replication, genetic recombination and DNA repair. They are nucleotidyltransferase enzymes (NTases) that use an energetic source to catalyze phosphodiester bond formation in a three-step reaction mechanism[49,62,63] (Figure 3).

The ligation reaction has a high energetically yield, in which an adenylate group (adenosine 5'monophosphate - AMP) is sequentially transferred from ATP or NAD^+ to a highly conserved lysine residue in the active site of the DNA ligase enzyme (Step 1), with the formation of a covalent enzyme-adenylate intermediate. This first step occurs independently of DNA whereas the subsequent

steps involve interaction between the DNA ligase and its DNA substrat. Formerly, the AMP is transferred to the 5'-PO_4^{3-} DNA end to generate a covalent DNA-adenylate intermediate (Step 2). In the final step, the non-adenylated DNA Ligase catalyzes the formation of a phosphodiester bond, in a reaction that involves a nucleophilic attack by a 3'-OH on the 5' end of the DNA adenylate and the release of AMP (Step 3)[49,62]. By the high favorable reaction equilibrium, each chemical step makes this reaction sequence effectively irreversible, proving once again the importance of DNA repair[49].

DNA ligases have the capability to change their conformation during the DNA joining reaction in order to accommodate the multiple reactions that catalyze. These enzymes have multiple domains that provide the necessary flexibility to completely encircle their DNA substrates as well as the capacity to open and close around DNA[49].

In the human genome there are three genes that codify four DNA Ligases: *LIG1, LIG3* and *LIG4*, with the DNA Ligases Ⅱ and Ⅲ being expressed by alternative splicing of mRNA from *LIG3*[49,64]. Consistent with the common evolution, all of the eukaryotic enzymes are ATP-dependent and are related in terms of sequence and structure[49,62,63].

LIG4 gene, located in 13q33 chromosome, codify an exclusively nuclear protein, with approximately 100 kDa, which shares homology with the other ligases in N-terminal region but not in the C-terminal region[62,65]. Its catalytic domain (CD) comprises six conserved sequence motifs (Ⅰ, Ⅲ, Ⅲα, Ⅳ, Ⅴ, Ⅵ) that define the nucleotidyltransferase family. Motif I includes the lysine residue that is adenylated in the first step of the ligation reaction. The non-CD, which is poorly conserved between the different family members, does not have a known function yet[63,64].

LIG4 enzyme is characterized by a C-terminal extension that includes two tandem copies of the BRCT homology domain, which are found in other DNA repair and checkpoint-associated proteins[65-67]. These motifs are separated by a short linker sequence that contains a conserved binding site presumably necessary to the interaction with XRCC4 in NHEJ pathway[68-70]. XRCC4 is responsible for the stabilization and stimulation of the ligase activity by LIG4, such as adenylation, as well as to protect LIG4 from degradation[68,71]. Furthermore, the stability of LIG4 is also regulated by phosphorylation at a serine residue (Ser650)[49]. Structural studies suggest that in the XRCC4-LIG4 complex, the stoichiometric proportion is one molecule of LIG4 to two molecules of XRCC4[70,72].

Despite the fact of being essential in the DSB repair, the XRCC4-LIG4 complex is DNA-PK dependent and because of that is an exclusive complex of the NHEJ pathway. However, LIG4 appears to function in specialized cells of the immune system where it also completes V(D) J recombination[69].

Under normal conditions, the human genome is replicated and stabilized by highly accurate complex replication and repair machinery. The increased incidence of certain pathologies, like cancer, associated with DNA repair-deficient human syndromes, illustrates the crucial role of these pathways in protection against genomic instability[73]. The LIG4 importance in the maintenance of genomic stability appears to be associated with the fact that mutations in this gene are associated with a rare autosomal syndrome (OMIM 606593) characterized by microcephaly, several immunodeficiency, spontaneous genomic instability and a higher susceptibility to complex diseases such as cancer[50,60,74,75]. Cells from LIG4 patients display increased radiosensitivity and are defective in NHEJ DSB repair[60,75,76]. Knocking out DNA Ligase IV in mice results in late embryonic lethality with massive neuronal apoptosis and lymphocyte development arrest due to lack of V(D)J recombination[77,78].

To date, several polymorphisms have been identified in *LIG4* gene, some of them potentially capable to modulate LIG4 activity. For example, LIG4 polymorphism rs1805388 C/T was found in N-terminal region and has been linked with a reduced adenylation and ligation activities of the enzyme[79]. Variations in enzymatic activity of LIG4 can conduct to a hyper-sensitivity to DNA damage, deregulation of repair and apoptosis mechanisms, affecting the susceptibility to cancer development as well as oncologic therapy response. The principal studies that have been developed to understand the LIG4 polymorphisms role in cancer are described in Table 2.

Radiotherapy and chemotherapy remain the core of conventional cancer treatment and it is necessary to understand how cells respond to DNA damage and determine whether DDR could be exploited or manipulated for therapeutic purposes. There is a growing interest in the identification of DNA repair inhibitors that will enhance the cytotoxicity of DNA damaging agents that, when used concomitantly, may have the capacity to increase the response to treatment[46,48].

Since DNA ligation is an ubiquitous stage in the majority of cellular processes and the last step of almost all DNA repair pathways, DNA ligases are attractive therapeutic targets since it is expected that cells defective in DSB repair will be more sensitive to chemotherapeutic agents[44,46,49].

Some studies suggest that LIG4 down-regulation could be a potential strategy to enhance the therapeutic effects of chemotherapy[44-46]. Kondo *et al*[45] designed a study to better understand the role of DSB repair pathways, including NHEJ, on cellular sensitivity to Temozolomide (TMZ) in glioblastoma. First, they evaluated the role of repair genes in the presence of TMZ-induced DNA damage. Within the cell lines evaluated, LIG4 -/- cells were the most sensitive to TMZ action. To test whether this result was pertinent to chemotherapy used against glioblastoma, LIG4 expression was silenced in A172 glioblastoma cells using siRNA. Results showed that LIG4 silencing increased cellular sensitivity to TMZ approximately three times. Therefore, the authors pro-

Table 2 Some studies that evaluate *LIG4* polymorphisms role in cancer

Authors	LIG4 SNP identification	Tumor model	Ethinicity	Result
Jakubowska *et al*[84]	rs1805386	Ovarian and Breast Cancer	Caucasian	The polymorphism was not associated with BRCA1-associated ovarian and breast cancer risk ($P = 0.16$ and $P = 0.97$, respectively)
Schildkraut *et al*[82]	rs10131	Ovarian Cancer	Caucasian	The polymorphism was significantly associated with invasive serous ovarian cancer risk ($P < 0.05$)
Pearce *et al*[83]	rs1805386	Ovarian Cancer	Mixed	The polymorphism was initially associated with ovarian cancer risk ($P = 0.007$) but replication results do not confirm this association
Yin *et al*[93]	rs1805388	Non-small cell Lung Cancer	Mixed	The polymorphism was significantly associated with the risk of severe radiation pneumonitis in non-small cell lung cancer patients who received radio(chemo)therapy ($P < 0.05$)
Tseng *et al*[94]	rs1805388	Non-small cell Lung Cancer	Asian	The polymorphism was significantly associated with lung cancer risk ($P = 0.038$) especially in smoking patients ($P = 0.015$), and with high fractional allelic loss ($P = 0.016$)
de las Peñas *et al*[89]	rs1805386	Non-small cell Lung Cancer	Caucasian	The polymorphism was not associated with survival in cisplatin/gemcitabine-treated non-small cell lung cancer patients ($P = 0.31$)
Sakiyama *et al*[95]	rs2232641	Lung Cancer	Japanese	The polymorphism was significantly associated with a diminish risk to develop lung cancer ($P = 0.03$)
Sobczuk *et al*[96]	rs2232641	Breast Cancer	Caucasian	The polymorphism was not associated with breast cancer risk ($P > 0.05$)
Han *et al*[97]	rs1805386 rs4987182	Breast Cancer	Mostly Caucasian	No statistically differences in breast cancer risk according LIG4 C299T or T1977C. The polymorphism T1977C was significantly associated with breast cancer risk if the patients had a first degree family history of breast cancer ($P = 0.01$)
Goode *et al*[98]	rs1805386	Breast Cancer	Caucasian	The polymorphism was significantly associated with the breast cancer survival ($P = 0.002$)
Kuschel *et al*[99]	rs1805386	Breast Cancer	Caucasian	The polymorphism was significantly associated with a decrease in breast cancer risk ($P = 0.04$)
Liu *et al*[100]	rs3093739	Glioma	Asian	The polymorphism was significantly associated with glioma risk ($P = 0.009$)
Liu *et al*[101]	rs7325927	Glioblastoma	Caucasian	The polymorphism was significantly associated with glioblastoma survival ($P = 0.008$)

SNP: Single nucleotide polymorphism.

posed that LIG4 down regulation can potentially be a useful strategy for enhance the therapeutic effects of TMZ, becoming LIG4 a new molecular target for chemotherapy[45]. In a study designed by Friesen *et al*[80], they investigated the role of LIG4 in deficient caspases activation by doxorubicin. The results showed that doxorubicin strongly induced apoptosis and caspases activation in LIG4 defective cells suggesting that LIG4, as a key enzyme for NHEJ repair, also plays an important role in deficient caspases activation in cancer cells[80].

In a last view, it may be useful the combination between LIG4 inhibitors with the individual's LIG4 profile since observations suggest that in a partially defective genetic background, additional reduction in ligase levels additionally compromises the cellular ability to repair DSBs[81].

LIGASE IV IN OC

In which concerns to LIG4 polymorphisms and their role in OC risk just a few studies have been made[82-84] (Table 2). Due to contradictory results obtained to some polymorphisms and OC risk, Ovarian Cancer Association Consortium has been formed with the purpose to evaluate the evidence for association in SNPs, which had already been genotyped by multiple studies by combining the existing data. This collaboration has shown that 1977 T/C polymorphism in *LIG4* gene (rs1805386) was not associated with OC risk, although the initial results proposed a significantly positive association[83].

OC remains a treatment challenge. Although the initial response of the OC patients to chemotherapy is good, many patients recur and develop, possibly, cell clones resistant to therapy. Platinum analogs, as cisplatin or carboplatin, are one of the most widely used anti-cancer drugs due to its broad-spectrum of activity against human tumors, namely OC. Platinum compounds react with DNA molecules, forming inter and intrastrand DNA crosslinks, and consequently blocking the movement of DNA replication and transcription machinery along DNA, which results in the arrest of the cell cycle and the activation of DNA repair pathways[85-88]. Nucleotide excision repair is the main mechanism responsible for platinum-DNA adducts removal although some studies proposed that these adducts can inhibit NHEJ repair pathway and consequently influence the patient's overall survival since survival is longer in patients with higher levels of platinum-DNA adducts[18,52,89]. Clinical use of platinum compounds in OC treatment is conditioned by the development of resistance which can result from reduced intracellular accumulation, increased drug inactivation, increased repair of damaged DNA, increased activation of pro-survival pathways or inhibition of pathways that promote cell death[90]. Besides the importance of NHEJ, and specifically of LIG4, to platinum treatment response, to the best of our knowledge no study evaluated the as-

sociation between LIG4 polymorphisms and the chemotherapy response of OC patients. It would be interesting to evaluate the role of LIG4 polymorphisms in platinum resistance and relate them to LIG4 mRNA expression in order to predict the clinical outcome of OC patients and possibly use this marker to guide chemotherapy selection in woman with OC.

Following the PARP inhibitors treatment applicability, LIG4 inhibitors may be concomitantly used with standard therapy for OC treatment in order to enhance its effect and to exploit intrinsic defects in specific DNA repair pathway. This approach might create a large therapeutic window and help to overcome chemotherapy failure in OC treatment. Potential strategies to inhibit the LIG4 action can be by the use of siRNA, as mentioned by Kondo *et al*[45], or by the use of small molecules in silico designed, as mentioned by Chen and collaborators[46].

CONCLUSION

Besides the strong link between DNA repair and OC, the knowledge about LIG4 role in ovarian carcinogenesis is still very limited and one of the aims of this review was to compile all the available information. In last years, translational research has reached an essential role in oncology and the identification of an individual SNP profile, which used in combination with risk factors, can lead to the establishment of potential susceptibility and prognosis factors. In this way, it became clear that the development of new studies are essential to better understand the functional role of polymorphisms in *LIG4* gene and how they can be linked to OC development, namely which concerns with repair-associated ovulation. In other perspective, the definition of a SNP profile could be a useful manner to implement screening and prevention strategies and consequently decrease the OC mortality. To the best of our knowledge, no study has been done regarding *LIG4* polymorphisms and their influence in OC treatment response. However, in this review, we described the dual role of LIG4 enzyme in cancer. Some studies have associated high levels of this enzyme with a good response to carcinogenic damage repair and the consequent genomic stability maintenance. However, in an opposite view, high levels of LIG4 enzyme can lead to worse treatment response due to the higher capability to repair the damage induced by chemo or radiotherapy. It is known that genetic polymorphisms are capable to affect the functional activity of DNA repair enzymes and affect significantly the effectiveness and therapy outcome. Besides this aspect, studies are made in order to discover and develop new treatment strategies to overcome therapy resistance and improve OC survival rates, namely using DNA repair as treatment target. The inhibition of LIG4 can possibly be an useful strategy to overcome the chemotherapy failure associated with OC standard treatment.

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CASE REPORT

Uterine intravenous leiomyomatosis with cardiac extension: Imaging characteristics and literature review

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Abstract

Intravenous leiomyomatosis (IVL), showing unusual growth patterns of uterine leiomyoma, is a rare neoplasm characterized by intravascular proliferation of a histologically benign-looking smooth muscle cell tumor mass, but not invading the tissue. To date, less than 300 cases have been reported and fewer than 100 cases with cardiac involvement. Imaging characteristics of IVL are still not clear so it is usually misdiagnosed before surgery. A 36-year-old woman, who had undergone hysterectomy due to hysteromyoma, presented with shortness of breath after activities. Imaging showed IVL with mass involvement of the left ovarian vein, left renal vein, left external and common iliac vein, as well as within the inferior vena cava (IVC), extending into the right atrium. The operation demonstrated that the mass had no stalk and had well-demarcated borders with the wall of the right atrium and IVC. The patient underwent a one-stage combined multidisciplinary thoraco-abdominal operation under general anesthetic. Subsequently, the pathology report confirmed IVL. IVL should be considered in a female patient presenting with an extensive mass in the right side of the heart. Imaging technology, such as echocardiogram, contrast-enhanced computed tomography and magnetic resonance imaging, can provide important information to reveal the mass, the range and path of the lesion, and relates to the surgical plan decision. Consequently, perfect and exact image examination is very necessary pre-operation.

Key words: Intravenous leiomyomatosis lower; Hysterectomy; Computed tomography; Echocardiogram; Imaging

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INTRODUCTION

Intravenous leiomyomatosis (IVL), showing unusual growth patterns of uterine leiomyoma, is a rare neoplasm characterized by intravascular proliferation of a histologically benign-looking smooth muscle cell tumor mass, but not invading the tissue. Birch-Hirschfeld[1] first presented a case of IVL in 1896 and Durck first presented a case of intracardiac extension of IVL in 1907; cases of intracardiac extension account for about 10%[2]. To date, less than 300 cases have been reported in the English literature.

Although histologically benign, IVL might sometimes be malignant in behavior, with not only involvement of pelvic veins, the inferior vena cava (IVC), adrenal and renal veins, but sometimes reaching as far as the right cardiac chambers and the main pulmonary artery, and may result in cardiac symptoms and a cardiac murmur, fainting and even, in some cases, sudden death[3]. Because of its rarity, IVL is usually misdiagnosed pre-operation and diagnosed late, and is subsequently not treated properly. The correct preoperative diagnosis of IVL depends on a

huge amount of information, especially a comprehensive imaging examination. Here, we present a case of IVL diagnosed by transthoracic echocardiography, abdominal contrast computed tomography (CT) and CT angiography (CTA) and verified by histopathological evaluation, and we review the literature.

CASE REPORT

Clinical manifestation and physical examination

A 36-year-old woman presented with a 1 mo history of shortness of breath after activities with no obvious cause, and not accompanied by palpitation, fever, cough and paroxysmal nocturnal dyspnea, *etc*. Two years ago, she had undergone hysterectomy due to hysteromyoma and was not found to have any heart disease or relevant history. On examination, there was no abnormality in the physical examination except for cardiac auscultation and mild edema of both lower extremities. Ⅱ/Ⅵ level of systolic blowing murmur and diastolic rumbling murmur were heard in the 4th and 5th intercostals on the left sternum. In addition, incomplete right bundle branch conduction block, increased right heart load and mildly lowered S-T segment were found on the electrocardiogram check. The laboratory examinations revealed normal results, including tumor markers, liver and kidney function, blood and urine examination, *etc*.

Echocardiogram

Transthoracic two-dimensional echocardiography showed a mild increase of the right atrium and ventricle and the atrium was filled with an echogenic oval tumor mass which was approximately 6.8 cm × 4.0 cm and moved back and forth through the tricuspid orifice into the right ventricle (Figure 1A and B). The mass size was about 10.7 cm × 1.2 cm and involved the IVC and extended through the lumen (Figure 1C). The mass of the right atrium and IVC was stretched and had well-demarcated borders with the wall of the right atrium and IVC. The presumptive diagnosis of left atrial myxomas was made by an ultrasonic practitioner.

CT and CTA

Contrast enhanced CT and CTA of the thoracic, abdominal and pelvic cavity were performed. The pelvic CT revealed a lobulated mass with local heterogeneity contrast enhancement (Figure 2). A low attenuation-filling defect without contrast enhancement was present in the left ovarian vein, left renal vein, left external and common iliac vein, as well as within the IVC, extending into the right atrium (Figure 3). The mass within the vein and right atrium was wide. Hence, the presumptive diagnosis of sarcoma with invasion in to the vein system and right atrium was made; the mass was not in the vein system, except for the thrombus formation.

Digital subtraction angiography

Digital subtraction angiography of the thoracic and abdominal cavity was performed. The imaging demonstrated an irregular mass, like a filling defect from the IVC to the right atrium, and presented as a large tumor within the atrium (Figure 4).

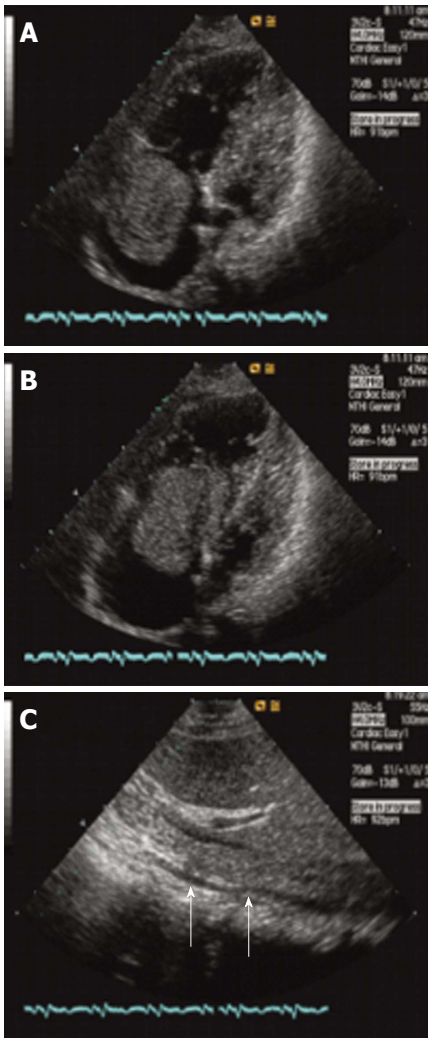

Figure 1 Echocardiogram of the heart and inferior vena cava. A, B: Axial image demonstrates a mass within the right atrium that moves back and forth through the tricuspid orifice into the right ventricle in the systolic and diastolic period; C: Axial image demonstrates a mass within the inferior vena cava (arrows).

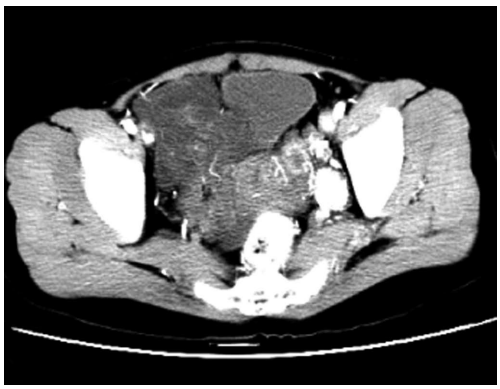

Figure 2 Preoperative pelvic contrast enhanced computed tomography shows a big irregular mass exhibiting heterogeneous enhancement.

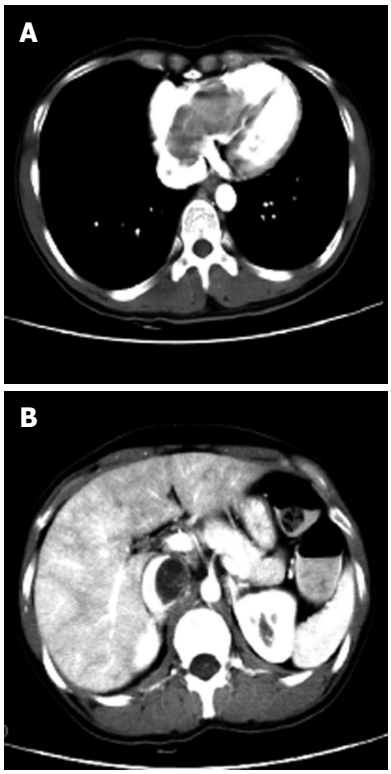

Figure 3 Preoperative chest and abdominal computed tomography. A: Axial image demonstrates a lobulated mass within the right atrium and ventricle; B: Axial image shows a tumor in the inferior vena cava and resulting lumen stenosis.

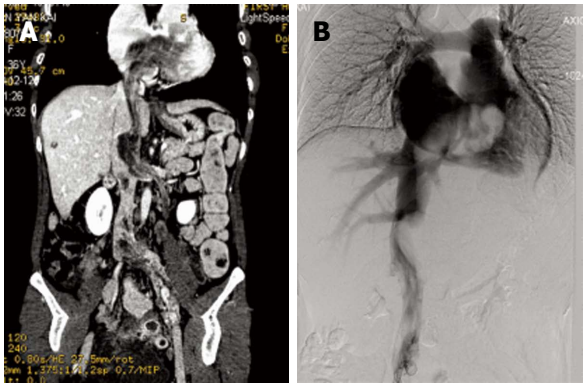

Figure 4 Coronal plane computed tomography and digital subtraction angiography image of intravenous leiomyomatosis. A: Preoperative coronal plane computed tomography demonstrates a mass was present in the left ovarian vein, left renal vein, left external and common iliac vein, as well as within the inferior vena cava, extending into the right atrium; B: Digital subtraction angiography image demonstrates an irregular mass filling defect from the inferior vena cava to the right atrium.

Operation and pathology

Cardiac and vascular surgery was carried out under general anesthetic and with circulatory arrest. The pelvic masses were partially resected because of extensive lesions, including bilateral accessories. The left ovarian vein was cut off and the IVC and right atrium opened; the size of tumor within the atrium was about 8 cm × 6 cm × 6 cm. The surgeons separated the adhesion between the mass and the wall of the vascular structure and heart and peeled the lesions completely. The length of tumor within the vascular structure and heart was 30 cm. All the masses looked red and had the same pathological features, showing a spindle cell tumor, size consistency, less karyokinesis and with a thick wall vessel in the mesenchyme. Subsequently, the pathological report confirmed IVL.

DISCUSSION

The neoplastic smooth muscles of IVL are histologically and cytogenetically similar to benign leiomyomata but might behave in a "malignant" fashion, with not only involvement of pelvic veins, IVC, renal veins, pulmonary artery and right cardiac chamber, but also distant metastasis, such as lung, brain, lymph nodes and so on[3-5]. Although cardiac involvement is present in up to 10% of cases[3], in the last 10 years the literature contains more reports of IVL and fewer than 100 cases with cardiac involvement.

So far, the pathogenesis of IVL is still unclear; one possibility is that the tumor originated from smooth muscle in the vessel wall and the other that it is the uterine leiomyoma which invaded the uterine vein and extended extensively. The current patient had a history of hysterectomy 5 years ago. CT imaging revealed an irregular enhancing tumor in the pelvis invading into the left ovarian vein, renal vein and common iliac vein, as well as within the IVC, extending into the right atrium. The mass was not adhesive with the wall of the vascular structure and heart and was resected completely. Therefore, the case was inclined to support the second theory of pathogenesis.

Clinical characteristics of IVL mainly depend on the location and scale of lesion. More distal intravascular extension of the tumor can result in various cardiorespiratory symptoms. Symptoms of abdominal pain, shortness of breath, palpitation and edema of the lower extremities are the most common and occasionally present with Budd-Chiari syndrome, pulmonary embolism and sudden death[3,5]. The prominent clinical manifestation of the present case was shortness of breath and mild edema of the lower extremities. The reason for misdiagnosis preoperation may be because it is not a typical symptom for IVL diagnosis.

Previously, many case reports described characteristic imaging features, including nodular, enhancing tumors that appear to originate in the uterus and extend into the venous system, causing expansion of the involved vascular structures and heart[6-8]. Echocardiographic features of IVL with cardiac extension include a hyperechoic elongated mobile mass extending from the IVC and an irregular mass in the atrium. The tumor in the atrium usually is misdiagnosed as myxoma, although some key points of a differential diagnosis are advanced[9], as happened in the present case. Usually, more imaging examinations are needed. Contrast-enhanced CT, CTA and post-processing (multi planar reformation, maximum intensity projection) can directly show the tumor and full-scale path of extension, which is the key for the establishment of an opera-

tive plan[6]. A pelvic irregular tumor usually presents with inhomogeneous distinct enhancement and the iliac vein, ovarian veins, IVC and right atrium are usually involved, distended and filled with an enhancing mass. In addition, IVC can be distended with a non-enhancing thrombus. In the present case, CT imaging revealed a lobulated inhomogeneous enhanced mass in the pelvis and a long, serpentine and elongated mobile mass extending from the left ovarian veins, left internal iliac vein, continued to the IVC and extending into the right atrium. However, it is sad that it did not raise the presumptive diagnosis of IVL. In retrospect, once this disease is discovered, the diagnosis of IVL should be considered by combining the CT features with the history of uterine myoma. Pre-surgical CT examination is very important. Magnetic resonance imaging (MRI) features of IVL were also reported, similar to the findings of CT but with advantages of superb soft tissue contrast resolution, direct multi-planar imaging capability and unique ability to assess blood flow.

According to imaging features, the differential diagnosis of IVL mainly includes intravenous thrombus, leiomyosarcoma, right atrial myxoma and tumor thrombosis with malignant carcinoma, for example, renal carcinoma, hepatocellular carcinoma, adrenal cortical carcinoma, *etc*. The differential diagnosis points of them have been described by previous reports[3,6,8]. Therefore, here, we summarize the diagnosis points of IVL: (1) Middle-aged women, usually with a history of hysterectomy; (2) Pelvic irregular mass with inhomogeneous enhancement, invasion in the pelvic vein and extending to the IVC, sometimes to the right atrium; (3) Lesions widely infringing the venous system is an important feature; (4) The mobile mass within the right atrium is always accompanied with a large mass in the IVC and continued; and (5) The mass in the heart and vein structures have no adhesion with the wall of heart and vein. According to these features, the present case was a typical IVL case but it was misdiagnosed pre-operation; to our knowledge, it was the first case confirmed in our hospital. Although these features strongly suggest the diagnosis of IVL, the final diagnosis depends on histopathology.

Surgical excision is still the best treatment of choice for IVL and complete removal of the tumor is considered essential to prevent a recurrence. In fact, it has a high rate of recurrence because complete resection is a difficult thing to achieve[10]. Once IVL involves the right heart chamber, a combined multidisciplinary thoraco-abdominal operation is required. In the present case, the intra-cardiac and intra-vascular mass was free-floating without involvement of the cardiac structure and vein wall tissue. The left ovarian vein was cut off and then the mass was resected by opening the IVC and right atrium. Only a partial resection of the pelvic tumor was performed because of the widespread tumor and involvement of small vessels in the pelvis. Estrogen would stimulate the tumor to grow[10] and therefore a bilateral oophorectomy was performed.

In conclusion, intracardiac leiomyomatosis should be considered in a female patient presenting with an extensive mass in the right side of the heart. Imaging technology, such as echocardiogram, contrast-enhanced CT and MRI, can provide important information to reveal the mass, the range and path of the lesion, and relates to the surgical plan decision. Consequently, perfect and exact image examination is very necessary pre-operation.

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EDITORIAL

Molecularly targeted therapies for advanced or metastatic non-small-cell lung carcinoma

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Abstract

Non-small-cell lung cancer (NSCLC) remains the leading cause of cancer-related death in both men and women in the United States. Platinum-based doublet chemotherapy has been a standard for patients with advanced stage disease. Improvements in overall survival and quality of life have been modest. Improved knowledge of the aberrant molecular signaling pathways found in NSCLC has led to the development of biomarkers with associated targeted therapeutics, thus changing the treatment paradigm for many NSCLC patients. In this review, we present a summary of many of the currently investigated biologic targets in NSCLC, discuss their current clinical trial status, and also discuss the potential for development of other targeted agents.

Key words: Non-small cell lung cancer; Molecular targeted therapy; Vascular endothelial growth factor; Epidermal growth factor receptor; Tyrosine kinase inhibitors; BRAF; Anaplastic lymphoma kinase

Core tip: Targetable molecular abnormalities have not yet been identified in approximately 80% of non-small-cell lung cancer patients. In addition to anaplastic lymphoma kinase, epidermal growth factor receptor, vascular endothelial growth factor targeted therapies, the results from ongoing trials will determine if the newer targeted agents will be incorporated into clinical practice.

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INTRODUCTION

Non-small-cell lung cancer (NSCLC) remains a therapeutic challenge. Despite some progress, it remains the leading cause of cancer-related death in the United States in both men and women. The estimated incidence of NSCLC is 226160 cases with 160340 deaths in the United States in 2012. The 5-year survival rates for advanced and metastatic NSCLC are only 24% and 4%, respectively[1].

The core drug and backbone of treatment in locally advanced and metastatic settings of NSCLC has been a platinum agent. In a large randomized clinical trial, Schiller *et al*[2] compared the efficacy of three commonly used regimens (cisplatin and gemcitabine, cisplatin and docetaxel, carboplatin and paclitaxel) with that of a reference regimen of cisplatin and paclitaxel. No significant difference in survival was observed among the four commonly used regimens, although the regimen of carboplatin and paclitaxel had a lower rate of toxic effects than the other regimens. On the basis of these results, Eastern Cooperative Oncology Group had chosen carboplatin and paclitaxel as its reference regimen for future studies; and it is still the most commonly used taxane-platinum combination in the United States[3] which produces 15%-32% objective response rates (ORR), with 7.9-10.6 mo median overall survivals (OS)[4-6].

Further attempt at subclassification is now accepted as a standard of care; separating squamous cell carcinoma from adenocarcinoma and large-cell carcinoma as the distinction carries implications for prognosis and treatment decisions. For example, a phase Ⅲ study in patients with advanced NSCLC treated with cisplatin plus pemetrexed (an inhibitor of purine and pyrimidine synthesis), showed no improvement in tumor response rate and survival over cisplatin plus gemcitabine for all histologies; however, an improvement in survival was noted in the non-squamous histology subset while a decrement in the squamous histology subset was observed[7]. Due to safety concerns observed in the phase Ⅱ trial, the addition of bevacizumab to carboplatin/taxol was subsequently studied in phase Ⅲ trial and improved efficacy was observed in patients with non-squamous histology (ORR, 35%; OS, 12.3 mo)[5].

In addition to making distinction in cytotoxic chemotherapy based on histology, over the past decade, a large number of studies have been published that aimed to target the molecular abnormalities implicated in NSCLC tumor growth, invasion, metastasis, angiogenesis and resistance to apoptosis. Currently, detection of the presence of mutations involving the epidermal growth factor receptor (*EGFR*) gene and fusion of the N-terminal portion of the protein encoded by echinoderm microtubule-associated protein-like 4 (*EML4*) gene with the intracellular signaling portion of the receptor tyrosine kinase encoded by anaplastic lymphoma kinase (*ALK*) gene - that is, EML4-ALK - has become routine in many centers because patients having tumors harboring such alterations benefit from novel targeted inhibitors as part of their treatment regimen. This review describes some of the important developments and targeted agents that have been tested in clinical trials; and the potential future biologics in the treatment of advanced or metastatic NSCLC.

MOLECULARLY TARGETED THERAPIES IN ADVANCED OR METASTATIC NSCLC

EGFR inhibition

EGFRs are a group of transmembrane proteins that regulate key processes in the cell, such as proliferation, division, migration, and differentiation. This family has 4 different members: EGFR (HER1 or ErbB1), HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4); all of which share a similar structure[8]. Upon binding to its ligands, EGFR induces receptor homo- or hetero-dimerization and results in the activation of an intracellular tyrosine kinase domain. Receptor activation cause downstream signaling events through activation of the Ras/Raf/MEK/MAPK and PI3K/AKT/mTOR pathways that regulate cell proliferation, differentiation, and survival[9]. The two most common EGFR mutations are short in-frame deletions of exon 19 and a point mutation in exon 21[10]. Tumors with EGFR mutations occur at a higher frequency in East Asians than in non-Asians (30% *vs* 8%), in women than in men (59% *vs* 26%), in never-smokers than in ever-smokers (66% *vs* 22%), and in adenocarcinoma than in other NSCLC histologies (49% *vs* 2%)[11]. In the United States, activating EGFR mutations are estimated to occur in 15% of patients with primary lung adenocarcinoma[12].

Monoclonal antibodies against EGFR: Cetuximab is a chimeric monoclonal antibody against EGFR. One of the first phase Ⅱ studies assessing combination chemotherapy with cetuximab (cisplatin or carboplatin and gemcitabine with or without cetuximab) showed an increased ORR, progression-free survival (PFS), and OS in the cetuximab group[13]. A similar phase Ⅱ study in which cisplatin and vinorelbine were administered with or without cetuximab also showed enhanced survival indices in the cetuximab arm[14]. However a subsequent large phase Ⅲ trial investigating paclitaxel or docetaxel and carboplatin, with or without cetuximab in 676 patients with NSCLC did not find any notable differences in PFS or ORR[15].

The recently published FLEX study demonstrated that adding cetuximab to cisplatin-based chemotherapy resulted in a small but significant improvement in median OS in patients with advanced NSCLC [11.3 mo *vs* 10.1 mo; hazard ratio (HR): 0.87; $P = 0.04$][16]. A retrospective analysis of FLEX data showed that 31% of patients with high EGFR expression, adding cetuximab increased the median OS from 9.6 to 12 mo (HR: 0.73; $P = 0.011$)[17]. Ultimately, a meta-analysis looking at the four trials in which 2018 previously untreated NSCLC patients were analyzed concluded that cetuximab improved OS and ORR regardless of the presence of EGFR mutations[18]. In accordance with the above results, a more in-depth analysis of these subgroups in phase Ⅲ trials revealed that specific activating mutations in the tyrosine kinase domain of the *EGFR* gene were associated with sensitivity to gefitinib but not to cetuximab[19]. In addition, no significant cetuximab treatment-specific correlations between EGFR or K-RAS mutation status and PFS, OS, or ORR were observed in the phase Ⅲ trials[20,21]. Therefore, we can conclude that EGFR or K-RAS mutations may not be useful as biomarkers in cetuximab therapy. At present, a number of clinical trials are still evaluating the efficacy of cetuximab in combination with other treatment modalities in combination with tyrosine kinase inhibitors (TKIs), and other chemotherapeutic drugs. Most of these trials are also assessing biomarker status that could be predictive or prognostic in value.

EGFR-Tyrosine kinase inhibitors: EGFR-TKIs are small molecules administered orally and are subdivided in reversible, gefitinib and erlotinib, and irreversible, afatinib on the basis of their straight binding with the specific site of the EGFR intracellular domain. These drugs inhibit the phosphorylation and tyrosine kinase activity of the intracellular adenosine triphosphate (ATP)-binding domain of the EGFR through competitive binding to this site, and were initially investigated in unselected patients reporting contrasting results depending on the type of population/enrolled in each study. However, the discov-

ery that response to EGFR-TKIs is associated with the presence of activating EGFR mutations in NSCLC has led to the design of clinical trials in which patients were selected on the basis of the EGFR mutational status. Almost all patients who respond to EGFR-TKIs have been shown to carry activating mutations usually found in exons 18 through 21 of the TK domain of EGFR, and are either point mutations or in-frame small deletions or insertions[22]. Although more than 250 mutations of the EGFR have been described up to now, two mutations, one single point mutation in exon 21, the L858R, and a series of small in-frame deletions in exon 19 account for approximately 90% of all EGFR mutations.

Erlotinib: EGFR mutations have been defined "activating" and "sensitizing" and both definitions are correct. In fact, EGFR mutations lead to increased response of the EGFR to exogenous growth factors, thus producing a more significant and more persistent activation of intracellular signaling pathways, resulting in increased cell proliferation and survival. On the other hand, the mutant receptor is more sensitive to EGFR-TKIs as compared with wild type EGFR, since lower concentrations of drugs are required to inhibit its phosphorylation. Retrospective analyses have demonstrated that patients with EGFR mutations have high ORRs to EGFR-TKIs in any line of treatment[23]. These findings sustain the hypothesis that tumors with EGFR mutations are addicted to the EGFR pathway, *i.e.* depend on these pathways for their growth. In agreement with this hypothesis, tumors with EGFR mutations have shown to homogeneously carry this molecular alteration in all tumor cells[24]. As discussed above, erlotinib was first studied in unselected patients with NSCLC, and a subsequent analysis of the patients who had experienced dramatic tumor responses were found to have the activating mutations in the kinase domain of EGFR[25]. The response rate was as high as 81% in patients harboring EGFR tyrosine kinase domain mutations, but less than 10% in patients with wild-type EGFR[26]. The OPTIMAL trial was the first phase Ⅲ study directly comparing erlotinib with standard chemotherapy in the first-line setting of advanced NSCLC in Chinese patients with an activating EGFR mutation. That trial showed a PFS of 13.1 mo with erlotinib compared with 4.6 mo with gemcitabine-carboplatin chemotherapy (HR: 0.16; 95%CI: 0.1-0.26; $P < 0.001$)[27]. An updated analysis also showed median PFS of 13.7 mo *vs* 4.6 mo; HR: 0.164; $P < 0.0001$[28]. A second trial called EURTAC, the first to involve a Western European population, randomized patients to a platinum-based doublet chemotherapy regimen (docetaxel-gemcitabine) or to erlotinib in patients with an EGFR activating mutation. Patients treated with erlotinib experienced a PFS advantage (9.7 mo *vs* 5.2 mo; HR: 0.37; 95%CI: 0.25-0.54)[29]. Based on these results, erlotinib was approved as a first-line treatment in patients with advanced or metastatic NSCLC harboring the EGFR mutations.

Recent phase Ⅱ/Ⅲ trials have shown single agent activity of erlotinib in the second-line setting in either selected or unselected patients with metastatic NSCLC[30,31]. In the TITAN phase Ⅲ trial, the efficacy and tolerability of second-line erlotinib was compared with either pemetrexed or docetaxel in 425 patients with advanced NSCLC who were treated with first-line platinum doublet chemotherapy and had disease progression during or immediately after chemotherapy. The second-line erlotinib was associated with a similar median OS duration to pemetrexed or docetaxel in patients with advanced NSCLC (5.3 mo *vs* 5.5 mo; HR: 0.96 in the overall population; 95%CI: 0.78-1.19). Similarly, there was no difference in OS between the treatment groups (HR: 0.85; 95%CI: 0.59-1.22) in 149 patients with EGFR wild type tumors[32].

The phase Ⅲ SATURN trial examined erlotinib as maintenance therapy after platinum-based chemotherapy. That trial met the primary endpoint of significantly longer PFS in patients treated with erlotinib (12.3 wk) than in patients receiving placebo (11.1 wk; HR: 0.69; 95%CI: 0.58-0.82; $P < 0.0001$). The overall response rate was 11.9% in the erlotinib arm compared with 5.4% in the placebo arm ($P = 0.0006$)[33]. Importantly, the benefit of erlotinib maintenance on PFS and OS was also seen in EGFR wild-type patients (HR: 0.78, 95%CI: 0.63-0.96, $P = 0.0185$, and HR: 0.77, 95%CI: 0.61-0.097, $P = 0.008$, respectively).

Gefitinib: Two large phase Ⅲ studies highlighted the role of gefitinib in tumors harboring EGFR mutations[34,35]. In IPASS trial, the efficacy of gefitinib was compared with carboplatin/paclitaxel in previously untreated never-smokers and light ex-smokers with advanced pulmonary adenocarcinoma. Of 1217 enrolled patients, OS was similar for gefitinib and carboplatin/paclitaxel (HR: 0.90; 95%CI: 0.79-1.02; $P = 0.109$) in overall, or in EGFR mutation-positive (HR: 1.00; 95%CI: 0.76-1.33; $P = 0.990$) or EGFR mutation-negative (HR: 1.18; 95%CI: 0.86-1.63; $P = 0.309$) subgroups. Of importance, PFS was significantly longer with gefitinib for patients whose tumors had both high *EGFR* gene copy number and EGFR mutation (HR: 0.48; 95%CI: 0.34-0.67) but significantly shorter when high *EGFR* gene copy number was not accompanied by EGFR mutation (HR: 3.85; 95%CI: 2.09-7.09)[34]. Likewise, another multicenter phase Ⅲ trial demonstrated that patients with advanced-stage NSCLC containing EGFR mutations and treated with first-line gefitinib (compared with standard chemotherapy) had improved PFS[35]. Based on these results, the American Society of Clinical Oncology recommended EGFR mutation testing for patients with advanced NSCLC who are being considered for first-line therapy with an EGFR-TKI[12].

Two phase Ⅲ clinical trials suggested that gefitinib was more efficacious and less toxic than docetaxel as a second-line treatment in patients with previously-treated advanced NSCLC[36,37]. In the ISTANA trial, the primary endpoint of PFS was longer with gefitinib than

Table 1 Selected phase Ⅲ and randomized phase Ⅱ trials comparing epidermal growth factor receptor tyrosine kinase inhibitor and chemotherapy as first-line therapy in patients with advanced non-small cell lung cancer

Trial	*n*	Type of study	Study design	OS (mo) HR (95%CI)	*P* value	PFS (mo) HR (95%CI)	*P* value	ORR (%) HR (95%CI)	*P* value
Fukuoka *et al*[34]	261	Retrospective	Gefitinib *vs* PC	21.6 *vs* 21.9 1.00 (0.76-1.33)	0.99	9.6 *vs* 6.3 0.48 (0.36-0.64)	0.0001	71.2 *vs* 47.3 2.75 (1.65-4.6)	0.0001
Han *et al*[98]	42	Retrospective	Gefitinib *vs* Cis + G	27.2 *vs* 25.6 1.04 (0.49-2.18)	NA	8.0 *vs* 6.3 0.54 (0.26-1.1)	0.086	84.6 *vs* 37.5 9.16 (2.10-39.84)	0.002
Mitsudomi *et al*[99]	172	Prospective	Gefitinib *vs* Cis + D	35.5 *vs* 38.8 1.18 (0.76-1.8)	0.44	9.6 *vs* 6.6 0.52 (0.37-0.71)	0.001	62.1 *vs* 32.1 3.44 (1.60-7.37)	0.0001
Maemondo *et al*[35], Inoue *et al*[100]	228	Prospective	Gefitinib *vs* PC	27.7 *vs* 26.6 0.88 (0.63-1.24)	0.48	10.8 *vs* 5.4 0.32 (0.23-0.43)	0.001	73.7 *vs* 30.7 6.32 (3.55-11.25)	0.001
Chen *et al*[28]	154	Prospective	Erlotinib *vs* C + G	22.7 *vs* 28.85 1.04 (0.69-1.58)	0.69	13.7 *vs* 4.6 0.16 (0.10-0.26)	0.0001	83 *vs* 36 NA	0.0001
Rosell *et al*[29]	173	Prospective	Erlotinib *vs* platinum-based doublets	19.3 *vs* 19.5 1.04 (0.65-1.68)	0.87	9.7 *vs* 5.2 0.37 (0.25-0.54)	0.0001	58[1] *vs* 15[1] NA	NA
Yang *et al*[2][101]	345	Prospective	Afatinib *vs* Cis + P	NM		11.1[3] *vs* 6.9[3] 0.58 (0.43-0.78)	0.0004	56.1[3] *vs* 22.6[3] NA	0.001
Jänne *et al*[102]	345	Prospective	Erlotinib *vs* erlotinib + PC	24.6 *vs* 19.8 NA	NA	5.0 *vs* 6.6 NA	NA	35 *vs* 46 NA	NA

[1]Intention-to-treat population; [2]Only lung adenocarcinoma patients; [3]By independent review. PC: Paclitaxel and carboplatin; Cis: Cisplatin; C: Carboplatin; G: Gemcitabine; D: Docetaxel; P: Pemetrexed; OS: Overall survival; HR: Hazard ratio; NM: Not yet mature; NA: Not available; PFS: Progression-free survival; ORR: Objective response rate; *n*: Number of patients enrolled in the study.

docetaxel (HR: 0.729; 90%CI: 0.533-0.998; $P = 0.0441$), and the secondary endpoints showed superior ORR (28.1% *vs* 7.6%; $P = 0.0007$), good tolerability, and similar quality-of-life (QoL) improvement rates for gefitinib compared to docetaxel[37]. In the INTEREST trial, of 1433 patients analyzed (723 in gefitinib group and 710 in docetaxel group), non-inferiority of gefitinib compared with docetaxel was confirmed for OS (593 events *vs* 576 events; HR: 1.020, 95%CI: 0.905-1.150). Interestingly, superiority of gefitinib in patients with high *EGFR*-gene-copy number was not proven (72 *vs* 71 events; HR: 1.09, 95%CI: 0.78-1.51; $P = 0.62$; median survival 8.4 mo *vs* 7.5 mo)[36]. Table 1 summarizes the selected phase Ⅲ and randomized phase Ⅱ trials comparing EGFR-TKIs and chemotherapy as first-line therapy in patients with advanced NSCLC.

Vascular endothelial growth factor inhibition

Bevacizumab, a monoclonal antibody against circulating vascular endothelial growth factor (VEGF), was approved by Food and Drug Administration for the treatment of NSCLC in 2006. The combination of bevacizumab with carboplatin and paclitaxel was shown to prolong OS compared with chemotherapy alone (median OS, 12.3 *vs* 10.3 mo, respectively) in patients with nonsquamous advanced NSCLC[5]. Bevacizumab has also been combined with gemcitabine and cisplatin, with a modest benefit observed in PFS but no differences seen in OS[38]. Many other antiangiogenic agents have been under development.

Triple angiokinase inhibitors, which inhibit VEGF, platelet derived growth factor and/or fibroblast derived growth factor were thought to have the potential to improve the therapeutic outcomes for patients with NSCLC. Clinical trials have been ongoing involving several new antiangiogenic therapies, including ramucirumab, aflibercept, vandetanib, cediranib, nintedanib, sunitinib, pazopanib, brivanib, linifinib, axitinib, and motesanib (http://www.clinicaltrials.gov). To date, none of these agents in combination with chemotherapy have resulted in improvements in OS for patients with advanced NSCLC. Moreover, in a phase Ⅱ trial (ESCAPE), patients with squamous histology treated with chemotherapy plus sorafenib had a shorter OS than those receiving chemotherapy plus placebo (HR: 1.85; 95%CI: 1.22-2.81)[6]. A recent meta-analysis comparing the efficacy and toxicity of chemotherapy plus multitargeted antiangiogenic TKI with chemotherapy alone in patients with advanced NSCLC showed that chemotherapy plus a TKI significantly increased the ORR (HR: 1.71, 95%CI: 1.43-2.05) and PFS (HR: 0.83, 95%CI: 0.76-0.90], but not OS (HR: 0.93, 95%CI: 0.83-1.03). The toxicity was comparable between the two therapies[25]. Table 2 summarizes the phase Ⅲ clinical trials testing antiangiogenic TKIs in combination with chemotherapy in NSCLC.

There is evidence from the 3 phase Ⅱ clinical trials supporting the potential use of sorafenib as a monotherapy in chemotherapy refractory NSCLC[26,27]. Particularly, the BATTLE trial showed a promising response rate (8-wk disease control rate in 58% of patients) in heavily pretreated patients with single agent sorafenib. More impressively, in patients whose tumor harbored a KRAS mutation, sorafenib had a disease control rate of 79% while on a separate phase Ⅱ trial in NSCLC, the response rate to erlotinib was only 14% ($P = 0.016$)[28]. This indicates that the significant disease control rate in KRAS mutant NSCLC patients may be due to sorafenib's effects on KRAS downstream pathways such as Raf inhibition rather than its antiangiogenic effects. The randomized,

Table 2 Phase Ⅲ clinical trials testing antiangiogenic tyrosine kinase inhibitors in combination with chemotherapy in non-small cell lung cancer

Trial	*n*	Study design	PE	OS (mo)	PFS (mo)	ORR (%)
Vandetanib second-line						
ZEAL[103]	534	PV *vs* P	PFS	10.5 *vs* 9.2	17.6 wk *vs* 11.9 wk	19 *vs* 8
ZEST[104]	1240	EV *vs* E	PFS	6.9 *vs* 7.8	2.6 *vs* 2.0	12 *vs* 12
ZODIAC[105]	1391	DV *vs* D	PFS	10.6 *vs* 10.0	4.0 *vs* 3.2	NA
Vandetanib second or third-line						
ZEPHYR[106]	924	V *vs* placebo	OS	8.5 *vs* 7.8	NA	2.6 *vs* 0.7
Sorafenib first-line						
NEXUS[107]	904	G + Cis + S f/b S *vs* G + Cis f/b placebo	OS	376 d *vs* 379 d	183 d *vs* 168 d	28 *vs* 26
Motesanib first-line						
MONET[6]	1090	PC + M *vs* PC	OS	13.0 *vs* 11.0	5.6 *vs* 5.4	40 *vs* 26
Cediranib first-line						
BR29 (active, no longer recruiting, NCT00795340)	750	PC + Ced *vs* PC	OS	NA	NA	NA
Nintedanib second-line						
LUME-Lung 1 (active, no longer recruiting, NCT00805194)	1300	D + Nin *vs* D	PFS	NA	NA	NA
LUME-Lung 2 (active, no longer recruiting, NCT00806819)	1302	P + Nin *vs* P	PFS	NA	NA	NA

PC: Paclitaxel and carboplatin; P: Pemetrexed; E: Erlotinib; D: Docetaxel; V: Vandetanib; DV: Docetaxel-vandetanib; EV: Erlotinib-vandetanib; G: Gemcitabine; Cis: Cisplatin; S: Sorafenib; f/b: Followed by; M: Motesanib; Ced: Cediranib; Nin: Nintedanib; OS: Overall survival; PE: Primary endpoint; PFS: Progression-free survival; ORR: Objective response rate; NSCLC: Non-small cell lung cancer; NA: Not available.

placebo-controlled, multicenter international phase Ⅲ trial (NCT00863746 MISSION Trial) is currently underway to evaluate single agent sorafenib as third- or fourth-line therapy in patients with NSCLC. The enrollment for MISSION Trial has been concluded and data should be available later this year.

EML4-ALK inhibition

Rearrangements of the *ALK* gene are felt to be mutually exclusive of EGFR and KRAS mutations and occur in approximately 4% of NSCLC. The ALK mutations are more common in adenocarcinomas and in light smokers or non-smokers[39]. The phase I trial of the ALK-inhibitor crizotinib in advanced ALK-positive NSCLC revealed a response rate of 57% (95%CI: 46%-68%) and an estimated 6-mo PFS probability of 72% (95%CI: 61%-83%)[40]. A retrospective review of 82 ALK-positive patients (including patients who had received multiple lines of therapy) treated with crizotinib revealed an impressive 1-year survival of 74% (95%CI: 63%-82%) and 2-year survival of 54% (95%CI: 40%-66%)[41]. Crizotinib was approved in the United States in 2011, primarily based on response rates of 50% on the first 136 patients with *ALK*-rearranged NSCLC enrolled on PROFILE 1005[42] and secondarily on a response rate of 61% from the first 119 patients with *ALK*-rearranged NSCLC enrolled on PROFILE 1001[43]. Table 3 lists the major ongoing trials with crizotinib for advanced NSCLC.

New ALK inhibitors are under investigation, with phase I trials of LDK378 (not yet recruiting) and AP26113 (currently recruiting). NCT01449461, a phase I trial of AP26113, will be conducted in two parts, with the second part including expansion cohorts. The 4 cohorts include ALK mutations with no previous exposure to ALK inhibitors, ALK mutation with resistance to an ALK inhibitor, EGFR mutation with resistance to EGFR inhibitors, and non-lung malignancies with ALK mutations.

KRAS and BRAF mutations and MEK inhibition

Mutations in KRAS have been found in 15%-30% of patients with NSCLC and are considered to be one of the more frequent mutations in these tumors[44,45]. Approximately 97% of K-RAS mutations in NSCLC involve codons 12 or 13[46]. As with EGFR mutations, KRAS mutations are detected mainly in lung adenocarcinomas and are less frequently observed in squamous cell carcinomas of the lung[47,48]. In contrast with lung adenocarcinomas harboring EGFR mutations, tumors having KRAS mutations are seen at a higher frequency (20%-30%) in Caucasian patients than in East Asian patients (5%)[49]. Also, compared with EGFR mutations, KRAS mutations are more common in current or former smokers than in never-smokers[50].

Although the value of KRAS status as a prognostic and predictive biomarker for anti-EGFR therapy is less clear in NSCLC, several studies have demonstrated that KRAS mutations are a factor correlated with poor survival in patients with NSCLC[51-53]. A recent prospective biomarker-driven phase Ⅲ trial conducted in 889 patients comparing placebo with sequential erlotinib maintenance in unresectable NSCLC (SATURN, BO18192) showed that the presence of KRAS mutations was not predictive for erlotinib efficacy and was prognostic significantly associated with reduced PFS[54]. The predictive significance of KRAS mutation status is being further evaluated in BATTLE-2 clinical trial.

Table 3 Major ongoing clinical trials with crizotinib for advanced non-small cell lung cancer[1]

Trial number	Phase	Study design	Key entry criteria	PE
PROFILE 1007 (NCT00932893)	Ⅲ	Crizotinib *vs* Pem or Doc as second-line	ALK(+) and 1 prior platinum-based chemo	PFS
PROFILE 1014 (NCT01154140)	Ⅲ	Crizotinib + Pem + Cis/Carbo *vs* Pem + Cis/Carbo as first-line	ALK(+) and chemotherapy-naive	PFS
PROFILE 1005 (NCT00932451)	Ⅱ	Crizotinib *vs* placebo as third-line	ALK(+) and PD in arm B of study PROFILE 1007	RR
PROFILE 1001 (NCT00965731)	Ⅰ/Ⅱ	Crizotinib + erlotinib *vs* erlotinib as second or third-line	Adenocarcinoma NSCLC and 1-2 prior chemo	MTD
PROFILE 1001 (NCT01121575)	Ⅰ	Crizotinib + PF0299804	Acquired resistance to erlotinib or gefitinib	MTD

[1]Data available at URL: http://www.cancer.gov/clinicaltrials. chemo: Chemotherapy; Pem: Pemetrexed; Doc: Docetaxel; Cis: Cisplatin; Carbo: Carboplatin; PD: Progressive disease; NSCLC: Non-small cell lung cancer; ALK: Anaplastic lymphoma kinase; PFS: Progression-free survival; RR: Response rate; MTD: Maximum tolerated dose; PE: Primary endpoint.

BRAF encodes a non-receptor serine/threonine kinase that is a member of the Ras/MAPK signaling pathway downstream of Ras protein. Upon activation, BRAF directly phosphorylates MEK, which in turns phosphorylates ERK, thereby regulating cellular responses to growth signals[55]. BRAF mutations were first identified in melanoma cells, with 80% of mutations involving the Val600 residue in the kinase domain. By contrast, BRAF mutations account for only 1%-3% of NSCLC and they are mostly non-Val600Glu mutations including Gly468Ala and Leu596Val[56,57]. BRAF mutations were shown to be mutually exclusive with EGFR mutations within exons 18-21, KRAS codon 12 mutations, ERBB2 codon 20 mutations, and translocations in ALK[58]. Furthermore, V600E mutated NSCLCs showed a more aggressive tumor histology characterized by micropapillary features and were associated with poor prognosis[59].

A number of studies are currently examining the effect of MEK inhibitors on BRAF or KRAS-mutated solid tumors. As a downstream effector of the EGFR pathway that signals through K-RAS, MEK inhibition has also been suggested to play a role in patients who become resistant to EGFR inhibitors. A number of trials to examine MEK inhibitors alone or in combination with other targeted treatments are currently recruiting. The NCT00888134 phase Ⅱ trial is examining the effects of MEK inhibitor AZD6244 in patients with metastatic malignancy and a BRAF mutation. Dasatinib was shown to selectively induce senescence in NSCLC cells with inactivating BRAF mutations[60]. The NCT01514864 phase Ⅱ trial is now recruiting patients to examine the effect of dasatinib in patients with NSCLC or melanoma harboring a BRAF mutation (Clinicaltrials.gov).

GSK2118436 is a potent MEK inhibitor that has been shown to have preclinical activity in BRAF mutant NSCLC and melanoma. A phase Ⅱ trial (NCT01336634) is currently recruiting patients with previous exposure to platinum chemotherapy, and will examine GSK2118436 in advanced NSCLC patients with a BRAF mutation. The primary outcome will be ORR, and the trial is expected to be completed in late 2013. A phase Ⅰ trial (NCT01324258) of GSK1120212, another potent MEK inhibitor, in combination with gemcitabine is currently recruiting patients with solid tumors in Japan. An Open-Label, Phase Ⅰ / Ⅰb Dose Escalation Study to assess the safety and tolerability of GSK1120212 in combination with docetaxel, erlotinib, pemetrexed, pemetrexed + carboplatin, pemetrexed + cisplatin, or nab-paclitaxel in patients with advanced metastatic lung and/or pancreatic cancers is currently recruiting patients (NCT01192165). A number of phase Ⅰ trials are currently examining the combination of MEK162, a MEK1/2 inhibitor, with PI3K (BYL719) or Raf (Raf265) inhibitors in advanced solid tumors with documented KRAS or BRAF mutations (NCT01449058, and NCT01352273). Selumetinib (AZD6244, a potent MEK inhibitor) is being investigated in NSCLC patients with tumors harboring KRAS mutations[52]. Table 4 lists the ongoing clinical trials involving targeted agents for patients with advanced or metastatic NSCLC.

OVERCOMING ACQUIRED DRUG RESISTANCE TO EGFR TARGETED THERAPIES IN NSCLC

Despite the significant improvement in outcomes for these highly selected patients, treatment failures secondary to resistance have been described since 2005[61]. Known mechanisms of resistance include secondary EGFR mutations (T790M mutant) or persistent phosphorylation of EGFR that reduces the inhibitory ability of gefitinib or erlotinib, and MET amplification with subsequent activation of downstream pathways[61,62]. The discovery of resistance to the EGFR-TKIs has led to the development of second-generation EGFR-TKIs, or the use of combination of EGFR inhibitors with other targeted therapies. Moreover, a third generation of EGFR-TKIs is now entering clinical trials; these compounds bind covalently to the ATP-binding cleft of mutant EGFR and appear to have selective activity against the T790M mutant[63].

Second-generation EGFR-TKIs

Many trials have studied intensification of EGFR inhibition through use of second-generation TKIs such as neratinib, afatinib, and dacomitinib[64]. These inhibitors are different from erlotinib and gefitinib in 2 main ways: each forms a covalent, irreversible bond with the EGFR protein, and each also inhibits other members of the ERBB family of kinases[64].

Dacomitinib (PF0299804): PF0299804 is an oral irreversible inhibitor of the EGFR/HER1, HER2, and

Table 4 Ongoing phase Ⅱ/Ⅲ clinical trials involving targeted agents for patients with advanced or metastatic non-small cell lung cancer

Study design	Clinical trial ID	Phase	Status	Key entry criteria
EGFR inhibition				
Erlotinib *vs* docetaxel	NCT00637910	Ⅲ	Recruiting	WT EGFR, prior platinum chemo, no prior taxanes
Erlotinib *vs* pazopanib	NCT01027598	Ⅱ	Active, not recruiting	1 prior chemo
Erlotinib + OSI-906	NCT01221077	Ⅱ	Recruiting	EGFR mutation (+), chemotherapy-naive
Erlotinib + ARQ197	NCT01377376	Ⅲ	Recruiting	WT EGFR, prior platinum-based chemo
Erlotinib + ARQ197	NCT01244191	Ⅲ	Recruiting	2 prior lines of chemo
Erlotinib + PC + Bev	NCT00976677	Ⅱ	Active, not recruiting	Non-squamous, nonsmokers
Gefitinib (maintenance)	NCT01404260	Ⅲ	Active, not recruiting	Stable disease after chemo, EGFR unknown, never or light smokers
Gefitinib *vs* Pem	NCT00891579	Ⅱ	Recruiting	WT EGFR, prior platinum-based chemo
Afatinib	NCT00525148	Ⅱ	Active, not recruiting	EGFR mutation (+)
Afatinib	NCT00711594	Ⅱ	Active, not recruiting	Prior platinum-based chemo, progressed after erlotinib or gefitinib
PF00299804	NCT01000025	Ⅲ	Recruiting	1 prior chemo
PF00299804 *vs* erlotinib	NCT01360554	Ⅲ	Recruiting	1 prior chemo
BRAF inhibition				
AZD6244 + erlotinib	NCT01229150	Ⅱ	Recruiting	KRAS WT or KRAS mutant
Dasatinib	NCT01514864	Ⅱ	Recruiting	Tumors harboring DDR2 mutation or inactivating B-RAF mutation
AKT inhibition				
MK-2206 + erlotinib	NCT01294306	Ⅱ	Recruiting	Progressed after initial response to erlotinib
MEK inhibition				
GSK2118436	NCT01336634	Ⅱ	Recruiting	BRAF mutation (+)
HDAC inhibitor				
Vorinostat + gefitinib	NCT01027676	Ⅱ/Ⅲ	Recruiting	prior platinum-based chemo
Vorinostat + bortezomib	NCT00798720	Ⅱ	Completed recruiting	2 prior chemo
Belinostat + Bev + PC	NCT01090830	Ⅱ	Recruiting	Chemotherapy-naive
LBH589 + Pem	NCT00907179	Ⅱ	Recruiting	1 prior chemo
KRAS mutations				
AZD6244 + erlotinib	NCT01229150	Ⅱ	Recruiting	Prior platinum-based chemo
Erlotinib + ARQ197 *vs* single-agent chemo	NCT01395758	Ⅱ	Recruiting	KRAS mutation (+)
GSK1120212 *vs* docetaxel	NCT01362296	Ⅱ	Recruiting	KRAS mutation (+)

PC: Paclitaxel and carboplatin; Bev: Bevacizumab; Pem: Pemetrexed; NSCLC: Non-small cell lung cancer; chemo: Chemotherapy; WT: Wild-type; EGFR: Epidermal growth factor receptor; HDAC: Histone deacetylase inhibitor.

HER4 tyrosine kinases. Preclinical data showed activity for PF0299804 against EGFR mutations and T790M[61,65]. Two phase Ⅱ studies highlighted the agent's clinical antitumor effect, both in first-line therapy and in treatment-refractory settings. In the first of the studies, PF0299804 was compared with erlotinib[66]. That trial enrolled a range of molecular subgroups, including a group of patients with wild-type KRAS. In all subgroups, PF0299804 showed a PFS advantage (12.4 wk *vs* 8.3 wk; HR: 0.704; $P = 0.030$). In the second phase Ⅱ trial, dacomitinib demonstrated significantly improved PFS over erlotinib (2.86 mo for patients treated with dacomitinib and 1.91 mo for patients treated with erlotinib, HR: 0.66; 95%CI: 0.47-0.91; $P = 0.012$), with an acceptable toxicity. PFS benefit was observed in most clinical and molecular subsets, notably KRAS wild-type/EGFR any status, KRAS wild-type/EGFR wild-type, and EGFR mutants[67].

Afatinib: Afatinib has been shown to suppress the kinase activity of wild-type and activated EGFR, including erlotinib-resistant isoforms with the T790M mutation. The phase Ⅱb/Ⅲ LUX-Lung 1 randomized, double-blind trial examined best supportive care plus afatinib or placebo in patients in whom chemotherapy and a reversible EGFR inhibitor had failed. No difference in OS was observed; however, PFS was significantly improved with afatinib (3.3 mo *vs* 1.1 mo; HR: 0.38; 95%CI: 0.306-0.475; $P < 0.001$), as were tumor-related symptoms and QoL[68]. The most exciting clinical trial of afatinib in the acquired-resistance setting was a phase I b study in the United States and Netherlands. Patients who had progressed on erlotinib or gefitinib were given afatinib and cetuximab. Approximately 94% of patients, regardless of T790M mutation status, had a partial response or stable disease[69].

A number of phase Ⅱ trials continue to examine the safety and efficacy of afatinib as a second-line therapy. LUX-Lung 2 phase Ⅱ trial (NCT00525148) has completed enrollment of patients with activating EGFR mutations in whom first-line chemotherapy has failed. Similarly, LUX-Lung 4 phase Ⅰ/Ⅱ Japanese trial (NCT00711594) has completed accrual; results are awaited from this group of patients with first generation EGFR-TKI-resistant advanced NSCLC.

The phase Ⅲ LUX-Lung 3 trial reported the efficacy and safety data of first-line afatinib *vs* cisplatin and pemetrexed (PC) in patients with EGFR mutation-positive tumors. Treatment with afatinib led to a significantly prolonged PFS *vs* PC (median 11.1 mo *vs* 6.9 mo; HR: 0.58; 95%CI: 0.43-0.78; $P = 0.0004$). In 308 patients with common mutations (Del19/L858R), median PFS was 13.6 *vs* 6.9 mo, respectively (HR: 0.47; 95%CI: 0.34-0.65; $P < 0.0001$). ORR was significantly higher with afatinib (56%

vs 23%; $P < 0.0001$). Significant delay in time to deterioration of cancer-related symptoms of cough (HR: 0.60, $P = 0.0072$) and dyspnea (HR: 0.68, $P = 0.0145$) was seen with afatinib *vs* PC. Drug-related adverse events led to discontinuation in 8% (afatinib; 1% due to diarrhea) and 12% of patients (PC). Given the promising results of this pivotal trial, afatinib is now being compared with gefitinib as first-line treatment in patients with stage ⅢB/Ⅳ lung adenocarcinoma with EGFR activating mutations (LUX-Lung 7; NCT01466660).

Dual inhibitors

Increasing evidence has suggested that solid tumors have multiple salvage and resistance pathways that allow them to circumvent inhibition of a single signaling pathway[70]. In fact, EGFR is known to regulate the production of VEGF and other proangiogenic factors[71], and increased VEGF expression has been associated with resistance to EGFR inhibition in a human tumor xenograft model of NSCLC[72]. Thus, it is likely that blocking only one of these pathways will be insufficient for providing any meaningful therapeutic outcomes. Based on the logical strategy for improving anti-tumor efficacy by inhibition of multiple signaling pathways, a number of clinical trials are currently dual-inhibition strategies [*e.g.* mTOR, c-MET, PIK3CA, insulin-like growth factor 1 receptor (IGF-1R) or histone deacetylase (HDAC) inhibitor plus EGFR inhibitor].

Combination of EGFR and VEGF inhibitors: There have been promising results from combination of sorafenib with erlotinib. The combination has shown encouraging disease stabilizing effects with tolerable toxicity profiles[73-75]. In a randomized, double-blind, placebo controlled, phase Ⅱ trial in 168 patients with previously treated advanced NSCLC, sorafenib plus erlotinib was compared with erlotinib plus placebo. Overall, there were no significant differences in OS, PFS, or ORR between these two groups. However, in 67 patients with tumors bearing wild-type EGFR, sorafenib/erlotinib group showed a superior median PFS (3.38 mo in sorafenib/erlotinib group *vs* 1.77 mo in placebo/erlotinib group; $P = 0.018$) and a superior mean OS (8 mo for sorafenib/erlotinib *vs* 4.5 mo for placebo/erlotinib; $P = 0.019$)[74]. Another phase Ⅱ study evaluated sorafenib in combination with gemcitabine or erlotinib in 60 elderly patients with previously untreated advanced NSCLC[52]. ORR and median OS were 6.5% and 6.5 mo with sorafenib plus gemcitabine, and 10.3% and 12.6 mo with sorafenib plus erlotinib[75]. Similarly designed randomized phase Ⅱ/Ⅲ trials failed to show any improvement in OS from the addition of sunitinib to erlotinib (9.0 mo *vs* 8.5 mo with placebo plus erlotinib; HR: 0.922; 95%CI: 0.797-1.067)[74]. In a phase Ⅲ trial, the addition of bevacizumab to erlotinib suggested a non-significant OS benefit with the combined inhibition therapy in patients with EGFR-mutant tumors (median OS: 18 mo for bevacizumab plus erlotinib *vs* 12 mo for erlotinib; HR: 0.44; 95%CI: 0.11-1.67)[76].

A recent meta-analysis[77] evaluated the safety and efficacy of the combined inhibition of the VEGFR and EGFR signaling pathways with single-targeted therapy. Patients receiving combined inhibition therapy had a significant longer PFS than the group with single-targeted therapy (HR: 0.80; 95%CI: 0.67-0.95; $P = 0.011$). The combined therapy was associated with a non-significant 3% improvement in OS (HR: 0.97; 95%CI: 0.89-1.05; $P = 0.472$) confirming the previous studies. Also, no difference in the ORR between the study groups were detected (HR: 1.44; 95%CI: 0.95-2.18; $P = 0.085$). Subgroup analysis revealed that combined inhibition therapy using combination regimens was associated with statistically significant improvement in both ORR and PFS in the expense of increased toxicity in combined inhibition therapy. Currently, there is no evidence to support the use of combined inhibition of the VEGFR and EGFR signaling pathways in unselected patients with advanced NSCLC. Nonetheless, combined inhibition therapy may have a potential advantage in the treatment of advanced NSCLC compared with single inhibition therapy if the subsets of patients who may benefit from this treatment are well identified.

MET inhibitors: Investigation of resistance to current EGFR inhibitors has highlighted the role of the c MET/ALK pathway. MET amplification leads to EGFR-independent activation of the PI3K/Akt pathway through the activation of erbB3-dependent signaling and thereby could lead to EGFR inhibitor resistance[78,79]. Thus, combinations of EGFR and c-MET/ALK inhibitors hold potential for overcoming resistance[80].

The addition of c-MET inhibitor to erlotinib has demonstrated promising clinical activity in phase Ⅱ studies[81,82] when compared with erlotinib alone, particularly among patients with MET overexpression and non-squamous histology. The subset analyses of the trial by Spigel *et al*[82] suggested that METMab plus erlotinib was associated with increased PFS and OS as compared with erlotinib alone in patients with MET overexpression. In the study[81] comparing ARQ 197-209 (c-MET inhibitor) plus erlotinib *vs* erlotinib alone, a statistically significant improvement in OS was also found in non-squamous patients favoring ARQ 197-209 and erlotinib combination. In another randomized phase Ⅱ study[83] investigating second-line erlotinib with or without ARQ-197 in patients with advanced NSCLC, primary objective of the trial (PFS) was met in 167 patients (HR: 0.68, 95%CI: 0.47 to 0.98; $P < 0.05$) and the phase Ⅲ trial is ongoing[84]. Furthermore, albeit in a small subgroup of patients, that trial showed an advantage in terms of PFS for the combination of erlotinib and ARQ-197 in K-RAS-mutated, EGFR wild-type and c-MET amplified subjects.

HDAC inhibitors: The HDACs act to tighten the bond between histones and DNA, thus inhibiting gene transcription by blocking binding sites on promoters[55]. Inhibition of HDAC leads to induction of apoptosis in ma-

lignant cells[56]. Vorinostat is currently the furthest along in the development. A phase I trial (NCT00702572) with carboplatin, paclitaxel, bevacizumab and vorinostat for patients with advanced NSCLC is recruiting patients. A number of other phase I clinical trials to examine the effect of vorinostat with other targeted treatments including inhibitors of EGFR, mTOR, and a proteasome inhibitor, NP10052 are ongoing.

PI3K-AKT-mTOR inhibitors: One downstream mutation that has been described in lung cancers with acquired resistance to TKIs is in PIK3CA, a gene encoding a protein in the PI3K/AKT/mTOR pathway[85]. The PI3K/AKT pathway up-regulates mTOR in response to stimulation by growth factors[86]. Loss of inactivating mutations of phosphatase and tensin homolog (PTEN) results in a gain in function of the *PI3KCA* gene[87]. Phosphorylated AKT overexpression and loss of PTEN expression in NSCLC was shown to confer poor prognosis[88]. Phase Ⅱ study of everolimus (an oral mTOR inhibitor) plus erlotinib in previously treated patients with advanced NSCLC yielded a 11% difference in disease-control rate at 3 mo favoring the combination but did not meet the prespecified threshold to support a phase Ⅲ study[89]. Preclinical trials of PI3K inhibitors have shown efficacy, and research is ongoing[90,91]. A phase Ib trial is going to evaluate the combination of BYL719 (a selective inhibitor of PI3Kα) and the MEK inhibitor (MEK162). This international multicenter trial is not recruiting patients yet, but is expected to be completed by 2014 (NCT01449058).

IGF-1R inhibitors: Activation of the IGF-1R pathway has been noted as a consequence of EGFR inhibition in a variety of NSCLC cell lines, leading to cellular proliferation and evasion of apoptosis[92]. Studies have also documented heterodimerization of EGFR and IGF-1R in response to stimulation with either EGF or IGF-1, the ligands for the two receptors[91]. In a preclinical study, coinhibition of EGFR and IGF-1R resulted in synergistic growth inhibition of H1299NSCLCxenografts *in vivo* compared with treatment with erlotinib alone[93].

Unfortunately, the clinical studies have not been promising. A randomized phase Ⅱ study of erlotinib in combination with R1507 (a recombinant monoclonal antibody against IGF-1R) did not provide PFS or survival advantage over erlotinib alone in an unselected group of patients with advanced NSCLC[94]. The absence of therapeutic benefit with EGFR inhibitor in combination with an IGF-1R-targeted agent was further substantiated by other phase Ⅲ clinical trials[95,96]. The study evaluating the use of OSI-906 (IGF-1R TKI) in combination with erlotinib in patients with advanced NSCLC with activating mutations of the EGFR is ongoing but not actively recruiting patients (NCT01221077).

CONCLUSION

Recent research in NSCLC has focused on understanding the molecular abnormalities associated with NSCLC cell growth and proliferation and their impact on response to treatment and survival. In addition to histology, testing EGFR mutation and ALK rearrangement has now become the standard of care for treatment selection in NSCLC patients. However, only 20% of Western NSCLC patients have an activating EGFR mutation or ALK translocation[97]. Targetable molecular abnormalities have not yet been identified in approximately 80% of NSCLC patients. Multiple targeted agents, including monoclonal antibodies and receptor TKIs, are at various stages of development and hold promise. The results from ongoing trials will determine if the newer targeted agents will be incorporated into clinical practice.

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Statin a day keeps cancer at bay

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Abstract

In addition to cholesterol reduction, statins, currently the most commonly prescribed drug in the world, have been shown to have anti-neoplastic and immunomodulatory effects. Several observational studies and meta-analyses have shown reduction in risk of multiple cancers. More recently there has been an increasing interest in the potential role of statins as adjuvant therapy after cancer diagnosis and in modifying cancer mortality. Although post-hoc analyses of randomized controlled trials of statins for cardiovascular outcomes have not shown reduction in the risk of cancer mortality with statin use, these studies lack sufficient power to detect a significant difference in cancer outcomes. Recently, in a Danish nationwide population-based cohort study, Nielsen *et al* showed a 15% reduction in all-cause and cancer-specific mortality in statin users as compared to non-users. Improved survival with statin exposure was seen in 13/27 cancer subtypes, including the 4 most common cancers - lung, prostate, colorectal and breast. In this commentary, we examine this important study, review its implications and limitations, and briefly discuss impact of other drugs like metformin and aspirin that also exhibit anti-neoplastic effects.

Key words: Statins; Cancer; Mortality; Chemoprevention; Metformin

Core tip: With increasing economic burden of cancer care, cost-effective, preventive strategies are in focus. Commonly used drugs like statins, metformin and aspirin have been shown to have anti-neoplastic effects and are attractive candidates for cancer chemoprevention and reducing cancer-related mortality. Recently, in a Danish nationwide population-based cohort study, statin users had 15% reduction in all-cause and cancer-specific mortality as compared to non-users. These results are encouraging and show that statin use may be associated with reduced cancer mortality across different subgroups and cancer sites. However, several confounding variables remain, which merit further evaluation before this can change clinical practice.

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STATINS AND CANCER MORTALITY

Statins or 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors have been used for primary and secondary prevention of cardiovascular diseases and currently are among the most commonly prescribed medications in the world. Besides cholesterol reduction, pre-clinical studies have shown that statins may exert anti-neoplastic effects, through both HMG-CoA reductase-dependent and HMG-CoA reductase-independent pathways. By competitive inhibition of HMG-CoA reductase, statins prevent post-translational prenylation of the Ras/Rho superfamily, which are important mediators of cell growth, differentiation and survival[1]. In addition, statins exert proapoptotic, antiangiogenic, and immunomodulatory

effects, which may prevent cancer growth[1,2]. Indeed, several observational studies and meta-analyses have shown that statin use may be associated with reduced risk of prostate cancer[3], hepatocellular cancer[4], gastric cancer[5] and esophageal cancer[6] but not others[7,8]. More recently, there has been greater interest in the potential role of statins in modifying cancer outcomes and mortality. Early data from post-hoc individual patient data meta-analysis of randomized controlled trials (RCTs) of statins for cardiovascular outcomes has not shown reduction in the risk of cancer mortality with statin use, but these studies are limited by short follow-up and insufficient power to detect a significant difference in cancer outcomes between placebo and statin group[9].

In the November issue of the *New England Journal of Medicine*, Nielsen *et al*[10] studied the relationship between statin use (prior to cancer diagnosis) and cancer-related mortality in the entire Danish population from 1995-2009 in adults > 40 years of age. Through record linkage between the Danish Registry of Medicinal Products Statistics (which records information on all drugs dispensed from Danish pharmacies), the Danish Cancer Registry (which tracks data on 98% of all incident cancers in Denmark) and the Danish Register of Causes of Death, in 1072503 person-years of follow-up on 295925 patients with incident cancer, they observed 195594 deaths, of which 162067 were cancer-related. As compared to statin non-users, patients using statins prior to cancer diagnosis were 15% less likely to die from any cause [adjusted hazard ratio (HR): 0.85; 95%CI: 0.83-0.87] or cancer specifically (adjusted HR: 0.85; 95%CI: 0.82-0.87). On evaluating risk of death from 27 individual cancers comparing 18721 statin users and 277204 statin non-users, they observed improved survival with statin exposure for 13 cancers, including the 4 most common cancers - lung (adjusted HR: 0.87; 95%CI: 0.83-0.92), colorectal (adjusted HR: 0.79; 95%CI: 0.75-0.85), prostate (adjusted HR: 0.81; 95%CI: 0.75-0.88) and breast (adjusted HR: 0.88; 95%CI: 0.80-0.99). The hazard ratios for cancer death in statin users ranged from 0.64 (95%CI: 0.46-0.88) for cervical cancer to 0.89 (95%CI: 0.81-0.98) for pancreatic cancer. These results were stable across a nested 1:3 matched case-control study of statin users *vs* statin non-users with matching for sex, age at cancer diagnosis, cancer type and year of diagnosis to adjust for the evolving cancer treatments and increasing use of statins over the follow up period. Their robust study design adjusted for multiple confounding factors including age at diagnosis, sex, level of education, residential area, cancer stage, presence of cardiovascular disease or diabetes before cancer diagnosis and whether they received chemotherapy and/or radiotherapy. They also accounted for probability of prescribing statins through propensity score analysis.

Despite the comprehensive nature of the analysis and well thought out adjustments for confounding factors, several important limitations remain. Firstly, no data was available on smoking that affects cancer incidence and related mortality. Conceivably patients may stop smoking after starting statin for a recent acute myocardial infarction, which may favorably modify the relationship between statin use and mortality from smoking-related cancers. Secondly, the healthy user effect and the healthy adherer effect needs to be considered while interpreting the results of this study. Studies[11] have shown that doctors may selectively under-prescribe lipid-lowering agents to smokers or obese patients, because of their unhealthy lifestyle, both of which are associated with increased all-cause and cancer mortality[12,13]. Statin users are more likely to be health-conscious and be more compliant with cancer screening leading to early cancer detection and treatment, translating into improved survival. This may partially be addressed by the study adjusting for cancer stage (tumor size and spread to the lymphatic system), but as nearly one-third of the patients in the statin use group and three-quarters of the no-statin use group had missing data pertaining to tumor size and lymphatic spread, residual confounding cannot be completely excluded. Also, no data is provided in terms of incident cancers or mode of cancer diagnosis - it is plausible that more cancers in the statin users were detected on screening exams in asymptomatic individuals. Besides early diagnosis, statin use prior to cancer diagnosis may also reduce the risk of cancer metastases. *In vitro* studies have shown that lipophilic statin use may reduce the formation and spread of metastatic prostate colonies[14]. This reduction in the risk of cancer metastases has also been observed with aspirin use, and has been implicated in the early reduction in cancer deaths observed in trials of daily aspirin *vs* control[15].

Thirdly, the study does not take into account the potential for concomitant use of other drugs with known anti-proliferative activity and anti-neoplastic potential. Statin users in the study had a significantly higher proportion of patients with cardiovascular disease (70% *vs* 21%, $P < 0.001$) and diabetes mellitus (18% *vs* 3%, $P < 0.001$) and conceivably would have a disproportionately higher use of aspirin or metformin that could have led to significant confounding. The authors do report that a sensitivity analysis excluding patients with cardiovascular disease (which is the only indication for aspirin use with statins in Denmark) produced results similar to the main finding, which adjusts for the impact of aspirin use. Aspirin as well as anti-diabetic medications like metformin use has been associated with reduced cancer-related mortality[15-18] and cancer risk[19-21]. In a post-hoc individual patient data meta-analysis of 51 RCTs, aspirin users were 15% less likely to die from cancer (OR = 0.85; 95%CI: 0.76-0.96), with a more profound effect seen with > 5 years of aspirin use (OR = 0.63; 95%CI: 0.49-0.82)[22]. Aspirin may inhibit cancer cell proliferation and promote apoptosis through cyclo-oxygenase 2 (COX2) mediated and COX2 independent effects[23]. Likewise, metformin use may improve colorectal cancer mortality in observational studies[17], with its anti-neoplastic effects being mediated by activation of adenosine monophosphate-activated protein kinase and consequent inhibition of the

mammalian target of rapamycin pathway, a downstream effector of growth factor signaling which is frequently activated in malignant cells[24]. In addition, metformin may also inhibit cell growth and promote cell senescence by inhibiting cyclin D1 expression and pRb phosphorylation[25].

Additionally, while Nielsen *et al* identified a consistent reduction in mortality across various cancer types and various sub-groups of patients, there was no clear dose-response relationship with statin use. The reduction in all-cause mortality was similar in patients with defined daily dose of statins of 0.01-0.75 (HR: 0.82; 95%CI: 0.81-0.85), 0.76-1.50 (HR: 0.87; 95%CI: 0.83-0.89) and > 1.50 (HR: 0.87; 95%CI: 0.81-0.91). This partially could be accounted for by the increased cardiovascular mortality of patients who were on higher defined daily dose of statins, however, there was similar lack of gradient even for cancer-related mortality. This could be secondary to a threshold effect but based on Hill's criteria for causality, presence of a biological gradient or dose-response effect helps to strengthen a causal association. Moreover, they have not explored the potential effects of statins as adjuvant therapy after cancer diagnosis and this merits further evaluation. Lastly, as 97% of their study population was comprised of white persons of Danish descent, their results are not generalizable to other ethnic populations, especially in United States.

In conclusion, statins are gaining traction for multiple non-cardiac indications including cancer. The results of this large nationwide observational study are encouraging and show that statin use may be associated with reduced cancer mortality across different subgroups and cancer sites. However, there are several confounding variables which merit further evaluation and it still is a long way from changing clinical practice. Although cancer risk and mortality have been studied in secondary analyses of many RCTs to assess the efficacy of statins for cardiovascular indications[9,26,27], clinical trials evaluating cancer as primary outcome are lacking. Well-designed, prospective, randomized trials of statins with cancer incidence or mortality as the primary endpoint are needed to confirm or refute these findings. These must take into account various other factors that tend to cluster in statin users and may independently modify cancer risk. Certainly, focusing on high risk populations or patients with pre-existing cancer may be a first step towards the right direction. Nonetheless, as we await data from ongoing RCTs where statins are being investigated for primary cancer prevention (NCT01500577), preventing recurrent cancer (NCT01011478) or reduced cancer mortality when combined with conventional chemotherapy for different cancers (NCT00433498 and NCT01238094), Nielsen *et al*' notable data moves us probably another step closer to broadening recommendations for statin use. Statins as well as other commonly used and safe drugs like metformin and aspirin may cause a paradigm shift in how we approach cancer prevention and treatment in the years to come.

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BRIEF ARTICLE

Combined chemo-radiotherapy in locally advanced nasopharyngeal carcinomas

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Abstract

AIM: To provide efficacy and safety data about the combined use of radiotherapy and chemo-radiotherapy in nasopharyngeal carcinoma (NPC).

METHODS: We reviewed data of 40 patients with locally advanced NPC treated with induction chemotherapy followed by concomitant chemo-radiotherapy (CCRT) (22/40 patients) or CCRT alone (18/40) from March 2006 to March 2012. Patients underwent fiberoscopy with biopsy of the primitive tumor, and computed tomography scan of head, neck, chest and abdomen with and without contrast. Cisplatin was used both as induction and as concomitant chemotherapy, while 3D conformal radiation therapy was delivered to the nasopharynx and relevant anatomic regions (total dose, 70 Gy). The treatment was performed using 6 MV photons of the linear accelerator administered in 2 Gy daily fraction for five days weekly. This retrospective analysis was approved by the review boards of the participating institutions. Patients gave their consent to treatment and to anonymous analysis of clinical data.

RESULTS: Thirty-three patients were males and 7 were females. Median follow-up time was 58 mo (range, 1-92 mo). In the sub-group of twenty patients with a follow-up time longer than 36 mo, the 3-year survival and disease free survival rates were 85% and 75%, respectively. Overall response rate both in patients treated with induction chemotherapy followed by CCRT and in those treated with CCRT alone was 100%. Grade 3 neutropenia was the most frequent acute side-effect and it occurred in 20 patients. Grade 2 mucositis was seen in 29 patients, while grade 2 xerostomia was seen in 30 patients. Overall toxicity was manageable and it did not cause any significant treatment delay. In the whole sample population, long term toxicity included grade 2 xerostomia in 22 patients, grade 1 dysgeusia in 17 patients and grade 1 subcutaneous fibrosis in 30 patients.

CONCLUSION: Both CCRT and induction chemotherapy followed by CCRT showed excellent activity in locally advanced NPC. The role of adjuvant chemotherapy remains to be defined.

Key words: Nasopharyngeal carcinoma; Induction chemotherapy; Concurrent chemoradiotherapy; Adjuvant chemotherapy; Locally advanced disease

Core tip: Clinical data of 40 patients (33 males, 7 females) with locally advanced nasopharyngeal carcinoma (NPC) treated at two participating institutions from March 2006 to March 2012 were reviewed. Patients received either induction chemotherapy followed by concomitant chemo-radiotherapy (CCRT) (22/40 patients) or CCRT alone (18/40). Patients underwent fiberoscopy with biopsy of the primitive tumor, and a computed tomography scan of the head, neck, chest and abdomen with and without contrast. Cisplatin was used both as induction and as concomitant chemotherapy, while 3D conformal radiation therapy was delivered to the nasopharynx and node areas (total dose, 70 Gy). A complete response rate of approximately 95% was achieved both in patients treated with induction chemotherapy followed by CCRT and in those treated with CCRT alone. In the sub-group of twenty patients with a follow-up time longer than 36 mo, the 3-year survival and disease free survival rates were 85% and 75%, respectively. These results showed that both CCRT and induction chemotherapy followed by CCRT have excellent activity in locally advanced NPC. The role of adjuvant chemotherapy remains to be defined.

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a rare malignancy that arises from the epithelium of the nasopharynx. It is particularly frequent in Southeast Asia and can be classified into three histological types, namely nonkeratinizing squamous cell carcinoma, keratinizing squamous cell carcinoma and undifferentiated carcinoma[1,2]. NPC presents several features that differentiate it from other head and neck carcinomas, such as its prognosis and its association with the Epstein-Barr virus (EBV)[3]. While radiotherapy alone is associated with a 5-year disease free survival (DFS) of 95/100% in patients with early stage disease (T1,2aN0M0), locally advanced disease requires combined use of chemotherapy and radiotherapy[4-6]. Two large meta-analysis studies showed superiority of concurrent chemo-radiotherapy (CCRT) compared to radiotherapy alone[7,8]. The role of adjuvant chemotherapy remains controversial. A significant survival advantage was reported for CCRT followed by adjuvant chemotherapy with respect to radiotherapy alone in some trials[9,10], but it was not confirmed by others[11]. Neoadjuvant chemotherapy also appears to be a feasible option, since it may control subclinical metastatic foci, especially patients with locally advanced disease (T4b and/or N2/3). Although several phase Ⅱ and Ⅲ trials of induction chemotherapy followed by radiotherapy have been carried out, no conclusive evidence in favor of its efficacy is presently available[12-15].

In this retrospective analysis, we reviewed data of 40 patients with locally advanced NPC treated with induction chemotherapy followed by CCRT or CCRT alone .

MATERIALS AND METHODS

Patients selection

Data regarding patients with a histologically confirmed diagnosis of locally advanced NPC (T2bN0M0-T4bN3M0) and treated with chemotherapy and radiotherapy from March 2006 to March 2012 at the participating Institutions, were retrieved from reviewed charts. Patients underwent fiberoscopy with biopsy of the primitive tumor, and computed tomography (CT) scan of head, neck, chest and abdomen with and without contrast. A 18-fluoro-2-deoxy-*D*-glucose positron emission tomography (FDG-PET) scan was performed in selected patients according to the physician's judgment.

Treatment plan

Patients were treated with the either induction chemotherapy followed by CCRT (22 patients) or with CCRT alone (18 patients). Several cisplatin-based regimens were used for induction chemotherapy (Table 1). After induction chemotherapy, a CT scan of head, neck, chest and abdomen and a fiberoscopy were performed for re-staging. Patients receiving CCRT were treated with cisplatin (100 mg/m^2 on days 1, 22 and 43) and 3D conformal radiation therapy, which was administered concurrently in cycle 1.

The nasopharynx and other relevant anatomic regions were included in the treatment plan. Gross tumor volume (GTV), clinical target volumes (CTVs), planning target volume and planning organ at risk volumes were defined for each patient according to the reports 50 and 62 of the International Committee on Radiation Units and Measurements. The CTV-T included the GTV-T, the posterior third of the nasal cavity, the maxillary sinuses, the inferior sphenoidal body, the clivus and the pterygoid fossae. CTV-N was defined as the volume encompassing GTV-N (if macroscopic nodal metastases were present) and bilateral cervical lymph node stations (levels Ib-V), the medial supraclavicular fossae and retro/parapharyngeal spaces. In order to account for set-up errors and patient movements, two sets of planning target volumes were also defined by adding a 5 mm margin to each corresponding CTV. A total dose of 70 Gy was planned. The treatment was performed using 6 MV photons of the linear accelerator administered in 2 Gy daily fraction for five days weekly. In all patients, an electron beam boost (8-10 MeV) was administered to limit the dose to spinal cord. Late toxicity was graduated according to the Radiation Therapy Oncology Group guidelines for toxicity.

Response assessment

Patients underwent a fiberoscopy and a FDG-PET scan 60-90 d after radiotherapy, while a CT scan of head,

Table 1 Patients characteristics

Characteristic	Patients
Total	40
Male	33
Female	7
Age, yr, median (range)	60 (24-82)
Stage	
Ⅱb	3
Ⅲ	18
Ⅳa	15
Ⅳb	4
ECOG performance status	
0	36
1	4
2	0
Treatment performed induction CT followed by	
CCRT[1]	22
CCRT[2]	18
Total	40
Induction chemotherapy scheme	
PF[3]	9
TPF[4]	3
TP[5]	9
BMC[6]	1
Total	22
Total radiation dose delivered	
70 Gy	36
68 Gy	3
66 Gy	1
Histology squamous cell	
G1	1
G2	2
G3	4
Undifferentiated	33

[1]Induction chemotherapy followed by concomitant chemo-radiotherapy; [2]Concurrent chemoradiotherapy; [3]Cisplatin (100 mg/m^2 every 3 wk) and 5-fluorouracil (5-FU) (1000 mg/m^2 per day, 4-d continuous infusion every 3 wk); [4]Docetaxel (75 mg/m^2 every 3 wk), cisplatin (75 mg/m^2 every 3 wk) and 5-FU (750 mg/m^2 per day, 4-d continuous infusion every 3 wk); [5]Docetaxel (75 mg/m^2 every 3 wk) and cisplatin (75 mg/m^2 every 3 wk); [6]Bleomycin (25 mg/m^2 on days 1 and 8 of a 21-d cycle), methotrexate (35 mg/m^2 weekly) and cisplatin (80 mg/m^2 every 3 wk). ECOG: Eastern Cooperative Oncology Group; CT: Computed tomography.

neck, chest and abdomen with and without contrast was performed 45-50 d after completion of radiotherapy. The response evaluation criteria in solid tumors criteria were used to define response.

This retrospective analysis was approved by the review boards of the participating institutions. Patients gave their consent to treatment and to anonymous analysis of clinical data.

RESULTS

Patients characteristics

Forty patients (33 males and 7 females) were included in this analysis. Median age was 60 years (range, 24-82 years). The majority of patients had an undifferentiated carcinoma (33 patients, 82.5%) and a stage Ⅲ-Ⅳ disease (37 patients, 92.5%). Patients' characteristics are detailed in Table 1.

Table 2 Results *n* (%)

Treatment performed	Results
ORR	40 (100)
Total (all group)	
Induction chemotherapy followed by	
CCRT[1] group	22 (100)
CCRT[2] group	22 (100)
CR rate	
Induction chemotherapy followed by	38 (95)
CCRT[1] group	21 (95.5)
CCRT[2] group	17 (94.4)
3-yr OS	
Total (all group)	17 (85)
3-yr DFS	
Total (all group)	15 (75)

[1]Induction chemotherapy followed by concomitant chemoradiotherapy; [2]Concurrent chemoradiotherapy. ORR: Overal response rate; CR: Complete response; OS: Overall survival; DFS: Disease free survival.

Response rate

All patients were evaluable for response after completion of the planned treatment. In patients receiving induction chemotherapy followed by CCRT, overall response rate (ORR) to induction chemotherapy was 90.9% (20/22), with a complete response (CR) rate of 36.4% (8/22). In this sub-group, after completion of chemoradiotherapy, ORR was 100% with a CR rate of 95.5% (21/22). In the CCRT only group, an ORR of 100% was obtained, with a CR rate of 94.4% (17/18).

Survival

Median follow-up time was 58 mo (range, 1-92 mo). At the time of the analysis, no patient had been lost to follow-up, six had died for the disease, twenty-eight were disease free, and the remaining six patients were alive with recurrent/persistent disease.

In the sub-group of 20 patients with a follow-up period > 3 years (12 treated with induction chemotherapy followed by CCRT, 8 treated with CCRT only), the 3 year overall survival and DFS rate were respectively 85% (17/20) and 75% (15/20). These results are detailed n Table 2.

Toxicity

Grade 3 neutropenia was the most frequent acute side-effect and it occurred in 20 patients. Grade 2 mucositis was seen in 29 patients, while grade 2 xerostomia was seen in 30 patients. Overall toxicity was manageable and it did not cause any significant treatment delay. In the whole sample population, long term toxicity included grade 2 xerostomia in 22 patients, grade 1 dysgeusia in 17 patients and grade 1 subcutaneous fibrosis in 30 patients.

DISCUSSION

NPC is highly chemo and radiosensitive, and an excellent disease control can be achieved using combined modal-

ity chemoradiation even in patients with locally advanced disease[1,2]. Presently, the benefit of adding neoadjuvant/adjuvant chemotherapy remains to be defined. Three large phase Ⅲ trials confirmed the superiority of CCRT followed by adjuvant cisplatin and 5fluorouracil *vs* radiotherapy alone[9-11]. Interestingly, a combined analysis of two large studies (NPC-9901 and the NPC-9902) revealed that the dose of cisplatin during the CCRT had a significant impact on locoregional control[16,17]. Despite patients included in this retrospective study did not receive adjuvant chemotherapy, a CR rate of approximately 95% a 3-year DFS rate of approximately 75% were obtained. These results are in line with published data and highlight the need of further phase Ⅲ trials to assess the role of adjuvant therapy.

One possible way to select better patients suitable for an adjuvant approach may be assessment of plasma EBV DNA levels. In fact, several data showed that EBV DNA levels correlated significantly with tumor load, recurrence rate and survival[18,19]. An early post-CCRT detection of high EBV DNA levels may be an indication to administer adjuvant chemotherapy.

One strategy to further improve the efficacy of chemotherapy is to use induction chemotherapy followed by radiation therapy alone or CCRT. Induction chemotherapy is generally better tolerated than adjuvant chemotherapy and might provide early eradication of distant micro-metastases[3], especially in patients with locally advanced disease (T4 and/or N2/3). In addition, induction chemotherapy could shrink the primary tumor to give wider margins for irradiation. In several phase Ⅱ clinical trials, induction cisplatin-taxane containing chemotherapy followed by radiotherapy or chemo- radiotherapy has been employed, with a median ORR of 94% and a 3-year DFS of 81%[20-22]. These results are in line with those reported here. One interesting strategy may include selection of NPC patients who are more likely to benefit from chemotherapy. Human papilloma virus positivity, high Ki-67 value, absence of p53 mutation are strongly related to chemo and radiosensitivity in head and neck squamous cell carcinomas[23,24]. These factors should be explored in NPC also.

Patients included in this retrospective analysis received 3D conformal radiation therapy. Of note, intensity-modulated radiation therapy (IMRT) can improve dose conformity for complex tumor targets and is able to obtain a better protection of adjacent organs[25,26]. It is likely that IMRT will become the standard technique employed for head and neck malignant tumors.

In conclusion, our study confirms that concurrent chemoradiotherapy represents the standard treatment for patients with locally advanced NPC. The role of adjuvant chemotherapy following CCRT is not well defined and requires to be investigated in phase Ⅲ trials. Assessment of EBV DNA titers in patients treated with CCRT may be helpful to select patients requiring adjuvant chemotherapy.

COMMENTS

Background

Nasopharyngeal carcinoma (NPC) is a rare malignancy that has several distinct features with respect to other head and neck tumors. While radiotherapy alone is associated with a 5-year disease free survival of 95/100% in patients with early stage disease (T1, 2aN0M0), locally advanced disease requires combined use of chemotherapy and radiotherapy.

Research frontiers

Adjuvant and neoadjuvant chemotherapy may have a role for the treatment of locally advanced NPC.

Innovations and breakthroughs

Three large phase Ⅲ trials confirmed the superiority of concurrent chemotherapy and radiotherapy followed by adjuvant chemotherapy *vs* radiotherapy alone in NPC.

Applications

Results obtained in this retrospective review confirm the effectiveness of combined use of chemotherapy and radiotherapy in locally advanced NPC. The role of adjuvant chemotherapy remains to be ascertained.

Terminology

Epstein-Barr virus is a virus of the herpes family that is best known as the cause of infectious mononucleosis, but it is also associated with human malignancies, such as NPC and lymphomas. Intensity-modulated radiation therapy is an advanced type of radiation therapy that uses multiple small radiation beams of varying intensities to radiate a tumor in a precise way. It is considered to be more accurate than 3D conformal radiation therapy.

Peer review

The article is a retrospective study about the efficacy of induction chemotherapy in the context of chemoradiotherapy for locally advanced NPC and serves to confirm what has been published in several large studies and in some meta-analysis.

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BRIEF ARTICLE

Non-AIDS-related Kaposi's sarcoma: A single-institution experience

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Abstract

AIM: To evaluate the outcomes and potential prognostic factors in patients with non-acquired immunodeficiency syndrome (AIDS)-related Kaposi's sarcoma (KS).

METHODS: Patients with histologically proven non-AIDS-related KS treated with systemic chemotherapy were included in this retrospective analysis. In some cases, the human herpes virus 8 status was assessed by immunohistochemistry. The patients were staged according to the Mediterranean KS staging system. A multivariable model was constructed using a forward stepwise selection procedure. A P value < 0.05 was considered statistically significant, and all tests were two-sided.

RESULTS: Thirty-two cases were included in this analysis. The average age at diagnosis was 70 years, with a male/female ratio of approximately 2:1. Eighty-four percent of the cases had classic KS. All patients received systemic chemotherapy containing one of the following agents: vinca alkaloid, taxane, and pegylated liposomal doxorubicin. Ten patients (31.5%) experienced a partial response, and a complete response was achieved in four patients (12.4%) and stable disease in sixteen cases (50%). Two patients (6.2%) were refractory to the systemic treatment. The median progression-free survival (PFS) was 11.7 mo, whereas the median overall survival was 28.5 mo. At multivariate analysis, the presence of nodular lesions (*vs* macular lesions only) was significantly related to a lower PFS (hazard ratio: 3.09; 95%CI: 1.18-8.13, P = 0.0133).

CONCLUSION: Non-AIDS-related KS appears mostly limited to the skin and is well-responsive to systemic therapies. Our data show that nodular lesions may be associated with a shorter PFS in patients receiving chemotherapy.

Key words: Kaposi's sarcoma; Human herpes virus 8; Paclitaxel; Pegylated liposomal doxorubicin; Vinblastine

Core tip: Non-acquired immunodeficiency syndrome (AIDS)-related Kaposi's sarcoma (KS) is usually relatively benign, with an indolent disease course. It appears to be highly responsive to a wide variety of chemotherapy agents, including pegylated liposomal doxorubicin, vinca-alkaloids, etoposide and taxanes. However, fac-

tors predictive of progression-free survival are lacking. In our series of 32 patients with non-AIDS-related KS, we showed that presence of nodular lesions (*vs* macular lesions only) was associated with a 3-fold increased risk of progression. If confirmed by further studies, such a finding may be useful to improve the therapeutic strategy for this disease at the individual level.

Rescigno P, Di Trolio R, Buonerba C, De Fata G, Federico P, Bosso D, Virtuoso A, Izzo M, Policastro T, Vaccaro L, Cimmino G, Perri F, Matano E, Delfino M, De Placido S, Palmieri G, Di Lorenzo G. Non-AIDS-related Kaposi's sarcoma: A single-institution experience. *World J Clin Oncol* 2013; 4(2): 52-57 Available from: URL: http://www.wjgnet.com/2218-4333/full/v4/i2/52.htm DOI: http://dx.doi.org/10.5306/wjco.v4.i2.52

INTRODUCTION

Kaposi's sarcoma (KS) is a multifocal angioproliferative disorder of the vascular endothelium that usually presents itself with multiple vascular, cutaneous and mucosal nodules[1].

The four described clinical variants, *i.e.*, classic, endemic, iatrogenic and epidemic KS, show a distinct natural history and prognosis[2], but all share a causal relationship with human herpes virus 8 (HHV-8)[3]. Infection with this virus is a necessary condition, but it is not sufficient alone to cause KS, highlighting how genetic and angiogenic factors and the production of several inflammatory cytokines play a role in the multistep pathogenesis of KS[4].

As KS can be considered to be an opportunistic tumour, the restoration of immune competence is associated with remission in organ transplant recipients[5] and in acquired immune deficiency syndrome (AIDS)-related KS[6]. In classic KS, the cause of the underlying immunodeficiency is more difficult to identify and therefore to target by treatment.

Classic KS is a rare and mild form of the disease, primarily affecting men over 50 years old in endemic areas[7]. Lesions present themselves as purplish-red pigmented nodules on the legs and arms and tend to spread to more proximal sites[8]. The reported male-to-female ratio is 17:1[9]. Patients with classic KS have a greater risk to develop solid or haematopoietic neoplasms[10].

Iatrogenic KS is associated with the use of corticosteroids and other immunosuppressive agents[11]. The duration of immunosuppressive therapy does not seem to affect the risk of KS[12]. Iatrogenic KS more frequently involves the lymph nodes and viscera compared with classic KS[1,2].

The definition of the therapeutic strategy for KS depends on a number of factors, which include the location and variant of the KS, the pace of disease progression, the presence and severity of the symptoms (*e.g.*, pain and oedema), the number of lesions, the degree of host immune competence and comorbidities[2,7,13].

We present data about the treatment, response and outcome of 32 patients with non-AIDS-related KS treated with chemotherapy at our institution from January 2008 to December 2012.

MATERIALS AND METHODS

A retrospective review study of patients who received systemic treatment for classic or iatrogenic KS from January 2008 to December 2012 at the Division of Dermatology and Oncology of University Hospital Federico Ⅱ, Naples was performed. Informed consent for the anonymous publication of the data was obtained for all patients.

Patients who had histologically proven KS lesions of the skin and were negative for human immunodeficiency virus (HIV)-1/2 by macro enzyme immunoassay were included in this study. The histologic diagnosis required the presence of proliferative miniature vessels and tumour-like fascicles composed of spindle cells and a vascular network[1,2]. The HHV-8 status was assessed by immunohistochemistry using a monoclonal antibody against the latent nuclear antigen 1. Positivity for HHV-8 confirmed but was not strictly necessary for the diagnosis.

The tumour staging was performed with an ultrasound of the abdomen and the superficial lymph nodes, a chest X-ray and/or a whole body computed tomography scan. An esophagogastroduodenoscopy and rectosygmoidoscopy were performed in fit patients. Demographic features, such as origin, age at onset and gender, of the patients were retrieved. Data regarding the type, response and duration of the first systemic treatment delivered at our Institution and its related progression-free survival (PFS), overall survival (OS), comorbidities, number and extent of lesions and the presence of complications, such as lymphoedema, haemorrhage, pain, functional impairment and ulcerations, were also extracted from a review of the charts. The staging was performed according to criteria by Brambilla *et al*[14].

Five levels of the response to treatment were defined according to the revised World Health Organization criteria[15]: complete response, major response, minor response, stable disease and progression. All levels were based on the number of lesions: complete response, 100% resolution of the lesions; major response, > 50% to < 100% decrease; minor response, > 25% to < 50% decrease; stable disease, < 25% decrease to < 25% increase; and progression, > 25% increase in the number of lesions or worsening of the tumour-associated pain/oedema. Cox proportional hazards regression was used to investigate the prognostic factors of PFS and OS. A multivariable model was constructed using a forward stepwise selection procedure. A P value < 0.05 was considered statistically significant, and all tests were two-sided. All results are considered hypothesis-generating and require independent validation.

Table 1 Patient characteristics *n* (%)

	Patients number
Sex	
Male	21 (65.6)
Female	11 (34.4)
Comorbidities	
Diabetes	6 (18.7)
Alzheimer's	2 (6.3)
Hypertension	15 (46.9)
Kaposi variant	
Classic	27 (84.3)
Iatrogenic	5 (15.6)
Anatomic site	
Limbs	24 (75)
Limbs and trunk	5 (15.6)
Scrotum	1 (3.1)
Glans	1 (3.1)
Lymph node involvement	1 (3.1)
Number of lesions	
1	0
2	0
3	0
> 3	32 (100)
Stage	
Stage Ⅱb	18 (56.2)
Stage Ⅲ-Ⅳ	14 (43.7)

Table 2 Chemotherapy agents employed in the sample population *n* (%)

	Patients number
Sytemic treatment[1]	
Vinblastine	17 (53.1)
Pegylated liposomal doxorubicin	8 (25)
Paclitaxel	5 (15.6)
Gemcitabine	1 (3.1)
Vinorelbine	1 (3.1)
Overall number of lines of systemic treatment received by the patient	
1 line	26 (81.2)
> 1 line	6 (18.8)

[1]The first systemic treatment delivered at our institution is reported.

Table 3 Response to treatment *n* (%)

Response	
Complete response	4 (12.4)
Partial response	10 (31.5)
Stable disease	16 (50)
Progressive disease	2 (6.2)
Progression-free survival, mo (range)	11.7 (3-48)
Overall survival, mo (range)	28.5 (12-48)

Disease control rate is 93.7%.

RESULTS

Thirty-two cases of non-AIDS-related KS were included in this study. The mean age at diagnosis was 70 years. Twenty-one patients (65.6%) were male, and 11 (34.4%) were female, with an approximate male:female ratio of 2:1. All patients were Italian. With respect to the clinical subtype, 27 (84.3%) cases of classic KS and five cases (15.6%) of iatrogenic KS were included in this analysis. Of note, two patients with classic KS suffered from tumour-induced immunosuppression: one had B-cell lymphoma, and the other presented with Good's syndrome associated with a thymic epithelial tumour[16].

In particular, three patients were on immunosuppressive therapy due to an autoimmune disease (rheumatoid arthritis or systemic lupus erythematosus). The medication used included systemic corticosteroids and cyclosporin A. Two patients were on systemic corticosteroids due to severe chronic obstructive pulmonary disease. All 25 cases tested for HHV-8 were positive.

In 90.6% (n = 29) of the cases, the KS was limited to the skin. One patient (3.1%) presented mucosal lesions of the glans, and another case had axillary lymph node invasion. The KS lesions were multiple (> 3) in all patients (n = 32). The patient characteristics are detailed in Table 1.

All patients received systemic chemotherapy. The most frequently used drugs were vinblastine, pegylated liposomal doxorubicin (PLD) and paclitaxel. One patient (3.1%) affected by thymoma and KS received gemcitabine, capecitabine and immunoglobulins. The treatments that were administered are detailed in Table 2.

We obtained a disease control rate (93.8%), as shown in Table 3. The median PFS was 11.7 mo (range, 3-48 mo) (Figure 1), and the median OS was 28.5 mo (range, 12-48 mo) (Table 3).

Of note, the presence of nodular lesions was related to a lower PFS compared with macular lesions in both the univariate and multivariate analyses. The results of the Cox proportional hazard analysis are detailed in Table 4.

No death was directly related to KS. One patient, affected by Good's syndrome, died as a result of an opportunistic infection.

DISCUSSION

Classic KS is a rare disease. Its incidence is affected by factors such as sex, age and immune status. Interestingly, the geographic origin may affect the female to male ratio, as shown by the male to female ratio reported in our case series (2:1) and in a case series of 874 classic KS patients from 15 Italian Cancer Registries (3:2)[17], which appear to be markedly different from that reported in other studies conducted in distinct geographic areas[9,10].

Different routes of transmission have been hypothesised for HHV-8[17]. In addition to sexual transmission, a number of studies support a role for saliva as an infection route. The copy numbers of HHV-8 were higher in the saliva then in the semen in patients with and without KS, and these differences were independent of the HIV status. Oropharyngeal epithelial cells may harbour HHV-8 and facilitate its replication[18]. A potential role in HHV-8 transmission could be played by haematophagous insects (*e.g.*, malaria vector Anopheles, black flies, sand flies, biting midges and mosquitoes), which could explain

Table 4 Cox proportional hazard regression for progression-free survival

Characteristic	Hazard ratio (95%CI)	*P* value
Univariable		
Stage (Ⅱ *vs* Ⅲ/Ⅳ)	1.63 (0.74-3.57)	0.22
Cutaneous lesion (macules *vs* nodules)	3.09 (1.18-8.13)	0.01
Extent (lower limb only *vs* other parts of the body)	1.61 (0.72-3.59)	0.24
Symptoms (no *vs* yes)	0.72 (0.32-1.62)	0.44
Age	0.97 (0.93-1.01)	0.16
Sex (female *vs* male)	0.73 (0.32-1.69)	0.47
Multivariable		
Cutaneous lesion (nodular/papular/macules *vs* macules only)	3.09 (1.18-8.13)	0.013

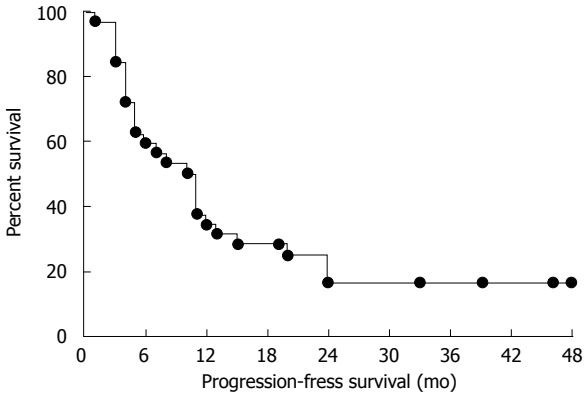

Figure 1 Kaplan-Meier plot of progression-free survival associated with the first line of systemic treatment delivered at our institution.

the high incidence in Italian areas where wetlands and swamps are widespread (*e.g.*, the Po delta and part of Sardinia) and malaria is epidemic[17]. Notably, the majority of our patients are elderly people from Campania, an area that used to be covered by wetlands.

Classic and iatrogenic KS mostly present themselves as multiple bilateral cutaneous lesions of the lower limbs[10]. We found that the lesions were multiple in 100% of the cases, as expected in a series of patients undergoing systemic treatment, and that the lesions involved the limbs in 75% of the cases. Only one patient with lymph-nodal disease was identified in our series.

One finding of interest was that the patients with nodular lesions appeared to display a more aggressive course of the disease, with an increased risk of progression compared with the patients with macular lesions in the multivariate analysis (hazard ratio: 3.09; 95%CI: 1.18-8.13; $P = 0.0133$). These data have not been reported previously in the literature.

A number of cytotoxic agents proved to be effective for the systemic treatment of recurrent, visceral, aggressive and widespread disease. These agents have not been tested in large, randomised-controlled trials[19]. The response rates (> 50% decrease in lesions) associated with the chemotherapy agents in classic KS ranged between 71% and 100% for PLD[20-22], 58% and 90% for vinca-alkaloids[23-25], 74% and 76% for etoposide[26], and 93% and 100% for taxanes[27,28]. Gemcitabine showed a response in 100% of the patients[29], and the combination of vinblastine and bleomycin was associated with a response rate of 97%[30].

All of these agents were employed in our patient population (PLD, vinca alkaloids, taxanes, and gemcitabine), with a remarkable overall disease control rate of 93.7%, which is in line with the literature data. At the time of the analysis, no patient had died as a direct consequence of KS, which confirmed the relatively benign behaviour of classic KS[31].

We performed immunohistochemical tests for HHV-8 staining on tissue samples of 25 patients (78.1%). All 25 patients (100%) were positive for infection.

These data suggest the high sensitivity of immunohistochemistry to detect HHV-8 infection, as previously reported in the literature[32].

In summary, in this study, KS nodular lesions appeared to be significantly associated with a decreased PFS in patients receiving chemotherapy. In sharp contrast to AIDS-related KS, classic and iatrogenic KS appear to have a more indolent course, being mostly limited to the skin and highly responsive to systemic therapeutic strategies.

The retrospective nature of this study and the small sample size mandate confirmation of our findings in further prospective trials.

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COMMENTS

Background

Non-acquired immunodeficiency syndrome (AIDS)-related Kaposi's sarcoma (KS) usually displays an indolent course, with a relatively benign behaviour of the disease. It is generally highly responsive to chemotherapy agents, including pegylated liposomal doxorubicin, vinca-alkaloids, etoposide and taxanes. However, some patients show a more aggressive course of the disease.

Research frontiers

Factors predictive of progression-free survival associated with chemotherapy are lacking and are required in this rare disease.

Innovations and breakthroughs

The multivariate analysis performed in our series of 32 patients with non-AIDS-related KS showed that the presence of nodular lesions (*vs* macular lesions only) is associated with a 3-fold increased risk of progression.

Applications

If confirmed by further studies, the presence of nodular lesions may be incorporated into the clinical decision-making process for the definition of the therapeutic strategy for this disease on an individual level.

Terminology

Juman herpes virus 8 stands for human herpes virus 8, a large double-stranded DNA virus that is the causative agent of KS.

Peer review

The paper by Rescigno *et al* evaluates outcomes and potential prognostic factors in patients with classic and iatrogenic KS. In this study the authors retro-

spectively reviewed all cases of non-AIDS related KS treated at their institution from January 2008 to December 2012. One finding of interest was that patients with nodular lesions appeared to display a more aggressive course of the disease, with an increased risk of progression compared to patients with macular lesions at multivariate analysis (HR: 3.09; 95%CI: 1.18-8.13; $P = 0.0133$). These data were not reported before in literature. The paper is well written and of interest for readers.

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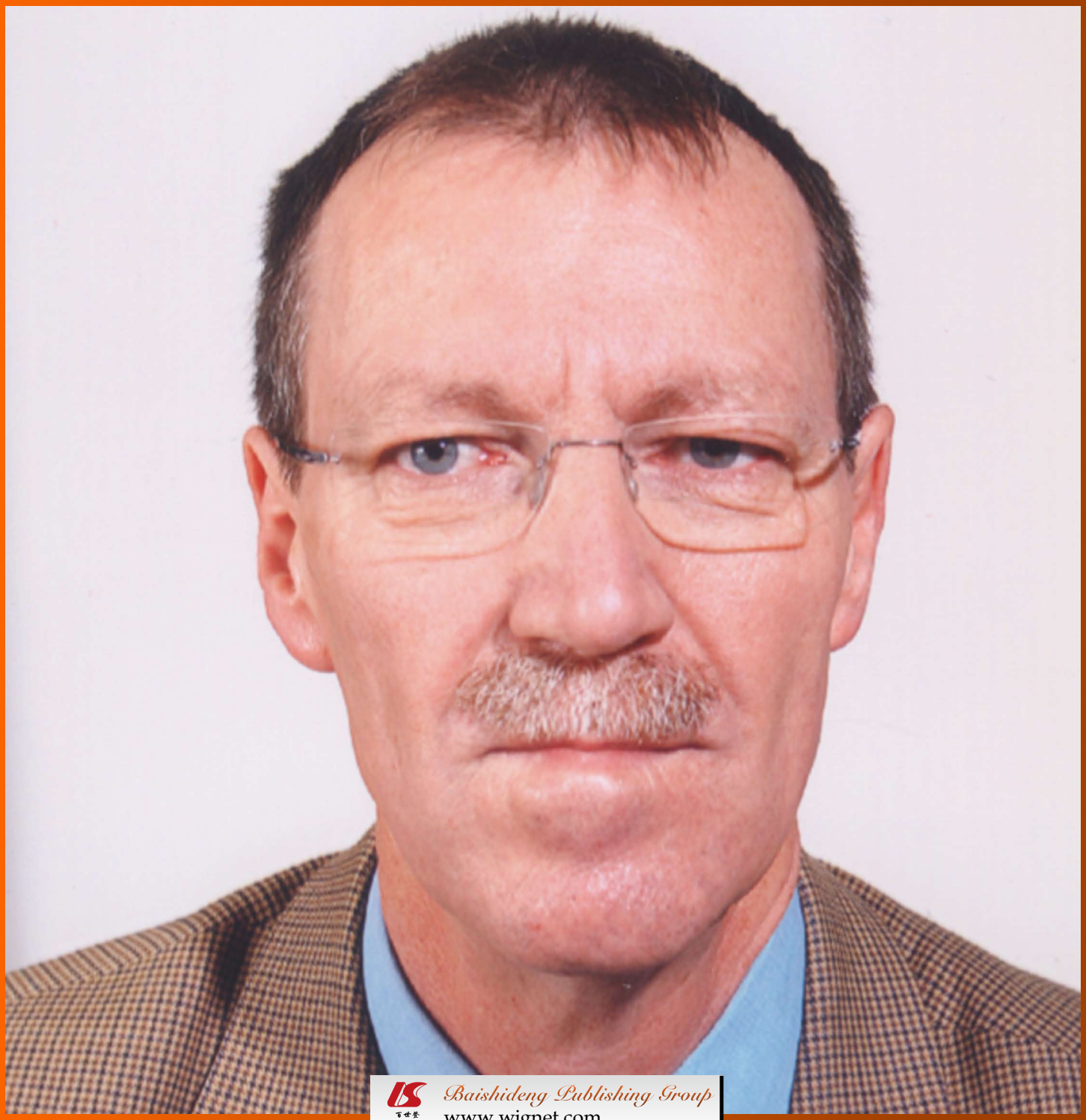

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REVIEW

Role of E3 ubiquitin ligases in lung cancer

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Abstract

E3 ubiquitin ligases are a large family of proteins that catalyze the ubiquitination of many protein substrates for targeted degradation by the 26S proteasome. Therefore, E3 ubiquitin ligases play an essential role in a variety of biological processes including cell cycle regulation, proliferation and apoptosis. E3 ubiquitin ligases are often found overexpressed in human cancers, including lung cancer, and their deregulation has been shown to contribute to cancer development. However, the lack of specific inhibitors in clinical trials is a major issue in targeting E3 ubiquitin ligases with currently only one E3 ubiquitin ligase inhibitor being tested in the clinical setting. In this review, we focus on E3 ubiquitin ligases that have been found deregulated in lung cancer. Furthermore, we discuss the processes in which they are involved and evaluate them as potential anti-cancer targets. By better understanding the mechanisms by which E3 ubiquitin ligases regulate biological processes and their exact role in carcinogenesis, we can improve the development of specific E3 ubiquitin ligase inhibitors and pave the way for novel treatment strategies for cancer patients.

Key words: E3 ubiquitin ligases; Lung cancer; Ubiquitin-proteasome system; Proteasome inhibitors; Bortezomib; Apoptosis; Gene regulation; DNA repair

Core tip: E3 ubiquitin ligases catalyze ubiquitination of proteins for degradation by the 26S proteasome. They are important for many biological processes including cell cycle regulation, proliferation and apoptosis. They are often overexpressed and deregulated in lung cancer, which contributes to cancer development. These processes underline their potential as anti-cancer targets. There is only one E3 ubiquitin ligase inhibitor in clinical trial. A better understanding of how E3 ubiquitin ligases regulate biological processes and of their exact role in carcinogenesis, will help to develop specific E3 ubiquitin ligase inhibitors to improve treatment strategies for cancer patients.

Snoek BC, de Wilt LHAM, Jansen G, Peters GJ. Role of E3 ubiquitin ligases in lung cancer. *World J Clin Oncol* 2013; 4(3): 58-69 Available from: URL: http://www.wjgnet.com/2218-4333/full/v4/i3/58.htm DOI: http://dx.doi.org/10.5306/wjco.v4.i3.58

INTRODUCTION

The ubiquitin-proteasome system (UPS) regulates multiple biological aspects of cell survival by mediating the degradation of targeted proteins and thereby maintaining cellular homeostasis[1]. In numerous cancer types, the deregulation of UPS components has been observed and their overexpression is often associated with chemoresistance and poor prognosis[2-5]. For example, the E3 ubiquitin ligase murine double minute 2 (MDM2), which is involved in the regulation of p53 levels, is frequently overexpressed in tumors and is predicted to be a nega-

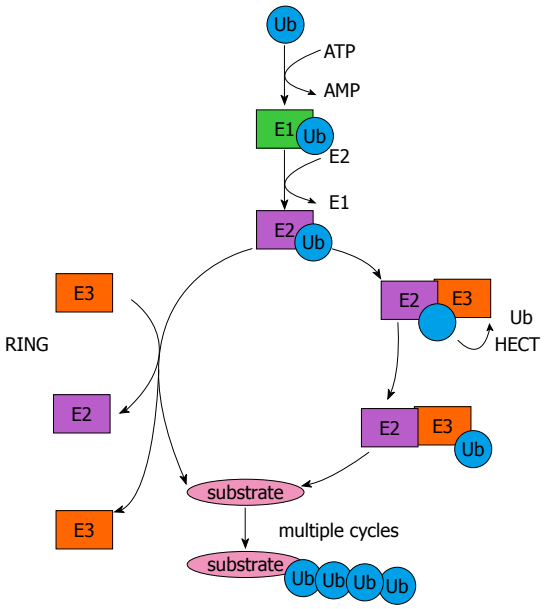

Figure 1 Ubiquitin molecules are covalently conjugated to targeted proteins in a three-step enzymatic cascade. First, an E1 ubiquitin-activating enzyme activates ubiquitin in an adenosine triphosphate (ATP)-dependent manner. Activated ubiquitin is then transferred to the E1 enzyme, followed by the transfer of ubiquitin to an E2 ubiquitin-conjugating enzyme. Finally, an E3 ubiquitin protein ligase recognizes the target proteins and mediates the conjugation of one or more ubiquitin molecules to a lysine residue on the targeted proteins. Really interesting new gene (RING) E3 ubiquitin ligases mediate the direct transfer of ubiquitin from E2 to the targeted substrate whereas Homologous to the E6-associated protein (E6-AP) carboxyl terminus (HECT) E3 ubiquitin ligases first interact with the cognate E2, followed by linkage with ubiquitin and subsequent transfer of ubiquitin to the targeted substrate. AMP: Adenosine monophosphate.

tive prognostic marker for the development of several human cancers including breast carcinoma and prostate carcinoma[6-9].

In the early 1980s, Ciechanover *et al*[10] and Hershko *et al*[11,12] obtained the initial understanding of ubiquitin-mediated protein degradation and identified several components of the ubiquitin system. A set of interconnected studies between 1984 and 1990 revealed the biological significance of protein degradation mediated by the ubiquitin system[13,14]. The mechanism by which ubiquitin molecules are covalently attached to targeted proteins can be delineated as a three-step enzymatic cascade (Figure 1). First, an ubiquitin-activating enzyme (E1) mediates the activation of the carboxyl-terminal glycine residue of ubiquitin in an ATP-dependent manner[10,15]. With the formation of a thiolester linkage, the activated ubiquitin is then transferred to E1 followed by the transfer of ubiquitin to a thiol site of an ubiquitin-conjugating enzyme (E2)[16]. Finally, an ubiquitin protein ligase (E3) confers substrate specificity by recognizing the target proteins and mediating the conjugation of (a) ubiquitin molecule(s) to a lysine residue on the targeted protein *via* an isopeptide bond[12]. Subsequently, the targeted protein is marked for degradation by the ATP-dependent 26S proteasome.

The addition of ubiquitin molecules onto targeted proteins is a modification that can be reversed. This reversal is called deubiquitination and is executed by proteases termed deubiquitinases (DUBs)[17]. DUBs specifically cleave ubiquitin after the terminal carboxyl group of ubiquitin and play a pivotal role in maintaining ubiquitin homeostasis[18,19]. Many DUBs have been shown to interact with E3 ligases, which suggests that a major function of DUBs is to control the stability of E3 ligases and subsequently destabilise the substrates of the cognate E3 ligase[20].

THE ROLE OF E3 UBIQUITIN LIGASES IN THE UPS PATHWAY

E3 ubiquitin ligases are often found overexpressed in a variety of human cancers, including lung cancer, and their deregulation has been shown to contribute to cancer development. As a result, increased attention is being paid to these E3 ubiquitin ligases and whether they can serve as potential anti-cancer targets. In targeted therapy, an ideal anti-cancer target should not only be overexpressed, but should meet additional criteria, such as its overexpression should be associated with poor prognosis, it plays a pivotal role in cancer genesis, inhibition induces apoptosis or growth reduction in the cancer cells, it is a "druggable" target (enzyme or cell surface molecule) that can be easily targeted, and finally, it is not expressed or is expressed at a very low level in normal cells.

E3 ubiquitin ligases can be divided in two major classes: the first class contains a C-terminal region Homologous to the E6-associated protein (E6-AP) carboxyl terminus (HECT), with an evolutionarily conserved cysteine residue required for the formation of a thiolester linkage with ubiquitin[21,22]. There are approximately 30 proteins containing the HECT domain. The second and largest class comprises E3 ubiquitin ligases that contain the really interesting new gene (RING) finger domain[23]. There are over 700 proteins containing the RING finger domain, but only a small part functions as an E3 ubiquitin ligase. Unlike RING proteins, most HECT proteins, if not all, are believed to function as E3 ubiquitin ligases. RING and HECT E3 ubiquitin ligases use different catalytic mechanisms to promote the transfer of ubiquitin to targeted substrates. RING E3 ubiquitin ligases can promote the direct transfer of ubiquitin from E2 to the targeted substrate, whereas HECT E3 ubiquitin ligases interact with the cognate E2, followed by the formation of a thiolester linkage with ubiquitin and subsequent transfer of ubiquitin to the targeted substrate (Figure 1).

The conjugation of one ubiquitin molecule to a protein is referred to as monoubiquitination, a process involved in protein trafficking, histone regulation, retrovirus budding and direct modulation of protein function[24]. As mentioned above, ubiquitin is attached to a lysine residue on the targeted substrate, however, ubiquitin itself also contains lysine residues that serve as self-conjugation sites. As a result, a chain of multiple ubiquitin molecules can be formed and appended to the targeted protein, which is referred to as polyubiquitination. Although monoubiquitination has been shown to be sufficient for the degra-

Table 1 A list of E3 ubiquitin ligases that have been found deregulated in lung cancer, along with their substrate(s) (when known) and the processes in which they are involved related to lung cancer, with corresponding references

Process	E3 ubiquitin ligase	Substrate	Ref.
Cell proliferation	c-Cbl	EGFR	61, 62
	Nedd4	PTEN	65
	Siah2	HIPK2	56, 128
Cell cycle regulation	APC	OLC1	37, 68
	Cul3-based ligase	Rho GTPase; Rho BTB2	96
	CCNB1IP1	Cyclin B	93
	Parkin	Parkin, CDCrel-1	86, 88
	SCF component: Fbxo7		79, 80
	SCF component: Skp2	CdK: p27, p21, p53	72, 73, 74
Apoptosis	MDM2	P53, pRb	108, 117, 118, 161
	Parkin		90, 91
	Pirh2	P53	115, 162
	SCF component: SAG	c-Jun	132, 134, 136, 137
	Siah2	HIPK2	128, 129
	Topors	P53	123
	TRAF2	RIP1	127, 163
	XIAP	XIAP, AIF	104
Gene regulation	Cul3-based ligase	IKBKb	140
DNA repair	FANCL	FANCD2	143-145

APC: Anaphase promoting complex; FANCL and FANCD2: Fanconi Anemia Complementation group type L and D2. HIPK2: Homeodomain-interacting protein kinase-2; SAG: Sensitive to Apoptosis Gene; OLC1: overexpressed in lung cancer 1; SCF: Skp, cullin, F-box protein; XIAP: X-linked inhibitors of apoptosis.

dation of some proteins, polyubiquitination accelerates the degradation of most proteins[25-27]. Self-conjugation of ubiquitin can occur through different lysine residues and it has been shown that polyubiquitin chains resulting from different ubiquitin linkages have distinct functions. Linkage through lysine-48 is the primary target signal for proteasomal degradation[28], whereas ubiquitin chains linked through lysine-63 execute many functions including protein synthesis[29], kinase activation[30] and DNA repair[31]. In addition, linkage through other lysine residues has been suggested including the involvement of lysine-6 linkage in the regulation of DNA repair[32]. Furthermore, linkage through lysine-29 has been shown to be involved in protein degradation, however, its function is not similar to that of linkage via lysine-48[33].

E3 ubiquitin ligases can execute their function as a single peptide or they can act as multi-component complexes that function as RING-finger type E3 ubiquitin ligases. The distinct superfamily of E3 ubiquitin ligase complexes consists of the skp, cullin, F-box protein (SCF) family, the anaphase-promoting complex (APC) family, and the VHL-elongin C/elongin B (VCB) family[34]. The SCF family makes use of adaptor subunits called F-box proteins that control substrate recognition through distinct protein-protein interaction domains[35], whereas the APC family uses different adaptors and targets proteins that regulate mitosis[36]. The APC complex is composed of at least 10 subunits including yeast Apc2 and Apc11p[37], which are thought to show homology to subunits of the SCF complex[38]. The VCB-like complexes possess a similar architectural structure as the SCF and APC family and on that basis are referred to be E3 ubiquitin ligases.

LUNG CANCER

Lung cancer is the most commonly diagnosed cancer as well as the most common cause of cancer related deaths worldwide, and can be divided in small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC)[39,40]. The major problem in the treatment of lung cancer is the emergence of intrinsic and acquired drug resistance[41]. Despite increased knowledge on the molecular mechanisms contributing to drug resistance and the development of novel agents, the overall 5-year survival of patients diagnosed with lung cancer is less than 15%. This highlights the relevance and necessity of novel agents that can be used in combinational therapies to circumvent drug resistance. One of the strategies that is currently being investigated is targeting components of the UPS system.

In lung cancer, the deregulation of various UPS components has been observed[2,42]. For instance, the mRNA expression of E1-like ubiquitin-activating enzyme (UBE1L) is often reduced in lung cancer cells[43,44]. UBE1L conjugates IFN-stimulated gene, 15-kDa protein (ISG15) and was shown to promote a complex between ISG15 and cyclin D1, which results in cyclin D1 inhibition and subsequent lung cancer growth suppression[45]. In addition, mRNA expression levels of E2 ligases UBE2C and UBE2T were found to be significantly upregulated in lung cancer tissues relative to normal lung tissues[46,47], whereas mRNA expression of E2 ligase Hrad6B was found to be significantly decreased[48]. These E2 ligases are involved in multiple biological processes: UBE2C (also known as UBCH10) initiates the degradation of APC/cyclosome (APC/C) substrates thereby regulating progression through mitosis[49], while UBE2T exerts its function in the fanconi anemia pathway by promoting monoubiquitination of the FANCD2 protein-a key step for efficient DNA repair[50]. In addition, Hrad6B is involved in UV mutagenesis, DNA repair[48,51] and was shown to be involved in histone methylation by promoting the ubiquitination of histone H2B[52].

In this review, we provide an overview of E3 ubiquitin ligases that have been found to be deregulated in lung cancer and a few of them meet some of the criteria of being an ideal anti-cancer target (Table 1). Furthermore, we will discuss the biological processes in which these E3 ubiquitin ligases are involved related to lung cancer as well as their potency to function as "druggable" targets for the treatment of lung cancer.

CELL PROLIFERATION

Tumorigenesis requires abnormal cellular proliferation. Consequently, signalling pathways controlling this complex process are the subjects of intensive research efforts. The RAS/MAPK pathway is one of the best-characterized signal transduction pathways involved in cellular proliferation. The GTPase RAS transmits extracellular signals from receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR), to downstream effector proteins (*e.g.*, RAF, MEK and ERK) that besides cell proliferation also control differentiation, survival and apoptosis[53,54]. In drosophila, the RING E3 ubiquitin ligase SINA was shown to be a critical component in RAS signalling located most downstream in the pathway[55]. SINA has two human homologs: SIAH1 and SIAH2 that share 76% and 68% sequence similarity, respectively[56]. Interestingly, SIAH2 protein levels were increased in highly aggressive lung tumors compared to little or no expression in normal lung tissues[57]. Moreover, similar levels of SIAH2 mRNA transcripts were detected in multiple lung carcinoma cell lines. However, SIAH2 expression is not restricted to tumorigenic cells but is also expressed by cells lacking any tumorigenic potential suggesting SIAH2 to be involved in all human proliferative cells[58]. As expected, inhibition of SIAH2 suppressed proliferation and reduced the tumorigenesis of human lung cancer cells. Recently, researchers identified the SIAH2-specific substrate homeodomain-interacting protein kinase-2 (HIPK2) which is a serine/threonine kinase that promotes p53-regulated gene expression by phosphorylating p53 at serine 46[23]. They found that overexpression of HIPK2 in lung cancer cells reduced cellular proliferation. This is in line with the suggestion that HIPK2 is tightly regulated in a p53-dependent manner in order to prevent ERK-mediated cell proliferation in the presence of activated p53[59,60].

The RING E3 ubiquitin ligase c-Cbl is known for its function in cell signalling and protein ubiquitination of multiple substrates including EGFR[61]. By targeting EGFR for proteasomal degradation, c-Cbl negatively regulates EGF signalling and opposes cellular proliferation[62]. Recently, Tan *et al*[42] determined the genetic variation and functionality of c-Cbl in NSCLC. They found a significant loss of heterozygosity of the c-Cbl locus in tumor samples from lung cancer patients compared to normal lung tissues. In addition, they identified novel somatic missense mutations of c-Cbl in multiple regions of the protein including the catalytic RING finger domain and the N-terminal tyrosine kinase binding (TKB) domain, both of which are vital for its E3 ubiquitin ligase activity[63,64]. Furthermore, overexpression of these mutations in NSCLC cell lines resulted in enhanced proliferative potential and cell motility suggesting an essential role for c-Cbl in lung tumorigenesis and metastasis.

In NSCLC, loss of the PTEN tumor suppressor is frequently observed leading to constitutive activation of the AKT pathway which is involved in fundamental cellular processes including protein synthesis, cell proliferation and survival. Recently, the protein Neural precursor cell Expressed Developmentally Down-regulated 4-1 (Nedd4-1) was identified as the E3 ubiquitin ligase responsible for PTEN proteasomal degradation[65]. An additional study showed that Nedd4-1 is overexpressed in 80% of NSCLC tumors which correlates with the loss of PTEN protein[66]. Accordingly, knock-down of Nedd4-1 stabilized PTEN protein levels and, in addition, significantly reduced proliferation of NSCLC cells *in vitro* and tumor growth *in vivo*.

CELL CYCLE REGULATION

In order for a multicellular organism to develop normally, tight regulation of the cell cycle is required[67]. Key regulators of the cell cycle are cyclins, which bind and activate cyclin-dependent kinases (CDKs) resulting in cell cycle progression. The cell cycle consists of four distinct phases: G_1, DNA synthesis (S phase), G_2 and mitosis (M phase), with G_1 and G_2 functioning as "gap" phases separating S phase and M phase in time. Cells exit from mitosis upon degradation of mitotic cyclins, a process controlled by the RING E3 ubiquitin ligase anaphase-promoting complex (APC)[37]. As mentioned earlier, the APC complex consist of at least 10 subunits[37]. Among these subunits are the activator proteins cell-division cycle protein 20 (CDC20) and cadherin-1 (Cdh1), which regulate the activity and substrate specificity of APC. The E3 ubiquitin ligase APC, together with its regulatory subunits CDC20 and Cdh1, were found to be accountable for the degradation of the overexpressed in lung cancer 1 (OLC1) protein[68]. OLC1 is highly expressed in lung cancer tissues from patients with a history of cigarette smoking[68,69]. OLC1 degradation by the E3 ubiquitin ligase APC was compromised upon introducing cigarette smoke condensate (CSC). Several studies have revealed that OLC1 is involved in cytokinesis, a process following mitosis[70,71]. However, additional studies are required to clarify the exact role of OCL1 in lung tumorigenesis.

Another important regulator of cell cycle progression is the F-box protein Skp2. Skp2 is part of an SCF complex that targets cyclin-dependent kinase inhibitors p27, p21 and p57 for proteasomal degradation, thereby promoting G_1 to S phase transition[72-74]. Overexpression of Skp2 is frequently observed in lung cancer tissues and is associated with the invasive and metastatic potential of NSCLC cells[75,76]. Accordingly, several studies have demonstrated that inhibition of Skp2 suppresses the growth of lung cancer cells[5,77,78].

The F-box protein Fbxo7 is also a component of an SCF complex and was found to selectively enhance CDK6 thereby regulating cell cycle progression[79,80]. Fbxo7 was reported to be upregulated in human lung cancers and to have transforming activity through cdk6[80]. Surprisingly, Fbxo7 does not seem to increase the degradation of the proteins with which it interacts

but rather increases their assembly and activity. However, novel Fbxo7-interacting proteins have been identified and are currently being investigated as candidates for Fbxo7-mediated ubiquitination.

In an attempt to identify novel tumor suppressors, Cesari and colleagues identified increased mRNA levels of parkin in NSCLC cells[81]. Conversely, a different study revealed a loss of parkin transcripts in NSCLC tumor tissues and showed that parkin expression was able to inhibit tumorigenicity in mice[82]. The parkin gene is mainly studied due to its pivotal role in the onset of autosomal recessive juvenile parkinsonism (ARJP)[83]. The parkin protein contains a RING finger motif and an ubiquitin-like domain, and many alternatively spliced isoforms have been identified[84,85]. Interestingly, parkin has been shown to exhibit E3 ubiquitin ligase activity targeting itself[86] and several other substrates for proteasomal degradation thereby regulating apoptosis and cell cycle[87-91]. However, the exact role of parkin in lung tumorigenesis needs to be further elucidated.

In contrast with studies on Skp2 and Fbxo7, researchers found that low levels of the E3 ubiquitin ligase CCNB1IP1 in NSCLC correlates with a lower overall survival[92]. CCNB1IP1 contains a RING finger domain and regulates cell cycle by interacting with cyclin B and promoting its degradation[93]. The exact role of CCNB1IP1 in lung tumorigenesis is not known.

A major class of ubiquitin ligases are the Cullin-based E3 ubiquitin ligases, which are incorporated in the SCF and APC complexes[94]. In mammals, eight distinct cullin proteins have been identified; Cul1 to Cul7 and PARC[95]. The Cul1-based E3 ubiquitin ligases are the best characterized and have been shown to control the protein levels of tumor suppressors and oncogenes, and are involved in cell cycle regulation[94]. The Cul3-based E3 ubiquitin ligases have recently emerged as key regulators of mitosis[96]. The atypical Rho GTPase RhoBTB2 is one of the substrates of Cul3-based E3 ubiquitin ligase complexes and its gene expression is ablated in 50% of lung cancer cell lines[97]. It has been suggested that RhoBTB2 functions as a tumor suppressor by recruiting proteins to a Cul3 ubiquitin ligase complex for degradation. However, it is unknown whether the ablation of RhoBTB2 in lung cancer cells correlates with deregulated levels of the Cul3 ubiquitin ligase.

APOPTOSIS

The ability of cells to undergo apoptosis is vital for tissue homeostasis and development[98]. An essential step in apoptosis is the activation of caspases, a family of cysteine proteases[99]. The inhibitors of apoptosis (IAP) proteins are a family that negatively regulate caspases, with the X-linked IAP (XIAP) protein as the best-studied member[100-103]. XIAP contains a RING finger domain and has been characterized as an E3 ubiquitin ligase[104]. Surprisingly, it was observed that high levels of XIAP correlate with a significant longer overall survival of NSCLC patients and is suggested to associate with less aggressive NSCLC[105]. These observations are conflicting with a study in leukemia patients where they demonstrate a correlation between XIAP expression and a decreased overall survival[106]. This implies alternate functions of XIAP in different types of cancer.

In response to physiological stress, the p53 protein is activated and promotes either apoptotic cell death or cell arrest[107]. The levels of p53 are tightly regulated by the E3 ubiquitin ligase MDM2 through an auto-regulatory negative feedback loop; a p53-regulated gene induces MDM2 expression while MDM2 targets p53 for degradation by the 26S proteasome thereby controlling p53-mediated biological responses[108]. MDM2 is often overexpressed in several human cancers including lung cancer[109]. It has been shown that protein expression levels of MDM2 are overexpressed in 70% of NSCLC tissues compared to adjacent normal lung tissues[110,111]. Recently, a single nucleotide polymorphism-SNP309-was identified in the promoter region of MDM2 and was shown to induce MDM2 overexpression thereby influencing p53 activity[112]. Interestingly, a subsequent study revealed an association between SNP309 and increased NSCLC risk, which was predominantly seen among woman[113].

The E3 ubiquitin ligase Pirh2 is another protein that promotes p53 degradation[114]. The *Pirh2* gene is regulated by p53 and encodes a RING-finger containing protein that exerts the ubiquitination of p53 independently of MDM2. A study from Duan and colleagues showed that Pirh2 is overexpressed in the majority of lung cancer tissues when compared to normal lung tissues[115,116]. Furthermore, they found enhanced p53 ubiquitination which subsequently resulted in lower p53 expression in mouse lung tumors than in normal tissues. These results are consistent with their hypothesis that increased Pirh2 expression affects lung tumorigenesis by reducing p53 activity.

In addition to targeting p53 for proteasomal degradation, MDM2 has been shown to ubiquitinate the retinoblastoma protein (pRB) which plays a dual role in both apoptosis and cell proliferation[117,118]. Miwa and colleagues found that high expression levels of MDM2 correlated with low expression levels of pRB in a subset of NSCLC patients[117,119]. This correlation was mainly observed in NSCLC cells lacking wild-type p53. Miwa and co-workers suggest that MDM2-induced ubiquitination of pRB perturbs the pRB pathway and subsequently promotes carcinogenesis in a p53-independent manner.

Another protein that has been shown to interact with p53 is the RING finger protein topoisomerase I-binding protein (topors)[120-122]. This interaction results in p53 stabilization and consequent induction of either apoptosis or cell cycle arrest. Conversely, topors has been shown to possess E3 ubiquitin ligase activity targeting p53 for proteasomal degradation, although to a lesser extent than MDM2[123]. This insinuates that topors-induced p53 regulation does not only occur through ubiquitination but also by other mechanisms. Interestingly, preliminary studies have revealed an increase of the human topors

gene (also known as LUN) in various lung cancer cell lines[122,124]. Furthermore, expression of LUN was slightly downregulated along with progression of primary NSCLC tumors and strongly downregulated in nodal metastasis[124]. It is suggested that LUN might play a role in inhibition of nodal metastasis as well as the oncogenesis of NSCLC.

Besides the well known caspase inhibitor XIAP and E3 ubiquitin ligases that interact with p53 there are more E3 ubiquitin ligases alternatively expressed in lung cancer that are involved in the apoptotic pathway. For example, the E3 ubiquitin ligase Tumor necrosis factor receptor-associated 2 (TRAF2) was identified as a candidate radiosensitizing target in lung cancer[125]. TRAF2 belongs to a family of seven TRAF members (TRAF1-7) that play a role in a variety of biological processes including immunity, inflammation and apoptosis[126,127]. The TRAF2 protein contains a RING finger domain and mediates several signalling pathways involved in apoptosis protection[127]. It was found that TRAF2 is overexpressed in both lung carcinoma tissues and lung cancer cell lines[125]. In addition, downregulation of TRAF2 in radioresistant lung cancer cells caused growth suppression and radiosensitization, suggesting that TRAF2 may be an attractive drug target for anticancer therapy and radiosensitization.

Moreover, the RING E3 ubiquitin ligase SIAH2 has been shown to play a role in apoptosis. Activity of the recently identified pro-apoptotic SIAH2-specific substrate HIPK2 is considered to play a role in restraining tumor development by targeting tumor cells toward apoptosis upon genotoxic stress[60]. Inhibition of HIPK2 in lung cancer cells resulted in protection against UV-induced apoptosis[128,129]. In accordance, overexpression of HIPK2 sensitized lung cancer cells to UV-induced apoptosis and reduced cellular proliferation.

Finally, it has been shown that the Sensitive to Apoptosis Gene (SAG) is significantly overexpressed in NSCLC tumor tissues compared to adjacent normal lung tissues[130,131]. The SAG protein contains a RING finger domain and has been characterized as a component of SCF E3 ubiquitin ligases targeting several substrates for proteasomal degradation[132,133]. Interestingly, high mRNA levels of SAG correlate with poor survival of NSCLC patients suggesting SAG as a potential prognostic marker in NSCLC. Furthermore, inhibition of SAG sensitizes radioresistant NSCLC cells to ionizing radiation[131]. Under stress conditions, SAG has been shown to function as a proliferating factor that inhibits apoptosis and promotes cell growth[134-137].

GENE REGULATION

The nuclear factor-κB (NF-κB) is a key transcription factor that is thought to play a major role in carcinogenesis[138]. NF-κB controls genes that regulate a variety of biological processes including inflammation, innate and adaptive immunity, and stress responses. In lung cancer, NF-κB is frequently expressed and was found to be involved in the pathogenesis of lung cancer[139]. The inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta (IKBKB) activates NF-κB and is a substrate of a Cul3 ubiquitin ligase complex. Recently, it was shown that genetic disruption of components of a Cul3 ubiquitin ligase complex results in elevated IKBKB levels and represents a mechanism of NF-κB activation in NSCLC[140]. Furthermore, inhibition of NF-κB was earlier shown to sensitize NSCLC cells to chemotherapy-induced apoptosis suggesting a possible role for the inactivation of NF-κB-induced pathways in the treatment of lung cancer[141].

DNA REPAIR

Upon DNA lesions, DNA damage surveillance systems are triggered and subsequently promote the activation of a multitude of genome-protection pathways[142]. One of these pathways involves the fanconi anemia (FA) proteins that are found to form a multi-protein complex that functions as an E3 ubiquitin ligase[143]. This E3 ubiquitin ligase complex exerts its function by monoubiquitinating FANCD2 during DNA replication or following DNA damage, mainly triggered by DNA crosslinking agents such as mitomycin C or Cisplatin[143]. Monoubiquitinated FANCD2 can interact with FANCD1/BRCA2 and others to repair damaged DNA. It was found that the lung cancer cell line Calu-6 harbors an impaired FA-BRCA pathway resulting from alternatively expressed FANCL, a catalytic subunit of the E3 ubiquitin ligase complex[144,145]. The pathway integrity was re-established upon FANCL complementation and reduced the hypersensitivity of Calu-6 cells to mitomycin[145]. Based on these results, it is suggested that the status of the FA-BRCA pathway could play an important role in determining the sensitivity of cancer cells to DNA crosslinking agents.

OTHERS

Most of the E3 ubiquitin ligases described above are also involved in other biological processes. For example, besides its involvement in cell proliferation and apoptosis, Siah2 plays a role in the physiological responses to hypoxia and was shown to target a rate-limiting enzyme in the mitochondrial Krebs cycle[146,147]. In addition, c-Cbl is involved in cell proliferation but also plays a critical role in angiogenesis and is involved in immunity by targeting many protein substrates for proteasomal degradation[148-151]. However, at present the E3 ubiquitin ligases that have been found to be deregulated in lung cancer have not been described to be involved in biological processes other than the ones we have discussed in this review.

CONCLUSION AND PERSPECTIVES

Currently, the major issue in targeting E3 ubiquitin ligases is the lack of specific inhibitors in clinical trials. Over

the past years, many research efforts have focused on the development of proteasome inhibitors. At present, Bortezomib is the only selective and reversible proteasome inhibitor approved by the United States Food and Drug Administration (FDA) and the European Medicine Agency and it is being used for the treatment of relapsed/refractory multiple myeloma and mantle cell lymphoma[152,153]. However, its induced cytotoxicity is based on overall inhibition of proteolysis of many cellular proteins. By selectively inhibiting an E3 ubiquitin ligase the proteins are stabilized that are regulated by this E3 ubiquitin ligase and thereby circumvent undesired effects on other cellular proteins. The deregulation of E3 ubiquitin ligases has been shown to contribute to cancer development and they are often found overexpressed in lung cancer[4,5]. Altogether, targeting E3 ubiquitin ligases has gained increasing attention, which has led to the development of high-throughput screening assays to identify inhibitors of multiple E3 ubiquitin ligases[154,155]. For example, small molecule inhibitors of the E3 ubiquitin ligase MDM2 have been identified and developed such as cis-imidazolines, benzodiazepines and spiro-oxindoles[156,157]. These inhibitors selectively inhibit MDM2 E3 ubiquitin ligase driven polyubiquitination of p53 with barely any effect on other enzymes using ubiquitin. However, one major concern is the selectivity between normal and cancer cells. Although it is still unclear, the activation of p53 by these MDM2 inhibitors in normal cells induces growth arrest rather than apoptosis making it achievable to obtain a therapeutic window[158]. Excitingly, inhibitors targeting MDM2 are now in clinical trials and pave the way for novel treatment strategies for cancer patients including those diagnosed with lung cancer[157]. Eventually, these inhibitors can be utilized in combination with other therapies, *e.g.*, chemotherapy in order to circumvent drug resistance which is an important problem in the treatment of patients with lung cancer.

In addition to MDM2, there are more E3 ubiquitin ligases that meet some of the criteria of being an ideal anti-cancer target. For example, the HECT E3 ubiquitin ligase Nedd4-1 is overexpressed in the majority of NSCLC tumors and its inhibition reduces the proliferation of NSCLC cells[66]. Furthermore, the SCF component SAG is frequently overexpressed in NSCLC tissues[131]. Importantly, high SAG expression is correlated with poor survival of NSCLC patients and could be a useful prognostic marker. In addition, other SCF components have been described in lung cancer. For example, the F-box protein Skp2 is often overexpressed in lung cancer tissues and is associated with the metastatic and invasive potential of NSCLC cells[75,76]. However, targeting SAG or Skp2 is challenging, since they are part of an SCF complex containing multiple components. Therefore, a general inhibitor against SAG or Skp2 may not have the desirable specificity against any SCF complex. More ideal anti-cancer targets would be the RING E3 ubiquitin ligase Pirh2 and TRAF2. Pirh2 is often overexpressed in many cancer tissues including lung cancer[159]. In addition, TRAF2 is overexpressed in the majority of lung cancer tissues and its downregulation suppresses cell growth and sensitizes otherwise radioresistant lung cancer cells. However, there are no inhibitors targeting these E3 ubiquitin ligases that are currently being tested in clinical trials.

Like E3 ligases, DUBs can be considered as potential anti-cancer targets. Although DUB inhibitors or activators have yet to successfully enter the clinic, multiple DUBs have been implicated in neoplastic disease such as ubiquitin-specific protease 4 (USP4), USP6, and USP8[160].

Although the biological functions of many E3 ubiquitin ligases are still not fully understood, it has become clear that some E3 ubiquitin ligases are promising anti-cancer targets. Despite the fact that we are still facing issues such as selectivity between normal and cancer cells and specificity between E3 ubiquitin ligase and protein substrates, the approval of Bortezomib and the recent entry of MDM2 inhibitors into clinical trials will further stimulate the development of specific E3 ubiquitin ligase inhibitors for the treatment of many cancers including lung cancer.

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CASE REPORT

Behavior of advanced gastrointestinal stromal tumor in a patient with von-Recklinghausen disease: Case report

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Abstract

Gastrointestinal stromal tumors (GISTs) represent a malignant gastrointestinal tumor of neurofibromatosis type 1 (NF1) Von Recklinghausen disease. In the current case, we report a 27-year-old woman with NF1, who presented with a lower abdominal mass, symptomatic anaemia, and significant weight loss. We employed multiple approaches to assess the tumor behavior, including computed tomography (CT) scan, surgical tumor resection, histological and immunohistochemical analysis and gene sequencing. Additionally, the patient was given Imatinib mesylate (Gleevec) as adjuvant therapy. CT scan delineated a large thick wall cavity lesion connecting to the small bowel segment. Resection of the tumor yielded a mass of 17 cm × 13 cm with achievement of safety margins. The diagnosis was GIST, confirmed by immunohistochemical expression of CD117, CD34, and Bcl-2. Sequencing revealed no mutations in either *KIT* or platelet-derived growth factor receptor-alpha, genes which are mutated in over 85% of sporadic GIST cases. Further, there was no evidence of recurrence, metastasis or metachronous GIST for over three years in our patient. From our analyses, we believe selective genotyping is advisable for high risk patients to predict potential tumor behavior.

Key words: *KIT*; Gastrointestinal stromal tumor; Imatinib; Neurofibromatosis type-1; Platelet-derived growth factor receptor-alpha

Core tip: There are several differences between gastrointestinal stromal tumors (GISTs) in a neurofibromatosis type 1 (NF1) patient and sporadic GISTs with regard to the tumor site, tumor behavior, targeted therapeutic approach, survival, prognosis, and recurrence based on genomic mutations. Wild type GISTs, those that do not have mutations in *KIT* or platelet-derived growth factor receptor-alpha (*PDGFR-α*), are typically resistant to imatanib therapy. Mutational analysis is critical in predicting tumor behavior and might individualize targeted therapy. We show that sequencing for *KIT* and *PDGFR-α* provide a useful tool in predicting tumor behavior of GIST in a NF1 patient.

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract[1] and originate from specific primitive cells, the interstitial cells of Cajal (ICCs). Most sporadic GISTs contain mutations in the *KIT* gene (80%-85%)[1]. Some GISTs have mutations in platelet-derived growth factor receptor-alpha (*PDGFR-α*) as an alternate oncogenic mechanism[1]. The 10%-15% of GISTs without activating mutations in either *KIT* or *PDGFR-α* are referred to as "Wild type" (WT) which are resistant to imatinib (Gleevec, Novartis, Basel, Switzerland) therapy[2].

Neurofibromatosis type 1 (NF1), also called Von Recklinghausen disease, is an autosomal dominant disorder with a genetic abnormality at chromosome 17q11.2, affecting 1/3000 individuals worldwide[3]. GISTs occur in approximately 5%-25% of NF1 patients[4]. WT GISTs in NF1 patients tend to be multiple and are located predominantly within the small intestine[5]. Mutation of *KIT* and *PDGFR-α* genes do not occur in GISTs from NF1 patients[4]. The underlying molecular mechanism for oncogenesis in these tumors has not been elucidated. Recently, Yantiss *et al*[4] discovered the same *KIT* point mutation (V559G) in exon 11 occurred in 3 separate GISTs from 1 NF1 patient, whereas 2 patients showed WT KIT and PDGFR-α sequences. However, the possibility of a constitutional mutation was not definitively ruled out. From the case reviewed here, we shed light not only regarding clinical and pathological parameters, but also assess the mutation status of *KIT* and *PDGFR-α* genes that predict the prognosis, survival, relapse and the behavior of the GIST tumor in a patient with NF1.

CASE REPORT

A 27-year-old female patient with known NF1 was admitted to our hospital complaining of lower abdominal dragging pain, symptomatic anemia, with significant weight loss during the previous two years. Our patient appeared cachectic and pale. Physical examination revealed multiple cutaneous and subcutaneous nodules and Café'-au-lait pigmentation all over the body (Figure 1). There was a large solitary lower abdominal mass (17 cm × 13 cm) that was not tender, but was firm in consistency, had active bowel sound, and had no hepatosplenomegally or ascites. Laboratory tests including the tumor marker, serum metanephrine, were normal; however, low hemoglobin levels were detected.

Computed tomography (CT) scan with oral and intravenous contrast revealed a large thick wall cavity lesion with multiple surrounding tortuous vessels, making a percutaneous biopsy high-risk (Figure 2A). Barium follow-through revealed a large cavity connected to a small bowel segment located at the mid-pelvic region which gradually filled with contrast on subsequent films (Figure 2B). Pelvic magnetic resonance imaging was requested to confirm that the lesion did not involve the ovaries (Figure 2C). Informed consent for exploratory laparatomy was obtained from the patient. From this procedure, we found an exophytic mass originating from the jejunal wall and covered by hypervascular omentum with dilated and engorged vessels (Figure 3). There was no evidence of obstruction, or liver and peritoneal metastasis. Resection of the mass was performed with adequate safety margins. Analysis of the gross specimen showed a small intestinal segment measuring 15 cm in length with an attached mass measuring 17 cm × 11 cm × 7 cm. The mass showed congestion and hemorrhage on the external surface. Microscopically, the mass possessed an invasive tumor comprised of spindle shaped cells arranged in bundles and fascicles along with a large area of necrosis. The individual tumor cells were spindle in shape with oval to elongated hyperchromatic nuclei (Figure 4A). Many abnormal nuclei and multinucleated tumor giant cells were noted, with mitotic figures measured at 4/50 (high power field) (Figure 4B).The proximal and distal margins were free of tumor.

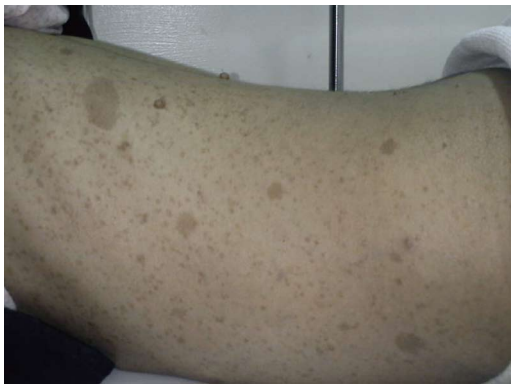

Figure 1 Cafe'-au-lait pigmentation with multiple cutaneous and subcutaneous nodules on the trunk.

An immunohistochemical analysis revealed that the tumor cells expressed KIT, CD34 (Figure 4C), Bcl-2 and focally expressed S-100 protein. The tumor was negative for desmin and smooth muscle-α actin. Genomic DNA analysis of the GIST sample was prepared from formalin-fixed tumor tissue (Qiagen). Direct sequencing of exon and intron-exon boundaries of the *c-KIT* and *PDGF-α* genes was performed (Applied Biosystems 3130 XL Genetic analyzer) (Table 1). There were no mutations in exons 9, 11, 13 or 17 in the *c-KIT* gene or in exons 12, 14, or 18 in the *PDGFR-a* gene.

The post-operative course was uneventful and the patient underwent a routine follow-up with CT scan and positron emission tomography (PET) scan every six months for three years. Imantinib mesylate (Gleevec; 400 mg) was given daily as adjuvant treatment throughout this time period. There was no evidence of recurrence, metastasis or metachronous GIST during the three year follow-up.

DISCUSSION

GISTs are typically classified into two subtypes: with or

Table 1 Sequencing of the primers used in this case study

Exon	Primers sequencing
c-kit gene	
9F	GTA GTG CGA TGG CCA GTATGCCACATCCCAAGTGTTT
9R	CAG TGT GCA GCG ATG ACTGACATGGTCAATGTTGGAA
11F	GTA GTG CGA TGG CCA GTTTTGTTCTCTCTCCAGAGTGCT
11R	CAG TGT GCA GCG ATG ACACCCAAAAAGGTGACATGGA
13F	GTA GTG CGA TGG CCA GTCATGCGCTTGACATCAGTTT
13R	CAG TGT GCA GCG ATG ACCAATAAAAGGCAGCTTGGACA
17F	GTA GTG CGA TGG CCA GTGTTTTCTTTTCTCCTCCAACC
17R	CAG TGT GCA GCG ATG ACGGACTGTCAAGCAGAG
PDGFA gene	
12F	GTA GTG CGA TGG CCA GTTCCAGTCACTGTGCTGCTTC
12R	CAG TGT GCA GCG ATG ACGCAAGGGAAAAGGGAGTCTT
14F	GTA GTG CGA TGG CCA GTTGGTAGCTCAGCTGGACTGAT
14R	CAG TGT GCA GCG ATG ACGGGATGGAGAGTGGAGGATT
18F	GTA GTG CGA TGG CCA GTCTTGCAGGGGTGATGCTATT
18R	CAG TGT GCA GCG ATG ACTGAAGGAGGATGAGCCTGAC

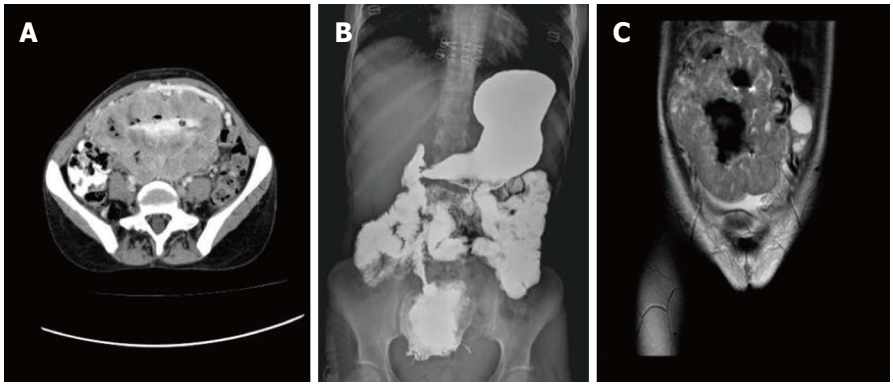

Figure 2 Results of image. A: Computed tomography scan: oral and IV contrast revealed a large thick wall cavity mass (17 cm × 11 cm) with the enhancing wall; B: Barium follow-through delineated a large cavity connected to a segment of small bowel located at the mid-pelvic region; C: Magnetic resonance imaging of T1 and T2 coronal image shows a large cavity lesion containing gas and fluids and connected to a segment of small bowel but not related to the ovaries.

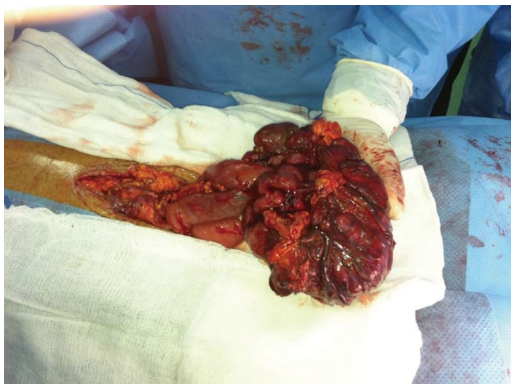

Figure 3 Congested exophytic mass measuring approximately 17 cm × 11 cm × 7 cm from the jejunal wall.

without mutations in *KIT* and *PDGFR-a* with the former being more malignant[6]. There are several differences between GISTs in a NF1 patient and sporadic GISTs with regard to the tumor site, tumor behavior, targeted therapeutic approach, survival, prognosis and recurrence based on genomic mutations. Many reports have demonstrated that NF1-GISTs are multicentric, and mainly found in the small intestine, a scenario which is rarely observed in sporadic GISTs[5], whereas our patient had a solitary tumor. Moreover, sporadic GISTs usually express a mutation of the *KIT* gene in exons 9, 11, 13 or 17; while NF1-GISTs lack *KIT* and *PDGFR-α* mutations[4]. There is evidence supporting the hypothesis that tumor mutational status may be a prognostic factor for GIST[6]. Patients with mutation-positive GISTs exhibit more frequent recurrence and higher mortality rates than patients without *KIT* mutations. Lau *et al*[7] found the 5-year relapse-free survival rate in patients with *KIT* mutations was 21% compared with 60% in patients without a *KIT* mutation.

Neurofibromin is a member of GTPase-activating protein family of ras regulatory proteins, and acts as a tumor suppressor[8]. Therefore, inactivation of neurofibromin in NF1-related GISTs is the mechanism leading to tumor formation[8]. In general, NF1 patients develop GISTs at a younger age compared to individuals with sporadic GISTs, as was the case with our patient. Definitive diagnosis of GISTs before surgery is not mandatory, especially because small biopsy samples can be inconclusive. GISTs are fragile and bleed easily, therefore if a suspected GIST is considered to be resectable, biopsy prior to surgery is not recommended because of the high risk of tumor dissemination. Surgery is the first-line therapy for patients with primary resectable GISTs. The object of surgical resection of a primary GIST is complete gross resection without rupturing the tumor

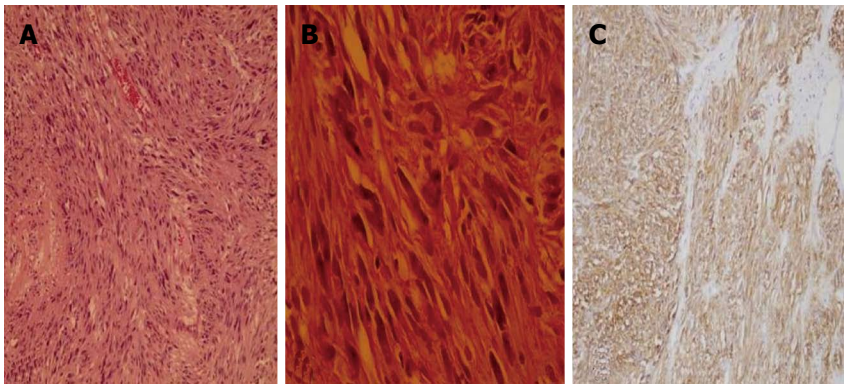

Figure 4 Histopathological examination. A: Invasive tumor comprising of spindle shaped cells arranged in bundles and fascicles; B: Many bizarre nuclei (oval to elongated hyperchromatic nuclei) and multinucleated tumor giant cells; C: Immunohistochemistry staining was positive for CD117.

psuedocapsule. Resection of adjacent organs in cases of adherent GISTs should be performed with the goal of obtaining negative margins. Lymphadenectomy is usually not warranted, with the exception of evident nodal involvement.

A multimodality approach for treatment combining surgery with systemic therapy is ideal. Marrari *et al*[9] recommended starting dose of imatinib (400 mg once daily) for patients with advanced GIST carrying mutations in *KIT* exon 11, *PDGFR-α*, or those with a WT genotype, with the option of dose escalation upon progression. Response to imatinib and dose escalation have been shown to correlate with the mutational status of *KIT* and *PDGFR-α* in GISTs[2]. Patients with *KIT* exon 9 mutations exhibit noticeably prolonged tumor-free survival when they start imatinib at 800 mg daily[10], but we decided to give our patient imatinib with a dose of 400 mg daily for three years, because she was considered a high risk patient. The risk classification stratifies tumors into very low, low, intermediate, and high-risk levels. Large tumors with high mitotic rates have the highest risk. However, a tumor greater than 10 cm with any mitotic rate, as in our case, or a tumor with more than 10 mitoses per 50 high powered fields, regardless of size, is considered to be high risk[11]. It is unclear if adjuvant therapy in high risk WT-GISTs yields benefit, and the effect of adjuvant imatinib on WT-GISTs may be variable. Regardless, adjuvant treatment with imatinib for three years was associated with a relapse-free survival and overall survival advantage in a randomized trial compared to one year of therapy in high-risk patients[12]. Supporting this, our patient also did not show resistance to imatinib or have tumor relapse within three years. Continuous treatment is recommended because often treatment interruption is followed by rapid tumor progression[13].

Clinical decision making should be individualized based on the mutational analysis (where available) in order to exclude resistant genotypes (*e.g.*, *PDGFR-α* D842V mutation) from imatinib therapy and permits the usage of an appropriate dose for *KIT* exon 9 mutations[14]. Marrari *et al*[9] demonstrated that patients with mutations in exon 11 of the *KIT* gene show more favourable clinical responses with imatinib treatment compared to those with exon 9 mutations or WT *KIT*. Surgery should not be delayed in those patients without mutations or who are imatinib resistant. Resistance to imatinib therapy can be primary (as with *KIT* exon 9 and *PDGFR-α* exon 18 mutations or WT GISTs[15]) or secondary (related to new kinase mutations). Sunitinib malate is approved for the treatment of advanced imatinib-refractory GIST and is associated with longer overall survival in patients with primary *KIT* exon 9 mutations and WT GIST compared with *KIT* exon 11 mutations in a retrospective study[16]. We believe that the clinical course of our patient may have also been attributed to the natural behavior of the tumor and not to imatinib. Tarn *et al*[17] recently published a study suggesting that WT GISTs are dependent on signalling through the insulin-like growth factor 1 receptor (IGF-IR), therefore monoclonal antibodies a against IGF-IR may be an option of targeted therapy in patients with WT GIST.

According to the European Society for Medical Oncology consensus recommendations, GIST tumors should be considered for molecular analysis for *KIT* or *PDGFR-α* mutations[14]. Experts recommend genotyping for all high-risk situations, such as our patient, primary imatinib resistance and metastatic GISTs[18]. Low-risk tumors and those fully resected do not require this type of testing. Furthermore, the optimal timing of mutational analysis to predict tumor behavior, at initial diagnosis or at time of surgery, remain unknown.

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CASE REPORT

Angioimmunoblastic T-cell lymphoma-associated pure red cell aplasia with abdominal pain

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Key words: Angioimmunoblastic T-cell lymphoma; Anemia; Pure red cell aplasia; Abdominal pain

Core tip: Angioimmunoblastic T-cell lymphoma (AITL)-associated pure red cell aplasia is a unique type of peripheral T-cell lymphoma with a constellation of clinical symptoms and signs; the histological features of AITL are also distinctive. AITL-associated pure red cell aplasia with abdominal pain has rarely been reported. Here, we report a rare case of AITL-associated pure red cell aplasia with abdominal pain. The diagnosis was verified by a biopsy of the enlarged abdominal lymph nodes with immunohistochemical staining.

Tao J, Zheng FP, Tian H, Lin Y, Li JZ, Chen XL, Chen JN, Shao CK, Wu B. Angioimmunoblastic T-cell lymphoma-associated pure red cell aplasia with abdominal pain. *World J Clin Oncol* 2013; 4(3): 75-81 Available from: URL: http://www.wjgnet.com/2218-4333/full/v4/i3/75.htm DOI: http://dx.doi.org/10.5306/wjco.v4.i3.75

Abstract

Angioimmunoblastic T-cell lymphoma (AITL) is a unique type of peripheral T-cell lymphoma with a constellation of clinical symptoms and signs, including weight loss, fever, chills, anemia, skin rash, hepatosplenomegaly, lymphadenopathy, thrombocytopenia and polyclonal hypergammaglobulinemia. The histological features of AITL are also distinctive. Pure red cell aplasia is a bone marrow failure characterized by progressive normocytic anemia and reticulocytopenia without leucopenia or thrombocytopenia. However, AITL with abdominal pain and pure red cell aplasia has rarely been reported. Here, we report a rare case of AITL-associated pure red cell aplasia with abdominal pain. The diagnosis was verified by a biopsy of the enlarged abdominal lymph nodes with immunohistochemical staining.

INTRODUCTION

Angioimmunoblastic T-cell lymphoma (AITL) is a unique type of peripheral T-cell lymphoma; AITL is rare, accounting for approximately 2%-5% of all non-Hodgkin lymphomas[1]. It is an uncommon type of non-Hodgkin's lymphoma and has a constellation of clinical symptoms and signs, including weight loss, fever, chills, anemia, skin rash, hepatosplenomegaly, lymphadenopathy, thrombocytopenia and polyclonal hypergammaglobulinemia[2,3]. The histological features of AITL are also distinctive. The lymph node is characterized by a polymorphic infiltrate, a marked proliferation of high endothelial venules, and a dense meshwork of dendritic cells, and the neoplastic cells display an atypical minimal cell. The neoplastic cells account for only a fraction of the infiltrate and are

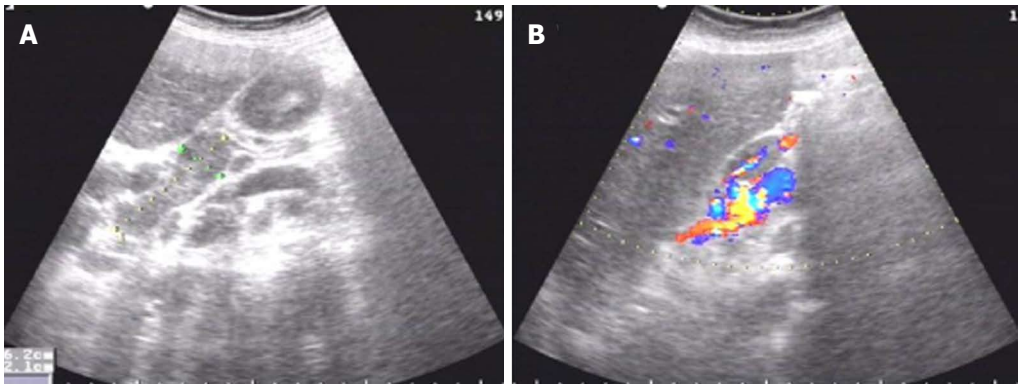

Figure 1 Ultrasound endoscopy showed cholecystitis and several enlarged abdominal lymph nodes. A: The enlarged lymph nodes were found in the porta; the lymph nodes showed a syncretic image with a clear boundary and uniform echo; B: A rich blood flow signal was observed in the lymph nodes.

admixed with a reactive population of small lymphocytes, eosinophils, histiocytes, plasma cells and a large lymphoid, sometimes composed of Reed-Sternberg-like B cells that are often infected with Epstein-Barr virus (EBV). It is believed that AITL derives from a follicular helper T-cell subset[4,5]. This subset of T-cells is located at the boundary between the germinal center light zone and the mantle zone. The tumor cells usually express CD3, CD10, CD21, CD23 and CXCL13, a phenotype that is unique among T-cell lymphomas. Pure red cell aplasia is a bone marrow failure characterized by progressive normocytic anemia and reticulocytopenia without leucopenia or thrombocytopenia. It is associated with various diseases, including thymoma, myeloproliferative disorders, infection and autoimmune diseases[6]. Compared with other non-Hodgkin lymphomas, AITL with abdominal pain and pure red cell aplasia is rare, and the prognosis is poor.

CASE REPORT

A 71-year-old woman presented with abdominal distension and fatigue that had developed two months previously. She also had a twenty-year medical history of hypertension but did not take medicine regularly. The patient suffered from cholecystolithiasis with cholecystitis for over ten years, and the cholecystitis with abdominal pain was relieved by antibiotic treatment. She had received a blood transfusion three years earlier because of severe anemia. Histories of hepatitis, infection, tuberculosis, wounds and surgery were denied. Two months prior to admission, the patient developed symptoms of abdominal pain with fullness not related to eating or posture, which sometimes showed spontaneous remission. She complained of a progressively poor appetite, fatigue, low-grade fever and nausea. On physical examination, severe pallor was observed, enlarged supraclavicular and inguinal lymph nodes were identified, and the liver and spleen were enlarged.

Laboratory blood examinations showed the following indexes (normal range in parentheses): hemoglobin, 50 g/L (120-140 g/L); peripheral white cell count, 4.61 $\times 10^9$/L (5-10 $\times 10^9$/L); neutrophils, 51.7% (40%-60%); reticulocyte count, 0.1% (0.5%-1.5%); platelet count, 229×10^9/L (100-300$\times10^9$/L); C-reactive protein, 67.3 mg/L (0-6.0 mg/L); erythrocyte sedimentation rate, 53 mm/h (0-20 mm/h); albumin, 30.7 mg/L (36-51 mg/L); total immunoglobulin (Ig), 38.3 mg/L (25.0-35.0 mg/L); lactate dehydrogenase, 208 U/L (71-231 U/L); total bilirubin, 10.58 μmol/L (4-23.9 μmol/L); alkaline phosphatase, 143 U/L (35-125 U/L); c-glutamyl transpeptidase, 104U/L (7-50 U/L); aspartate aminotransferase, 16 U/L (14-40 U/L); alanine aminotransferase, 22 U/L (5-35 U/L); creatinine, 76 μmol/L (31.8-91.0 μmol/L), and blood urine nitrogen, 33 g/L (31.8-91.0 g/L). A routine urine and stool test were normal. Autoimmune-related indicators and tuberculosis (TB)-related antibodies were not found in the blood, and the TB purified protein derivative (PPD) skin test was negative. Hepatitis B and C markers were also negative. The levels of CA-125, CA 19-9, CEA and AFP were normal.

A gastroscopy performed at another hospital indicated gastritis, and gastric polyp were removed by electrocision. *Helicobacter pylori* were negative. The histopathology of the gastric polyp showed a hyperplastic polyp. During the first two weeks of hospitalization, the patient received a blood transfusion of 800 mL, and the patient's symptoms were partly relieved. However, after two weeks, the patient experienced drastic upper abdominal pain, and abdominal ultrasonography demonstrated an acute onset of chronic cholecystitis and enlarged porta lymph nodes (Figure 1). An abdominal computed tomography (CT) scan revealed multiple enlarged coeliac and retroperitoneal lymph nodes, hepatosplenomegaly, and cholecystolithiasis with chronic cholecystitis (data not shown). Biopsies of the right cervical and left inguinal lymph nodes were performed at different times; the results revealed an inflammatory reaction only without lymphoma (Figure 2). On the sixth day of hospitalization, a bone marrow biopsy was performed; the bone marrow examination showed that the granulocyte series and megakaryocytes series were actively proliferating, whereas the erythrocyte series was marked by aplasia. The result demonstrated pure red cell aplasia with anemia (Figure 3). The patient accepted treatment with anti-

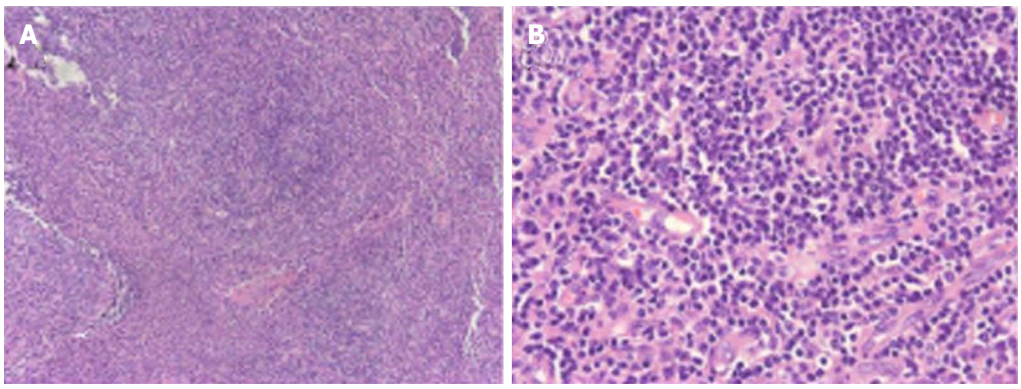

Figure 2 Photomicrograph of biopsy specimens of peripheral lymph nodes. Significant inflammatory cell infiltration was observed; however, tumor cells, such as lymphoma cells, were not found in the specimens. A: Hematoxylin and eosin (HE) staining, × 100; B: HE staining, × 400.

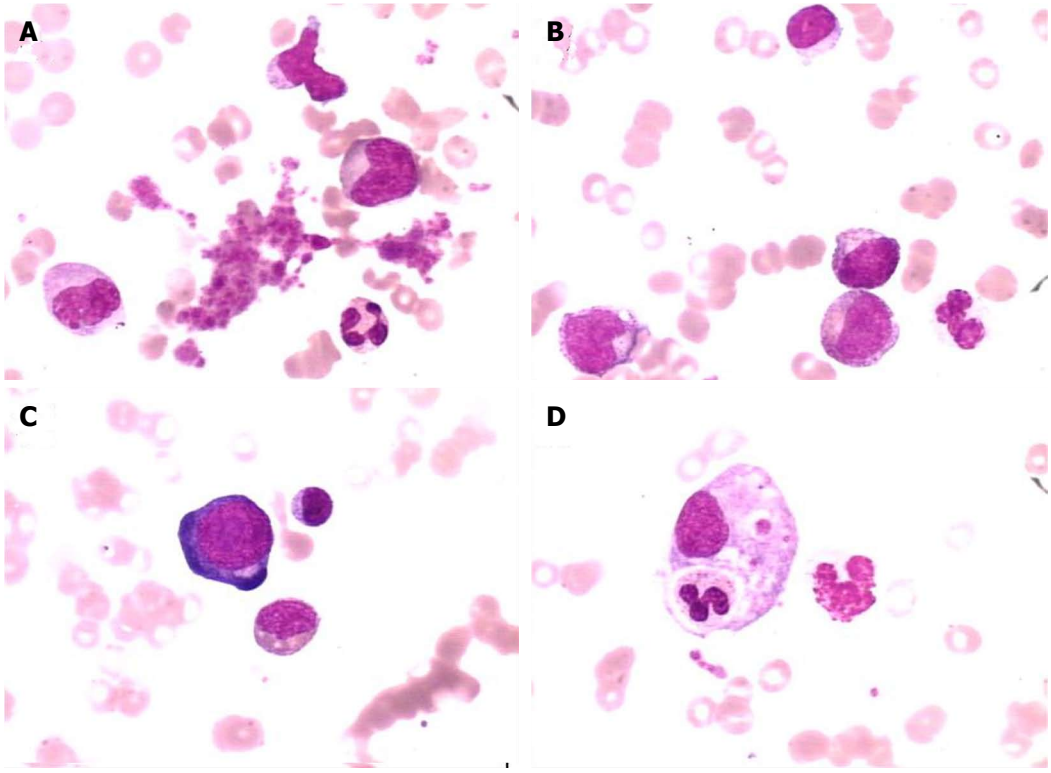

Figure 3 Representative images of bone marrow examination. The images showed anemia with marked red cell aplasia and the remarkable proliferation of granulocytes and megakaryocytes (Wright and Giemsa stain, × 1000). A: platelet; B: phagocyte; C: pronormoblast; D: megakaryocyte.

biotics and spasmolytics over one week, but the abdominal pain still worsened progressively.

The patient was examined again by abdominal CT scan at the sixth week. The images revealed that the number of lymph nodes had markedly increased and that the lymph node size was dramatically enlarged compared to the size observed six weeks earlier; the peritoneal cavity fat gap was fuzzy, and the peritoneum was thickening (Figure 4). To identify the cause of disease, the patient underwent a biopsy of the enlarged lymph nodes at both the porta hepatis and greater omentum with a cholecystectomy; a pathological examination and immunohistochemical staining showed only chronic inflammation without lymphomatous cells. However, the abdominal pain was not significantly alleviated after the cholecystectomy. Microscopically, the biopsy specimens revealed that the normal architecture was lost, except for the presence of a few depleted follicles with concentrically arranged follicular dendritic cells, and the architecture was effaced by some polymorphic infiltrate with marked vascular proliferation (Figures 5A and B). In addition, *in situ* hybridization showed a weakly positive nuclear labeling for Epstein-Barr virus-encoded small nuclear RNA (Figures 5C and D). The results of the immunohistochemical staining are shown in Figure 6. Evident positive staining was observed for CD3, CD4, CD21, CD10, CXCL13, Ki-67 and PAS, and marked follicular dendritic cell proliferation was also found around

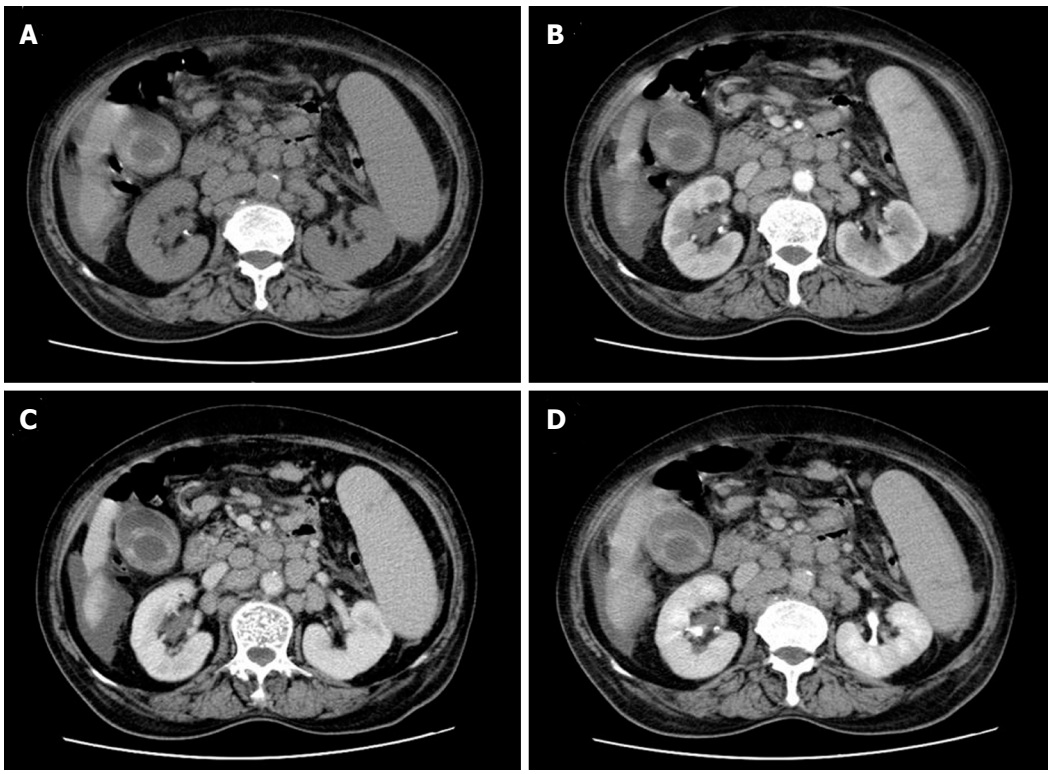

Figure 4 Abdominal computed tomography scan revealed a fuzzy peritoneal cavity and multiple enlarged portal peripheric lymph nodes with cholecystitis. A: Plain computed tomography (CT) scan image; B: CT image in the arterial phase of contrast enhancement; C: CT image in the portal venous phase of contrast enhancement; D: CT image in the parenchymal phase of contrast enhancement.

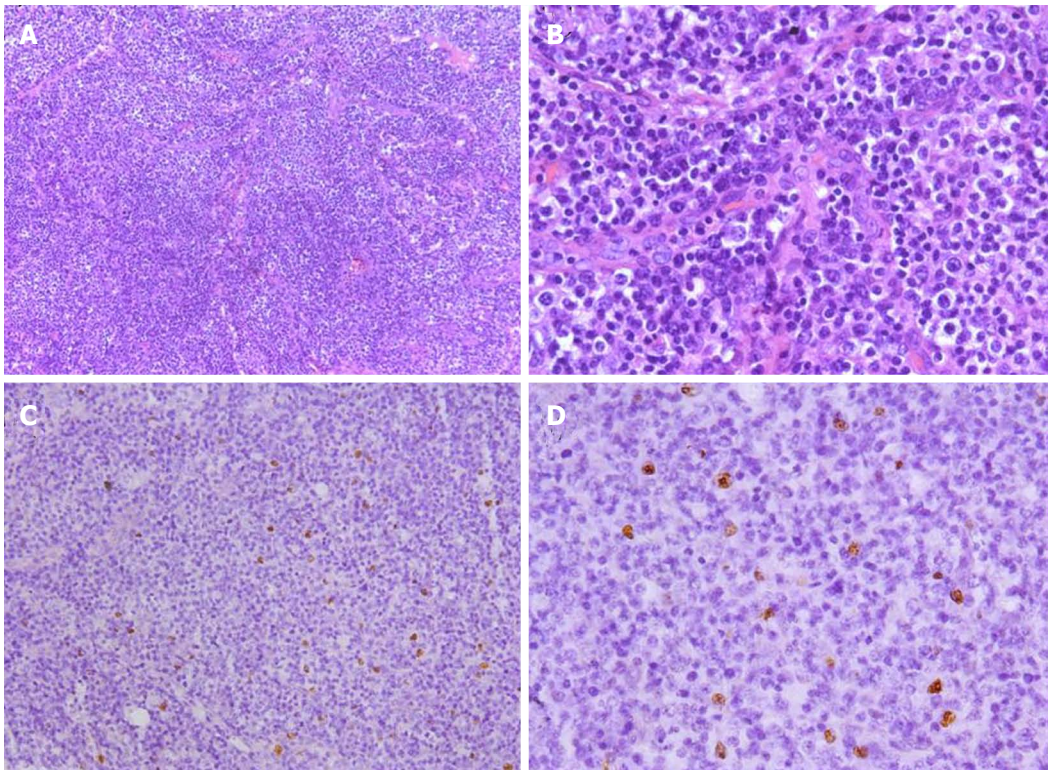

Figure 5 Photomicrograph of biopsy specimens in the celiac lymph nodes. The normal architecture was lost, except for the presence of occasional depleted follicles with concentrically arranged follicular dendritic cells, and the architecture was effaced by polymorphic infiltrate with marked vascular proliferation. A weakly positive nuclear labeling of Epstein-Barr virus-encoded small nuclear RNA was observed in the celiac lymph nodes by *in situ* hybridization. A: Hematoxylin and eosin (H and E) staining, × 100; B: H and E staining, × 400. C: *In situ* hybridization staining for Epstein-Barr virus-encoded small nuclear RNA, × 100; D: *In situ* hybridization staining, × 400.

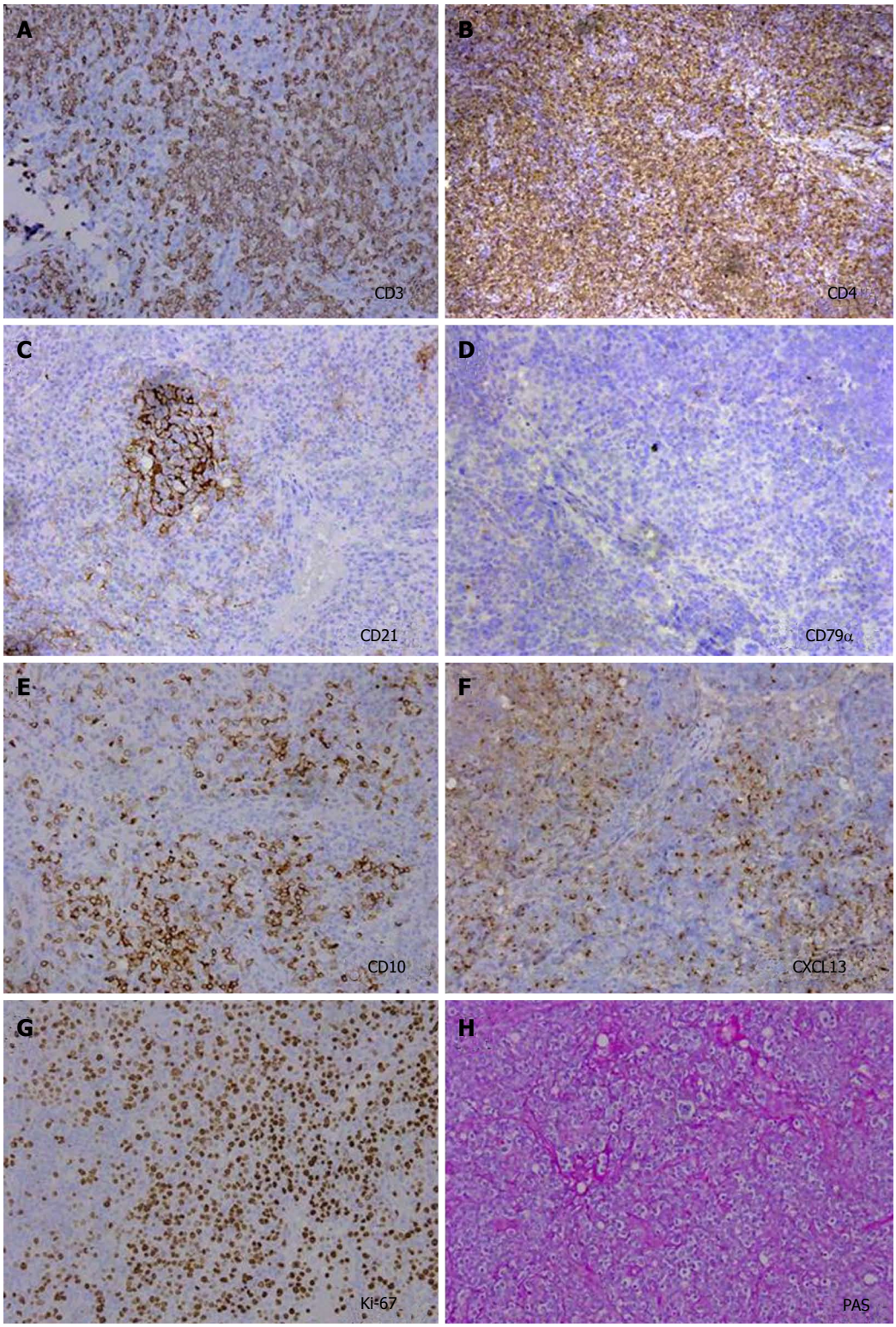

Figure 6 Immunohistochemical characteristics of the celiac lymph nodes. A: Neoplastic cells showed strong staining for cytoplasmic CD3; B: Strong staining for CD4; C: Strong vascular endothelial cell staining for CD21; D: Negative staining for CD79α; E: Strong staining for CD10; F: Moderate positive staining for CXCL13; G: Strong staining for Ki-67 over 70%; H: Strong staining for PAS.

small vessels. However, the staining showed negative staining for CD79α. Simultaneously, evident, positive CD23 staining was observed, whereas CD8 showed negative staining (data not shown).

Based on the pathological characteristics, the diagnosis of AITL was made. However, the patient was increasingly weak following deterioration and a consequential severe infection two weeks later, and the family requested that the patient return to a hometown hospital for sequential therapy.

DISCUSSION

AITL is a rare subtype of lymphoma and accounts for approximately 2%-5% of non-Hodgkin lymphomas. No consistent risk factors for development of the disease have been reported and to date, no etiological agent has been identified. Epstein-Barr virus is observed in most cases, and the virus has been found in the reactive B-cells that participate in the polymorphous infiltrate of this disease and in the neoplastic T-cells[7]. In our case, *in situ* hybridization showed a weakly positive nuclear labeling of Epstein-Barr virus-encoded small nuclear RNA. Immunodeficiency is also observed in this disease, but it may simply be a parallel state rather than a precipitating factor.

AITL is an age-related disease, with most patients presenting AITL within their sixth and seventh decades[8]. The presenting features of AITL span a spectrum ranging from asymptomatic lymphadenopathy to a syndrome associated with severe systemic symptoms. Nonetheless, the constellation of symptoms at diagnosis often involves diverse constitutional indicators, including fevers, night sweats, arthralgias or arthritis, and weight loss, but abdominal pain is rare. In the present case, though the patient had a ten-year medical history of cholecystolithiasis and cholecystitis, and her abdominal pain was not relieved after antibiotic treatment and gradually worsened; this outcome indicated that the abdominal pain was connected to the AITL but not the cholecystolithiasis and cholecystitis. The clinical syndrome of AITL overlaps with a wide range of inflammatory and neoplastic processes, and the changes in both peripheral blood and bone marrow are usually non-specific. AITL diagnosis can only be achieved by a biopsy with histological examination of the enlarged lymph nodes, through which the characteristic morphological features can be best appreciated.

AITL is usually accompanied by various immunologic and hematologic diseases, such as autoimmune hemolytic anemia, vasculitis, and autoimmune thyroid disease[9]. Pure red cell aplasia (PRCA), an autoimmune disorder resulting in selective aplasia of the erythroid series, is a bone marrow failure characterized by progressive normocytic anemia and reticulocytopenia without leucopenia or thrombocytopenia. PRCA is associated with various diseases, such as thymoma, lymphoma and myelo-proliferative disorders, autoimmune diseases, infection, and drugs, but PRCA is rarely associated with AITL. The pathogenesis has not been elucidated yet. Some reports suggest that the mechanism of lymphoma-associated PRCA is heterogeneous and that the durable maintenance-free remission of anemia can be obtained in some patients; moreover, some humoral factors causing inhibition of red cell precursors might play an important role in the pathogenesis of pure red cell aplasia associated with AITL[10,11]. Some researchers have noted that the patients had a dose-dependent inhibitor of colony-forming unit-erythroid but not of colony-forming unit-granulocyte and macrophage from a normal bone marrow cultures. Patients with AITL are known to produce antibodies against several autologous epitopes, and the most well-characterized mechanism of pure red cell aplasia involves antibodies to red cell precursors[12].

The diagnosis of AITL is difficult, and it is sometimes misdiagnosed as an infectious or other hematological disease. In particular, the histological appearance of extra nodal involvement, such as bone, skin spleen, marrow, and lung, is usually non-specific[13]. In our case, the diagnosis was made by biopsy of the enlarged lymph nodes at the porta hepatis and greater omentum with immunohistochemical staining but not of the peripheral lymph nodes because the lymphadenopathies existed only in the intraabdominal area. We and other groups have shown that CD10, CD21 and CXCL13 are expressed by neoplastic cells of AITL and that these specific immunohistochemical stainings contribute to AITL diagnosis.

Some researchers have found pertinent prognostic factors for AITL, such as male sex, mediastinal lymphadenopathy and anemia. However, these factors are not specific to AITL diagnosis. Few reports have attempted to investigate histological prognostic features, but none of them has been proven of clinical value[14,15]. Some studies have tried to identify the potential relationship between the increase in large cells and the disease prognosis, but these studies also show no definite outcome for the prognosis.

In summary, AITL- associated abdominal pain with pure red cell aplasia is a very rare disease and may be related to immune disorders. The diagnosis of AITL should rely on a combination of classical pathological criteria together with clinical and biological features; specific immunohistochemical study is especially pivotal. Because of gerontism and poor physical condition, the majority of patients cannot endure chemotherapy, the five-year survival rate is less than 20%, and the cause of death is usually severe infection.

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EDITORIAL

Are the data on quality of life and patient reported outcomes from clinical trials of metastatic non-small-cell lung cancer important?

Vera Hirsh

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Abstract

Majority of the patients with advanced non-small-cell lung cancer (NSCLC) experience two or more disease related symptoms, which may have a negative impact on their health-related quality of life (HR QOL). These patients prefer a therapy that would improve disease related symptoms, as opposed or treatment that slightly prolongs their survival without improving symptoms. The improvements of the symptoms augment the significance of improved response rates or progression free survivals. The choice of the questionnaires to evaluate patients-reported outcomes (PROs) and HRQOL benefits and methods of collecting the data and their interpretations are very important and are discussed in this manuscript. PROs and HR QOL outcomes are important in patients with advanced NSCLC only when the data are collected and analyzed correctly. Then they can be viewed as components of the total value of a treatment, providing a comprehensive picture of the benefits and risks of anticancer therapies. Enabling the patients to feel during the last months of their lives more comfortable and not be dependent on their loved ones is a very important task in the treatment of advanced NSCLC.

Key words: Quality of life; Clinical trials; Non-small cell lung cancer; Patient reported outcomes

Core tip: Are the data on quality of life (QOL) and patient reported outcomes (PROs) from clinical trials of metastatic non-small-cell lung cancer important? Yes, they are important if the data of PROs and QOLs questionnaires are collected appropriately with a good patient's compliance.

Hirsh V. Are the data on quality of life and patient reported outcomes from clinical trials of metastatic non-small-cell lung cancer important? *World J Clin Oncol* 2013; 4(4): 82-84 Available from: URL: http://www.wjgnet.com/2218-4333/full/v4/i4/82.htm DOI: http://dx.doi.org/10.5306/wjco.v4.i4.82

INTRODUCTION

Lung cancer is a leading cause of cancer death worldwide for both men and women[1].

Majority of the patients present at the time of diagnosis with metastatic disease. Of these patients with advanced non-small-cell lung cancer (NSCLC) approximately 90% of patients experience two or more disease related symptoms such as cough, dyspnea, pain and the general symptoms of fatigue and anorexia[2]. All these symptoms may cause psychological distress and may have a negative impact on a patient's health-related quality of life (HRQOL). High degrees of psychological distress influence the emotional well-being in both patients and their families. It is not surprising that 68% of patients would prefer a therapy that would improve disease-

related symptoms without prolonging life, as opposed to treatment that slightly prolonged survival without improving symptoms[3].

Treatment can affect a patient's well-being through both symptom control and treatment-related toxicity[4]. Therefore; treatments which can decrease tumour growth (achieve a tumour response) and at the same time be less toxic, are very important for these patients[4,5]. It is important for patients to preserve their independence and not be dependent on their loved ones, becoming a burden at the end for their lives[6-8].

The response to treatment can have an effect on disease-related symptoms and some studies suggest a link between tumour response and symptoms such as cough, dyspnea, chest pain and also systemic symptoms such as fever, anorexia and weight loss[9-11]. The improvements of these symptoms further augment the significance of improved response rates or progression free survivals (PFS). Median overall survival for most of the patients with metastatic NSCLC is modest, around one year; in epidermal growth factor receptor mutations positive tumors it approaches two years, thus HRQOL and patients-reported outcomes (PROs) carry high importance.

METHODS OF COLLECTING THE DATA

Patient-reported symptoms (outcomes) and HRQOL benefits are usually assessed using the self-administered cancer-specific European Organisation for Research and Treatment of cancer (EORTC) questionnaires QLQ C30[12] the lung cancer-specific EORTC QLQ-LC 13[13] and the Euro QOL EQ-5D[14] questionnaire or FACT-L[15] (functional assessment of cancer treatment in lung cancer) questionnaire. The QLQ-C30 questionnaire consists of five functional scales (physical, role, cognitive, emotional and social functioning), three symptom scales (fatigue, pain, nausea/vomiting), a global health status/QOL scale and single items, *i.e.,* dyspnea, loss of appetite, constipation, diarrhea, sleep disturbance and financial impact. The QLQ LC 13 questionnaire incorporates one multi-item scale to assess dyspnea and a series of single items assessing cough, pain, sore mouth, dysphagia, peripheral neuropathy, alopecia and use of pain medication. For each scale/item, a linear transformation was applied to standardize the raw score for a range from 0-100, with 100 representing best possible function/QOL for functional scales, and highest burden of symptoms for symptom scales and symptom items.

A 10-point change in an item or domain is perceived to be clinically meaningful[16]. The percentage of patients who are classified as improved ($\geqslant$ 10-point increase for functioning scales and $\geqslant$ 10-point reduction for symptom domains or items from baseline scores) with respect to each of the questionnaires is examined[16]. In addition, time to deterioration of an item/domain score is defined as the item from randomization to the first appearance of a score that is 10-points or more lower or higher than the baseline score ($\geqslant$ 10-point reduction for functioning scales and $\geqslant$ 10-point increase for symptom scales or items). The EQ-5D is a disease-generic questionnaire that comprises the EQ-5D and EQ-visual analogue scale (VAS). The EQ-5D measures five dimensions of health (mobility, self-care, usual activities, pain/discomfort and anxiety/depression). Each dimension comprises three levels (no problems, some/moderate problems and extreme problems). Utility scores range from 0-1 and were calculated from the five EQ-5D items scores using the United Kingdom preference weights[17]. The EQ-VAS records the patient's self-rated health status on a vertical, graduated (0-100) VAS. Functional Assessment of Cancer Therapy-Lung (FACT-L) questionnaire (version 4) comprises 36 items across 5 domains/categories, *i.e.,* physical, social, family, emotional and functional well-being. Lung cancer subscale consists of *i.e.,* symptoms, cognitive function and regret of smoking. Scores range from 0 (not at all) to 4 (very much)[15].

Each protocol specifies schedule for questionnaires to be completed, *i.e.,* at baseline, every 2-4 wk, at the end of treatment visit and during the first follow-up visit. The use of concomitant medications has to be assessed at the baseline and during the trial, especially the analgesic use, anti-anxiety, depression medication, O_2 use, *etc.*

RESULTS AND THEIR INTERPRETATION

In order to obtain reliable results, patients have to answer the questionnaires prior to meeting their physicians and finding out results of their tests (scans). Help with the questionnaires should be available by knowledgeable staff in the clinic/hospital. The questionnaire has to be filled out by the patients themselves, not by other family member. A supervision to ensure objectivity is important.

The attention has to be paid to baseline scores. In randomized trials, are they well balanced? Are they low (= low burden of symptoms) or high (= high burden of symptoms)? If the baseline scores are low, the percentage of patients with improved symptoms on certain anticancer treatment might be difficult to find. On the other hand, time to symptom deterioration (= delay of deterioration) might be of high importance. Also the longitudinal analysis looking at symptoms and HR QOL over time, at different visit intervals might be informative.

The compliance of the patients with the questionnaires should always be mentioned. One would like the compliance to remain through the study at $\geqslant$ 80%, in order to be able to analyse and interpret the results appropriately. In case of EORTC questionnaires, both EORTC QLQ LC 13 and QLQ C30 should be analysed to obtain a complete picture of not only lung cancer related symptoms, but also of symptoms related to cancer treatment toxicities.

The patient's symptoms are treated, especially the last months of life, by analgesics, cough suppressants, O_2, antidepressants, appetite stimulating agents and other supportive measures, which in final analysis, have to be incorporated. Other factors, such as performance status

(improving or deteriorating), weight loss and need for special emotional counselling are of great value in understanding the total value of lung cancer treatments.

CONCLUSION

In addition to efficacy and safety endpoints, PROs and HRQOL outcomes are important in patients in advanced NSCLC, when the data are collected and analysed correctly. They should be viewed as components of the total value of a treatment. They should provide, together with the other concern endpoints, a comprehensive picture of the benefits and risks of anticancer therapies. This position has been taken by Food and Drug Administration, (2003) and European Medicine Agency[18,19].

To collect and analyse the PROs and HRQOL data with high quality, completeness and an excellent patient' s compliance, a dedicated personnel is required. The process is time-consuming, it has to be a team work of knowledgeable, devoted workers, who are ready to participate in clinical trials and thus deliver reliable results of PROs and HR QOL questionnaires. Obtaining not only prolonged PFS, but enabling patients to feel during the last months of their lives more comfortable and independent, is a very important task in the treatment of advanced NSCLC.

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EDITORIAL

Squamous cell carcinoma of the skin: Emerging need for novel biomarkers

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Abstract

The incidence of non-melanoma skin cancers (NMSC) is rising worldwide resulting in demand for clinically useful prognostic biomarkers for these malignant tumors, especially for invasive and metastatic cutaneous squamous cell carcinoma (cSCC). Important risk factors for the development and progression of cSCC include ultraviolet radiation, chronic skin ulcers and immunosuppression. Due to the role of cumulative long-term sun exposure, cSCC is usually a disease of the elderly, but the incidence is also growing in younger individuals due to increased recreational exposure to sunlight. Although clinical diagnosis of cSCC is usually easy and treatment with surgical excision curable, it is responsible for the majority of NMSC related deaths. Clinicians treating skin cancer patients are aware that certain cSCCs grow rapidly and metastasize, but the underlying molecular mechanisms responsible for the aggressive progression of a subpopulation of cSCCs remain incompletely understood. Recently, new molecular markers for progression of cSCC have been identified.

Key words: Squamous cell carcinoma; Skin cancer; Biomarker; Matrix metalloproteinase; Serpin

Core tip: Several molecular markers for progression of cutaneous squamous cell carcinoma (cSCC) have been identified, but a clinically useful panel of biomarkers is still not available. Further studies are required to determine whether prognostic cSCC biomarker panel can be incorporated into clinical practice. In the meantime, while waiting for novel diagnostic and prognostic tools, clinicians must actively advocate public awareness on skin protection against excessive sun exposure in order to lower the increasing incidence of cSCC.

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INTRODUCTION

The incidence of cutaneous squamous cell carcinoma (cSCC) is increasing worldwide[1,2]. SCC of the skin is responsible for the majority of non-melanoma skin cancer (NMSC) deaths, as invasive cSCC displays a potential for recurrence and metastasis[3]. At present, cSCC is primarily a disease of the elderly, but the incidence is also increasing in younger individuals due to excessive recreational exposure to sunlight[4]. The rising incidence of cSCC is also a reason for increased demand for medical care related to skin cancer which is estimated to grow 5% annually in the Central Europe[5]. Public awareness of skin

Table 1 Risk factors for the development of squamous cell carcinoma of the skin

Exposure to ultraviolet (UV) radiation (UVA, UVB)
Therapy with methoxalen and UVA
Fair skin type
Ionizing radiation
Genodermatosis (albinism, xeroderma pigmentosum, epidermolysis bullosa)
Chronic inflammation of skin (lupus erythematosus, epidermolysis bul losa)
Chronically injured skin with ulcers (burn scars, leg ulcers, epidermoly sis bullosa)
Human papilloma virus infection
Exposure to chemical carcinogens
Immunosuppression
Immunosuppressive medications
Organ transplantation
Osteomyelitis
Sinus tracts
Precursor lesions (actinic and arsenical keratoses)
In situ squamous cell carcinoma (Bowen's disease and Erythro plasia Queyrat)
Tobacco smoking
Leukemia and lymphoma

Originated from Madan *et al*[2], with permission.

cancer as a potentially lethal disease should therefore be fostered, and the growing economical burden of rising skin cancer incidence to the societies should be taken into consideration in healthcare planning[6,7]. In addition to promoting avoidance of excessive sun-exposure, early lesional skin biopsy and treatment of premalignant lesions are essential in prevention of cSCC[8]. To ensure early diagnosis, dermatologic expertise is obviously needed, and this should be taken into account in planning of medical education[9].

The treatment of choice for primary cSCC is surgical excision, whereas Mohs micrographic surgery is recommended for high-risk cases[1,10]. Unfortunately, excision is not always curative, as cSCCs have an overall 5-year recurrence rate of approximately 5%[11]. Here, the challenge faced by clinicians is to identify the high-risk cases before the recurrence and metastasis of the tumor. There is an obvious demand for predictive molecular markers that could be used at the time of excision of the primary tumor for evaluation of the risk of recurrence and metastasis. Furthermore, if dissemination of the tumor has already taken place, novel targeted therapies are needed.

In this editorial, we discuss the molecular pathways involved in the development of invasive cSCC and the risk factors for cSCC progression, recurrence and metastasis. Finally, recent progress in the search for cSCC progression biomarkers will be discussed.

MORBIDITY AND ECONOMICAL BURDEN OF cSCC

NMSC, including cSCC, are among the most commonly diagnosed cancers in Europe, United States and Australia[12-14]. Moreover, the incidence of cSCC is steadily increasing worldwide, especially among the white population living in the proximity of the equator[1]. Interestingly, the incidence of cSCC is also rising in the less sun exposed regions of the globe, as approximately 4% annual increase in the incidence has been registered in Finland during the past decades[15]. The growing number of new cSCC cases, as well higher incidence of recurrent tumors makes cSCC one of the costliest cancers in many countries[7,16,17]. It is conceivable, that the cost of NMSC to the societies will continue to grow due to the extension of individual lifespan and aging of the population.

One of the main reasons for the rising incidence of cSCC is popularity of recreational sun-exposure despite growing public awareness of the harmful effects of solar ultraviolet (UV)-light[4]. On the other hand, the number of individuals receiving long-term immunosuppressive medication after organ transplantation has increased. As organ transplant recipients have a 65-fold higher risk of developing cSCC, regular follow-up of these individuals is mandatory and early diagnosis of cSCC, preferably at pre-malignant stage, is essential[18]. Even if the skin malignancies are diagnosed at premalignant stage, the treatment may be costly due to the field cancerization of the skin and the relatively high-cost of topical therapies available. Interestingly, kidney transplant recipients with a history of cSCC also have a higher risk for internal malignancies[19]. It has been proposed that switching the immunosuppressive medication from calcineurin inhibitors to inhibitors of the mammalian target of rapamycin, such as sirolimus could have an antitumoral effect among kidney-transplant recipients with previous cSCC[20] but this observation has been challenged[21].

For dermatologists, diagnosis of cSCC and its precursor, actinic keratosis and cSCC *in situ* (Bowen's disease), is often easy by visual inspection[22]. However, clinical diagnosis may sometimes be challenging, especially in patients suffering from severe generalized form of recessive dystrophic epidermolysis bullosa (gs-RDEB), with multiple chronic ulcers, mimicking malignant lesions[23].

RISK FACTORS FOR DEVELOPMENT OF cSCC

Sunlight has a vital role on the Earth as the primary source of energy, but it is also the primary cause of skin cancer[24]. Theoretically, UV-radiation as the major risk factor for cSCC could be avoided, but avoiding sun exposure is a challenge in daily life[4]. UVB (wavelength 280-320 nm) radiation can damage both DNA and RNA directly leading to the generation of mutagenic photoproducts such as pyrimidine-pyrimidine adducts and cyclopyrimidine dimers[25]. UVA (wavelength 320-400 nm) radiation damages DNA indirectly *via* a photo-oxidative-stress- mediated mechanism which results in DNA double-strand breaks[26,27]. As both UVB and UVA are carcinogenic, the sunscreen used should block both UVB and UVA rays[28]. The risk factors for the development of

Table 2 Risk factors for recurrence and metastasis of cutaneous squamous cell carcinoma

Variable	Approximate relative risk[1]	
	Recurrence	Metastasis
Rapid tumor growth	-	-
Tumor size > 2 cm	2	2
Tumor location (lip/ear)	2	3
Immunosuppression	-	2
Previous radiotherapy	-	-
Previously treated cSCC	3	4
RDEB -associated cSCC	-	-
Tumor depth > 4 mm	2	5
Poor differentiation	2	3
Acantholytic features	-	-
Spindle-cell features	-	-
Perineural invasion	5	5

Originated from Alam *et al*[1], with permission. [1]A relative risk of 1 is defined as the likelihood of recurrence or metastasis of a small primary cutaneous squamous cell carcinoma (cSCC). Dashes indicate an association with increased risk, but of which there are insufficient data to estimate the relative risk. RDEB: Recessive dystrophic epidermolysis bullosa.

cSCC are summarized in Table 1.

MOLECULAR PATHWAYS INVOLVED IN THE DEVELOPMENT OF cSCC

Major pathways involved in the pathogenesis of SCC of the skin are shown in Figure 1. Early inactivation of both alleles for tumor protein 53 gene (*TP53*) has an important role in the development of cSCC[29,30]. *TP53* mutations are observed in roughly 90% of all cSCCs and these mutations occur mainly due to UV radiation. Following inactivation of both *TP53* alleles, a marked expansion in simple mutations takes place making cSCCs the human cancer with highest mutation rate known[31]. Subsequently, epidermal keratinocytes undergo malignant transformation and clonal expansion will occur which is clinically manifested as the development of early *in situ* SCC, actinic keratosis[30,32]. In patients with xeroderma pigmentosum, mutations in xeroderma pigmentosum complementation group C, that lead to failure to repair DNA, are the key event of cSCC development[33]. Melanocortin-1 receptor variants are associated with fair skin, red hair and increased risk of developing melanoma, and they are also an independent risk factor for development of SCC of the skin[34]. In addition, telomerase activity may be elevated in cSCC leading to immortalization of tumor cells[35]. Moreover, inactivation of cyclin-dependent kinase inhibitor 2A locus has been detected in SCC of the skin[36]. Moreover, loss of function mutations of *NOTCH-1* and *NOTCH-2* genes have been noted in 75% of cSCCs emphasizing the importance of *NOTCH* genes as tumor suppressors in these epithelial malignancies[37].

Chronic skin exposure to UV light results in DNA damage and mutations in the genes mentioned above leading to malignant transformation of keratinocytes. Moreover, UV light can promote cSCC tumorigenesis and progression also *via* other mechanisms, such as immunosuppression and inhibition of macrophage migration[28,38].

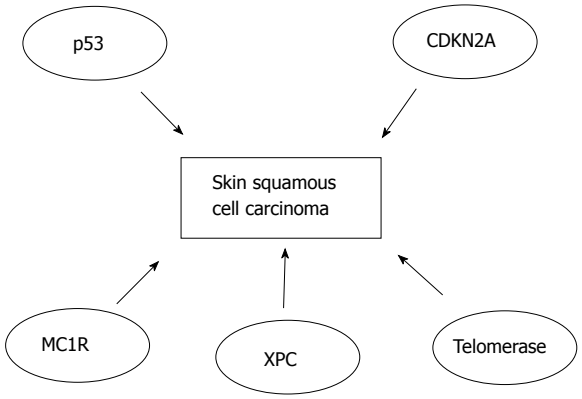

Figure 1 Pathways involved in the pathogenesis of cutaneous squamous cell carcinoma. Originated from Madan *et al*[2], with permission. p53: Tumor protein 53; CDKN2A: Cyclin-dependent kinase inhibitor 2A; MC1R: Melanocortin 1 receptor; XPC: Xeroderma pigmentosum complementation group C.

RECURRENCE AND METASTASIS OF cSCC

SCC of the skin is often relatively rapidly growing, locally invasive malignant tumor that has potential for recurrence and metastasis, with an overall 5-year recurrence rate of 8% and a 5-year rate of metastasis of approximately 5%[11]. The location and the size of the primary tumor are relevant in the assessment of the risk of recurrence and metastasis of a given tumor. Typical high-risk anatomical areas are lips and ears, as cSCC in these areas recur and metastasize at a rate of 10% to 25%. The relatively low general risk of recurrence and metastatic spread is markedly higher if the primary lesion is large, as tumors with a diameter > 2 cm show a recurrence rate of 15% and metastasis rate of 30%[11]. In certain cSCC subtypes, such as as in tumors arising in chronic ulcers or chronically injured skin, the risk of metastasis may be as high as 40%[39,40]. In addition, certain histological features of cSCC are known to be related to poor prognosis[1,41]. These features are shown in the Table 2.

Although these clinical and histological risk factors have been established, clinicians treating patients with cSCC still do not have access to molecular tools to assess the risk of recurrence and metastasis in a given patient. Thus, novel molecular biomarkers would be of great value in the risk assessment.

SEARCH FOR NOVEL BIOMARKERS FOR cSCC PROGRESSION

In our own studies, we have searched for novel biomarkers for progression of cSCC. We have utilized a diverse range of research methods to identify relevant candidate genes and tumor proteins. The first step has been genome-wide expression profile analysis of cSCC cell

lines *vs* normal human epidermal keratinocytes[42]. Secondly, we have validated the expression profiling data at the mRNA level with quantitative real-time polymerase chain reaction[43]. Then, we have confirmed the findings at the protein level with Western blotting[44]. As cultured tumor cells represent only a selected portion of a given tumor, we have collected a large panel of *in vivo* tissue samples containing normal human skin, actinic keratoses, cSCCs *in situ* (Bowen's diseases) and cSCCs[42-44]. These formalin fixed, paraffin embedded samples were used for immunohistochemical studies as tissue microarrays[42-44]. In addition, we have used chemically induced mouse skin carcinogenesis model for validation of our human data[42].

ROLE OF MATRIX METALLOPROTEINASE-7 IN cSCC PROGRESSION

Matrix metalloproteinases (MMP) contribute to the homeostasis of a variety of tissues and participate in many physiological processes, such as proteolysis of extracellular matrix in skin[45]. Upregulation of MMP expression has been seen in many different types of cancers, including cSCC[46,47].

In recent studies, we showed that the expression and production of MMP-7 is specifically elevated in cSCCs[43,44]. Interestingly, MMP-7 expression was even more abundant in the gs-RDEB-associated cSCCs representing an aggressive subtype of SCC of the skin[43]. Immunohistochemical studies revealed elevated MMP-7 expression especially in the invasive edge of the cSCC tumors[43].

Furthermore, we studied the mechanistic role of MMP-7 in cSCC and noted that MMP-7 activates heparin binding epidermal growth factor-like growth factor (HB-EGF) in cSCC cells[44]. In functional studies, proliferation of cSCC cells was suppressed when the activation of HB-EGF by MMP-7 was inhibited[44]. These findings provide mechanistic evidence for proposed therapeutic effect of epidermal growth factor receptor antagonists in treatment of advanced cSCCs.

SERINE PEPTIDASE INHIBITOR CLADE A MEMBER 1 AS A NOVEL BIOMARKER FOR PROGRESSION OF cSCC

Serine peptidase inhibitors (Serpins) constitute the largest and most broadly distributed superfamily of peptidase inhibitors described in humans[48,49]. We studied the gene expression levels of entire serpin family in cSCC cell lines *vs* normal keratinocytes and found that expression of SerpinA1, also known as 1-antitrypsin, was markedly elevated[42]. Furthermore, elevated SerpinA1 expression correlated with the tumorigenic potential of transformed keratinocytes[42]. Moreover, SerpinA1 expression in SCC tumor cells *in vivo* correlated with tumor progression[42]. Furthermore, SerpinA1 expression was clearly more abundant in gs-RDEB-associated cSCCs representing an aggressive subtype of cSCC[42]. To further verify the role of SerpinA1 in the progression of cSCC, we used chemically induced mouse skin carcinogenesis model that showed correlation with SerpinA1 expression and progression of mouse skin SCC[42]. Our findings clearly demonstrate that SerpinA1 may serve as a useful biomarker for progression of cSCC.

CONCLUSION

Although patients with cSCC in general do not have as poor prognosis as those with melanoma, the impact of cSCC to the quality of life of the patients, as well as to the societies in general will be greater in the near future due to the increased incidence of this malignant tumor and the longer life-span of the population. To improve the accuracy of diagnosis and the assessment of individual prognosis, there is a demand for novel biomarkers for progression of cSCC. We have identified potential biomarkers for this purpose, but further research is required to validate their feasibility in clinical practice. As cSCC is not a uniform disease but rather a heterogenous group of tumors, we assume that a single biomarker probably will not be sufficient, but a panel of biomarkers is needed for making clinical decisions. Finally, the power of preventive measures against skin cancer should not be underestimated. For this purpose, physicians, together with other healthcare professionals, must actively promote public awareness of skin protection against excessive sun exposure.

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ORIGINAL ARTICLE

Monitoring adenoviral based gene delivery in rat glioma by molecular imaging

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Abstract

AIM: To determine whether endothelial progenitor cells (EPCs) can be used as delivery vehicle for adenoviral vectors and imaging probes for gene therapy in glioblastoma.

METHODS: To use cord blood derived EPCs as delivery vehicle for adenoviral vectors and imaging probes for glioma gene therapy, a rat model of human glioma was made by implanting U251 cells orthotopically. EPCs were transfected with an adenovirus (AD5/carrying *hNIS* gene) and labeled with iron oxide and inoculated them directly into the tumor 14 d following implantation of U251 cells. Magnetic resonance imaging (MRI) was used to *in vivo* track the migration of EPCs in the tumor. The expression of gene products was determined by *in vivo* Tc-99m single photon emission computed tomography (SPECT). The findings were validated with immunohistochemistry (IHC).

RESULTS: EPCs were successfully transfected with the adenoviral vectors carrying hNIS which was proved by significantly ($P < 0.05$) higher uptake of Tc-99m in transfected cells. Viability of EPCs following transfection and iron labeling was not altered. *In vivo* imaging showed the presence of iron positive cells and the expression of transgene (hNIS) product on MRI and SPECT, respectively, all over the tumors following administration of transfected and iron labeled EPCs in the tumors. IHC confirmed the distribution of EPC around the tumor away from the injection site and also showed transgene expression in the tumor. The results indicated the EPCs' ability to deliver adenoviral vectors into the glioma upon intratumor injection.

CONCLUSION: EPCs can be used as vehicle to deliver adenoviral vector to glioma and also act as imaging probe at the same time.

Key words: Cord blood endothelial progenitor cells; Adenovirus; Human sodium iodide symporter; Single photon emission computed tomography; Magnetic resonance imaging

Core tip: Endothelial progenitor cells (EPCs) can be transfected with replication competent adenoviral vector carrying therapeutic/reporter gene and the transfected EPCs can be used to deliver the gene in tumor gene therapy. EPCs can be used as both imaging and therapeutic probes.

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INTRODUCTION

Glioblastoma (GBM) is one of the aggressive primary brain tumors with survival period falls from one to three years upon treatment[1,2]. Today's available treatments (chemotherapy, radiation and surgery) have little success and alternative or combinational approaches to control gliomas are in need[3]. In alternative treatments, gene therapy has shown promise due to development of various tools such as lentivirus, adenovirus, adeno-associated virus, conditional replicating viruses and tumor specific promoters[3-5]. In a gene therapy approach, therapeutic or suicidal genes or oncolytic viruses are delivered into the tumor cells to suppress or eliminate tumor growth[3,6]. Numerous studies have shown the ability of oncolytic viruses to suppress the tumor cells and have been proven to be safe in clinical trials[4,5,7]. The efficacy of the oncolytic agents, however, may be compromised due to their limited infection efficiency, mode of delivery to the tumors, and distribution into the entire tumor area[6]. Currently, different oncolytic viral vectors are being administered directly into the tumor sites by multiple intratumor injections[4,8,9]. Intratumor injection is advantageous compared with systemic injection of a virus to control entry into the circulation and to avoid unexpected side effects[10]. Strategies for improved gene delivery are highly desirable to overcome some of these short comings. In this context, genetically transformed cells are being considered as delivery vehicles to deliver genes or viruses directly into different tumors[11-14]. Due to their unique property to migrate to the pathological lesions, stem cells are considered a unique choice to be the delivery vehicle for therapeutic genes to the tumors, especially for glioma[15-17]. Endothelial progenitor cells (EPCs) are a subpopulation of pluripotent hematopoietic stem cells (HSC), which show active migration and incorporation into the neovasculatures of glioma when administered locally or systemically[18-21]. Based on the characteristics of EPCs, it is possible to use these cells as vehicles for the delivery of therapeutic genes to gliomas (using viral vectors)[14]. In addition, EPCs can be collected from a patient's own peripheral blood and bone marrow, which in turn eliminate the possibility of immune response[22].

Locally administered transfected EPCs can act as delivery vehicles for genes, viral vectors or both. For example, transgenic EPCs can carry either a therapeutic gene, such as growth inhibitory or inflammatory factors, or a suicidal gene, such as HSV-tk for antiviral drugs or human sodium iodide symporter (hNIS) for I-131 (radioiodine) or both[7,23,24]. The delivery of genes from transfected EPCs into adjacent tumor cells can be achieved by using replication competent viral vectors that will enable the virus to grow inside the EPCs, shed, and transfect the adjacent tumor and neovasculature cells[25]. Upon effective infection of the tumor and adjacent neovasculature cells, anti-tumor treatment can be started, especially if suicidal genes are used[5,7,23,24]. Local administration of virus-transfected EPCs will have advantages over the direct injection of viral particles to the tumors for the following reasons: (1) EPCs will not allow quick release of viral vectors to the circulation through blood brain barrier; (2) local administration will prevent accidental transfection of cells in non-target organs or tissues; and (3) locally administered EPCs may migrate not only to the periphery but also to the center of the tumor and incorporate into neovasculatures[14,25-27]. Therefore, there is a higher chance for the extensive transfection of tumor cells during the migration of transfected EPCs. That would enable the subsequent anti-tumor treatment (by targeting suicidal gene for example) to be more effective[7,23,24].

Reporter gene systems have been increasingly used for monitoring gene therapy in various tumor models to *in vivo* determine the delivery and expression of transgene products. hNIS is an intrinsic transmembrane glycoprotein that mediates the transport of iodides into the thyroid follicular cells[28,29]. A number of studies have demonstrated that active iodide uptake can be induced in a variety of cells[21,30,31]. This transport system also transports Tc-99m and can be imaged by gamma camera[32-34]. The expression of hNIS in the transfected cells is obviously higher than the non-transfected cells and higher signal associated with hNIS expression can be monitored with SPECT imaging. In our recent report we also showed the importance of hNIS in detecting *in vivo* gene expression[14].

The purposes of this study was to determine: (1) whether EPCs transfected with replication competent adenoviral vectors carrying hNIS can migrate to other parts of a tumor following injection into orthotopic glioma; and (2) whether migration of EPCs and the delivery of the gene product to the tumor cells can be determined by *in vivo* imaging.

MATERIALS AND METHODS

CD133$^+$ cell collection and isolation

Human cord blood was collected under Henry Ford Health System Institutional Review Board (IRB) approved protocol. The cord blood was collected from placenta using published method with minor modifications[35]. In brief, placental blood directly collected into 50 mL tube contains 1 × PBS (with penicillin/streptomycin and ethylene diamine tetraacetic acid) by opening the clamps. Collected cord blood samples were maintained in ice until reaching the laboratory. Once reaching the laboratory cord blood CD133$^+$ cells were isolated using our published method[14,21,36]. The mononuclear

fraction from cord blood was separated using Ficoll density gradient centrifugation. Immunomagnetic isolation kit (Miltenyi, CA) was used to isolate the $CD133^+$ cells. Isolated $CD133^+$ cells were maintained as suspension cultures using CellGenix SCGM media (CellGenix, Germany) supplemented with 40 ng/mL of stem cell factor (SCF), 40 ng/mL of FLT3 and 10 ng/mL of thrombopoietin (TPO) (Prospec, United States).

Production of replication competent viral vector

Replication competent adenovirus carrying *hNIS* gene was a gift from Dr. Barton, Henry ford Hospital[7]. The full details of the construction of the adenovirus were described in the published work of Dr. Barton[7]. The adenoviral construct used in this study was Ad5-yCD/mutTK(SR39)rep-hNIS (replication-competent adenovirus)[7]. The adenoviral vector carries therapeutic genes and reporter genes. The E1 region contains therapeutic gene [yeast cytosine deaminase (yCD) and mutant herpes simplex virus thymidine kinase mutTK (SR39) fusion gene] and the E3 region contains reporter gene (hNIS)[7]. Transgenes were expressed under the control of human cytomegalovirus (CMV) promoter[7]. We used adenovirus under approved Institutional Recombinant DNA Biosafety Committee (IRDBC) protocol. Adenovirus was produced using published method with minor modifications[37]. To produce adenovirus, 293 cells were seeded at a density of 1.6×10^6 per T75 flask. When they reached confluence, cells were split into three T75 flasks. When cells reached 80% confluence, the media was replaced with 5 mL of serum free Dulbecco's modified Eagle's medium (DMEM) containing adenovirus (2.6×10^9 viral particles per milliliter) and incubated at 37 ℃, 5% CO_2. After one hour incubation, 10 mL of complete media was added and incubated at 37 ℃, 5% CO_2. After 3 d of incubation supernatant containing adenovirus was collected and concentrated using polyethylene glycol (PEG) solution. Viral supernatants were mixed with PEG solution and incubated overnight at 4 ℃. After incubation, virus was collected by centrifugation at 1500 *g* for 30 min. Concentration of viral particles were measured using ultraviolet-visible (UV) spectrometer. In brief, 5 μL of sample (adenovirus) was added to 495 μL of 0.1% SDS solution. After vortex optical density (OD) was measured using UV spectrometer at 260 and 280 nm wavelength. Number of viral particle in concentrated samples were calculated using following formula: 1 $OD_{260} = 10^{12}$ viral particles per milliliter[37].

Transfection of EPCs

We used adenovirus in transfection experiments according to approved IRDBC protocol (Henry Ford Health system institutional recombinant DNA and biosafety committee). Adenoviral transfection of EPCs was performed according to published method with minor modifications[26]. To develop transfected EPCs, adenovirus carrying *hNIS* gene was added to sterile 1.5 mL microcentrifuge tube in 1:2000 ratio (cell:viral particle) and incubated for 1 h at 37 ℃, 5% CO_2. After one hour incubation, 1-3 mL of fresh media was added and transferred to a 6-well plate and further incubated for 24 h.

Tc-99m uptake assay

To test expression of hNIS gene in transfected EPCs, a Tc-99m uptake assay was performed according our published method[14]. In brief, 10 μCi of Tc-99m (Mallinckrodt, United States) was added to around 1 to 1.5×10^6 cells (in serum free media) and incubated at 37 ℃ fo-r 30 min followed by washing twice with phosphate buffered saline (PBS) and cell associated activity in pellet was measured using gamma counter (Wizard 1420, PerkinElmer, United States). We used the same method to test the Tc-99m uptake in iron labeled transfected EPCs.

Labeling of cells with ferumoxides

EPCs were labeled with ferumoxides using our published method[21,36]. In brief, ferumoxides (Fe) (Berlex Laboratories, United States) and protamine sulfate (Pro) were added to the cell suspension followed by 15 min incubation at 37 ℃, 5% CO_2. Upon incubation, complete stem cell media was added and incubated for 4 h followed by washing with PBS. Finally cells were resuspended in serum free media at the desired concentration for injection.

For cell viability, 100 μL cell suspension were mixed with trypan blue dye and observed under a microscope to determine cell viability. There were three types of cell preparations, which were injected in three separate groups of tumor bearing animals: (1) non-transfected non-FePro labeled (control cells); (2) transfected, FePro labeled; and (3) transfected, non-FePro labeled.

Animal model

Animal experiments in this study was approved by animal care and user committee at Henry Ford Health System. Human glioma cells (U-251, gift from Dr. Steve Brown, HFHS) were cultured with DMEM supplemented with 10% FBS, penicillin (100 IU/mL), and streptomycin (100 μg/mL). Upon reaching confluent, cells were collected and made a cell suspension (4×10^5 cells/5 μL) in serum free media before implanting in rat brain. Athymic nude rats 6-8 wk of age and 150-170 g of weight (Charles River Laboratory, Inc.) were used for the implantation[14,21,38]. Firstly, animals were anesthetized by intraperitoneal injection using ketamine/xylazine mixture (100 mg/kg ketamine, 10 mg/kg xylazine) and tumor cells were implanted according to our published methods[14,21,38]. There were at least 3 animals for each condition.

Intratumor injection of transfected EPCs

Intratumor injection was performed according to published method with modifications[39]. After 14 d of post implantation of U251 cells in the rat brain, animals were anesthetized and their skulls were exposed. Using a dental drill a hole was made at 3 mm to the right and

1 mm anterior to the bregma, exactly at the site of tumor implantation, and a 10 μL micro-syringe fitted with 26 s gauge-needle loaded with EPCs (around one or two million) in 5 μL was lowered to the depth of 4 mm, then raised to the depth of 3 mm. Either transfected labeled, transfected non-labeled EPCs or control EPCs were injected stepwise at a rate of 0.5 μL/30 s until the entire volume had been injected and syringe was withdrawn at one millimeter per minute. Bone wax was used to seal the drilled hole and finally the overlying skin was sutured.

Magnetic resonance imaging

Magnetic resonance imaging (MRI) imageries were obtained using a 3.0 Tesla clinical system (Signa Excite, GE health) using 50 mm diameter small animal imaging coil (Litzcage small animal imaging system, Doty Scientific Inc, Columbia, SC) according our published method[14]. Rats were anesthetized with 1.5%-2.0% isoflurane in oxygen and secured in a small animal imaging coil. MRI images were acquired before and 7 d post intratumor injection of EPCs carrying adenoviral vectors. Images were obtained with three dimensional (3D) isotropic Fast Imaging Employing Steady sTate Acquisition with parameters repetition time = 11.4 ms, echo time = 5.6 ms, using a 200 × 200 matrix, field of view = 60 mm and number of excitation = 2, effective slice thickness was 0.3 mm[14].

SPECT imaging

SPECT images were acquired based on our published method[14]. In brief, animals were anesthetized using ketamine/xylazine (100/10 mg/kg) and 1mCi of Tc-99m was injected through tail vein. After one hour of tail vein injection animals were subjected for SPECT imaging. Ketamine/xylazine (100/10 mg/kg) was used to achieve continuous state of anesthesia during l imaging period. SPECT was acquired with a dedicated PRISM3000 gamma camera fitted with mutli-pinhole rat collimators, 360 degree rotation with 36 degree increments, 180 s per projection, using 256 × 256 matrices, with a field of view of 4 cm × 6 cm[14]. We scanned the animals for 30 min to acquire SPECT images on the tumor area[14].

Histological analysis

Animals were euthanized and whole brain samples were collected as described in our previous publications[14,21,38]. For histological analysis, brain samples were fixed and processed for the frozen sections. The sections were strained with Prussian blue for the detection of iron labeled cells[14]. For the detection of transgene expression, sections were stained with anti-hNIS antibody (Genetex, TX, United States). For further analysis, we used anti-vWF antibody for the detection of endothelial cells and anti-EGFR (epidermal growth factor receptor) antibody for the detection of tumor cells. Some sections were double stained to determine double expression of hNIS/vWF (hNIS expression in administered EPCs) or hNIS/EGFR (hNIS expression in surround tumor cells)[14].

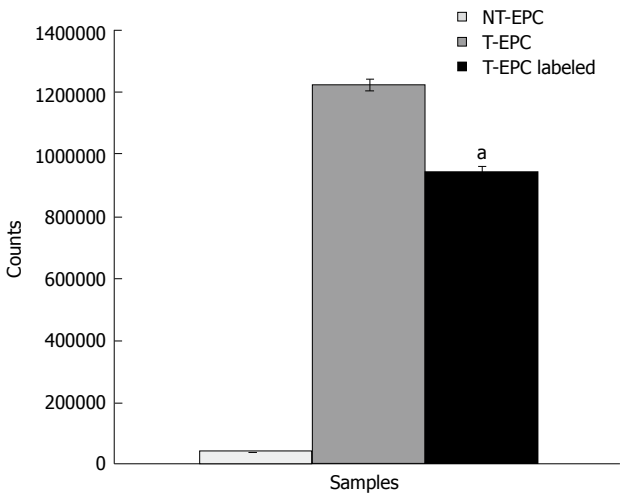

Figure 1 Transgene expression study using TC-99m uptake assay. Transfected and control endothelial progenitor cells (EPCs) were subjected to Tc-99m uptake assay to determine the transgene expression. Tc-99m uptake assay was performed with three type of conditions: (1) Non transgenic, non-labeled (without iron) EPC (control); (2) transgenic, non-labeled EPC; (3) transgenic and labeled (with iron) EPCs. Transfected EPCs showed higher Tc-99m uptake compared to control non transfected EPCs, which clearly indicates the functional expression of transgene. Data was indicated with Mean ± SD. $^{a}P < 0.05$ *vs* control cells.

Statistical analysis

All data are expressed as mean ± SD. A P value of < 0.05 was considered significant.

RESULTS

Reporter gene (hNIS) expression, viability and proliferation

EPCs were transfected with adenovirus to study the ability of EPCs as vehicle to deliver the adenovirus into glioma. Firstly, we optimized the transfection efficiency without compromising the cell viability, which is important to determine the cell to viral dose. We studied the viral dose versus cell viability by transfecting cells with different doses of viral particles. We determined cell to viral ratio (1:2000) was optimal dose. We further studied the expression of transgene in EPCs using Tc-99m uptake assay. Figure 1 shows Tc-99m uptake was significantly higher in transfected EPCs as well as in FePro labeled transfected EPCs compared to the control EPCs. These results indicate the functional expression of *hNIS* gene in the EPCs.

MRI and SPECT imaging

To study whether EPCs can deliver transgene to glioma, we injected transfected FePro labeled EPCs directly (intratumor) into glioma. Animals underwent MRI before and seven days after intratumor injection of EPCs. Figure 2 shows animals that received Fepro labeled EPCs into the tumor showed low signal intensity on MRI inside the tumor. All animals that received either labeled or non-labeled transfected EPCs showed accumulation of Tc-99m in the tumor. On the other hand, animals that received non-labeled non-transduced EPCs (control) showed neither low signal intensity nor Tc-99m activity

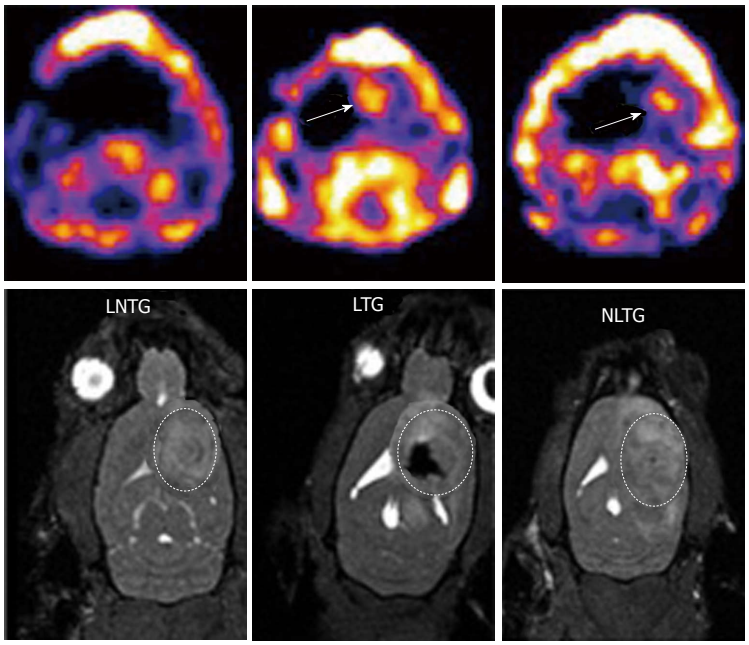

Figure 2 Magnetic resonance imaging and single photon emission computed tomography images for tracking of intratumor injected endothelial progenitor cells and transgene expression. Expression of transgene (*hNIS*) was detected by single photon emission computed tomography (SPECT) and magnetic resonance imaging (MRI) was used to detect the cell migration in the tumor. Upper panels (SPECT images): Left: Animals received non transgenic, non-labeled endothelial progenitor cells (EPCs) (control); Middle: Animals received transgenic EPCs labeled with iron; Right: Animals received non labeled transgenic EPCs. Corresponding MRI images are shown in the lower panels. Animals received labeled transgenic EPC or non-labeled transgenic EPC showed higher activities of Tc-99m in the tumors compared to control animals that received non-transgenic EPCs. MRI images clearly indicate the presence of iron labeled transgenic EPCs (lower panel, middle).

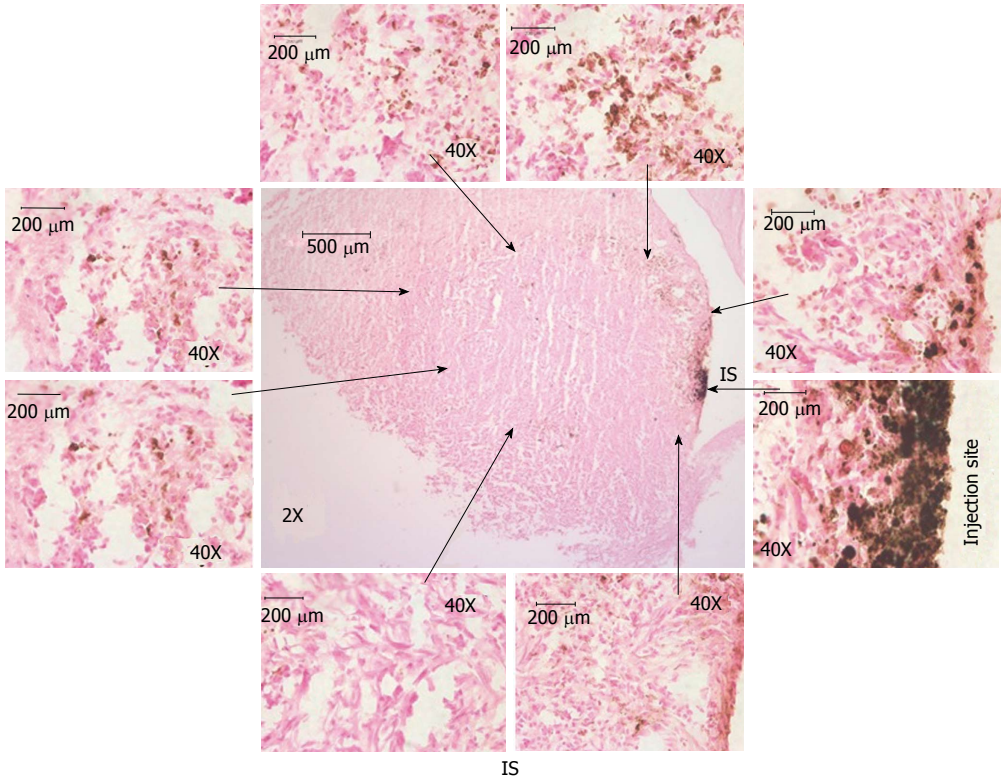

Figure 3 Histological analysis of intratumor injection and migration of cells. Animal brain sections were stained with Prussian blue and observed under light microscope. Center image (magnification × 2) shows the iron positive cells at injection site and migration around periphery and inside the tumor. Images × 2 shows the whole tumor area and injection sites and images × 40 shows iron labeled cell at injection site and around the tumor. Images × 40 were linked to images × 2 with arrow to show the region × is 40 near to the arrows (not exact match). These images indicate the migration of transgenic endothelial progenitor cells (EPCs) to periphery of the tumor from the injection site. Some of the EPCs were also migrated to the center of the tumor from the injection site. IS: Injection site.

in the tumor (Figure 2).

Histological and immunological analysis

Migration of intratumor injected iron labeled EPCs in and around the tumor was detected using prussian blue staining (Figure 3). These results indicate that the EPCs are migrating from the injection sites to the periphery as well as center of the tumors. The sections also stained with HE to observe the necrotic cells, we observed necrotic cells at periphery and central regions of the tumors (Figure 4). Immunohistochemistry staining revealed the expression of transgene (*hNIS*) at the injection site and at distal areas where injected EPCs migrated (Figure 5). Double labeling of sections with

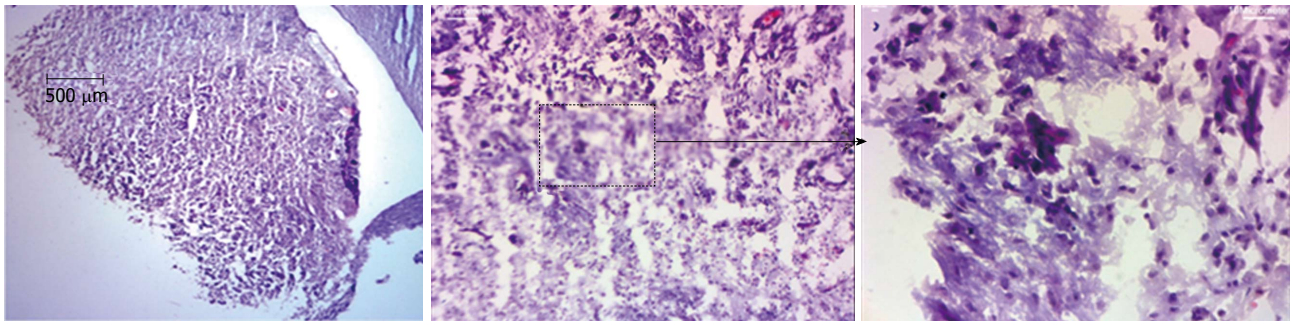

Figure 4 Hematoxylin and eosin staining of brain sections: Animal brain sections were stained with hematoxylin and eosin, In images × 2 show whole tumor area and images × 10 and images × 40 showing some the necrotic cells at injection site as well as at others regions of the tumor.

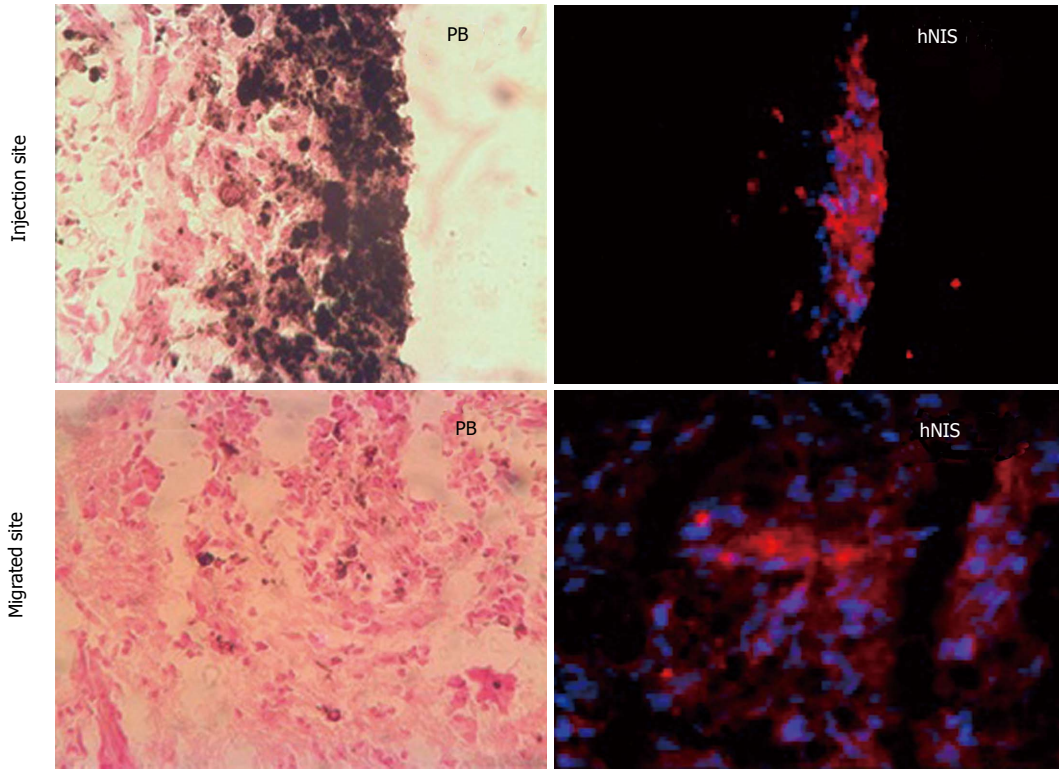

Figure 5 Immunohistochemical analysis of transgene expression at the site of injection and migrated areas. Red fluorescence (hNIS positive) is observed at site of injection of transgenic iron labeled endothelial progenitor cells (EPCs) and areas where injected EPCs migrated.

vWF and hNIS showed that endothelial cells migrated and expressed the transgene (Figure 6). To find out whether adenovirus also transfected the adjacent tumor cells, sections were also double stained for EGFR and hNIS. EGFR is highly expressed in glioma cells and can be used as a marker to detect the tumor cells. In double labeling, some of the hNIS positive cells also stained for the EGFR marker which indicates that adenovirus has transfected tumor cells (Figure 6). This indicates EPCs carrying replication competent adenoviral vector can transmit viral vector to the surrounding tumor cells and can act as delivery vehicles.

DISCUSSION

In this study, we used cord blood derived EPCs to deliver transgenes directly into glioma in rat models. Replication competent adenovirus was used to transfect EPCs and the transfected EPCs were directly injected into the tumors to deliver the transgene to glioma. Adenovirus has been used in gene therapy for the treatment of glioma due to various advantages[27,40,41]. Adenoviral vectors have capability to deliver therapeutic genes to tumor cells and at same time replicate in the tumor cells where by it destroys tumor cells[41]. However, the efficacy of using adenoviral vectors in tumor therapy is hampered due to volumes of distribution and blood brain barrier[42,43]. The migratory ability of the tumor cells and their infiltration into the normal brain parenchyma is the main limiting factor for adenoviral penetration and gene delivery[44,45]. New methods are warranted to enhance delivery of therapeutic genes to glioma *via* adenovirus for

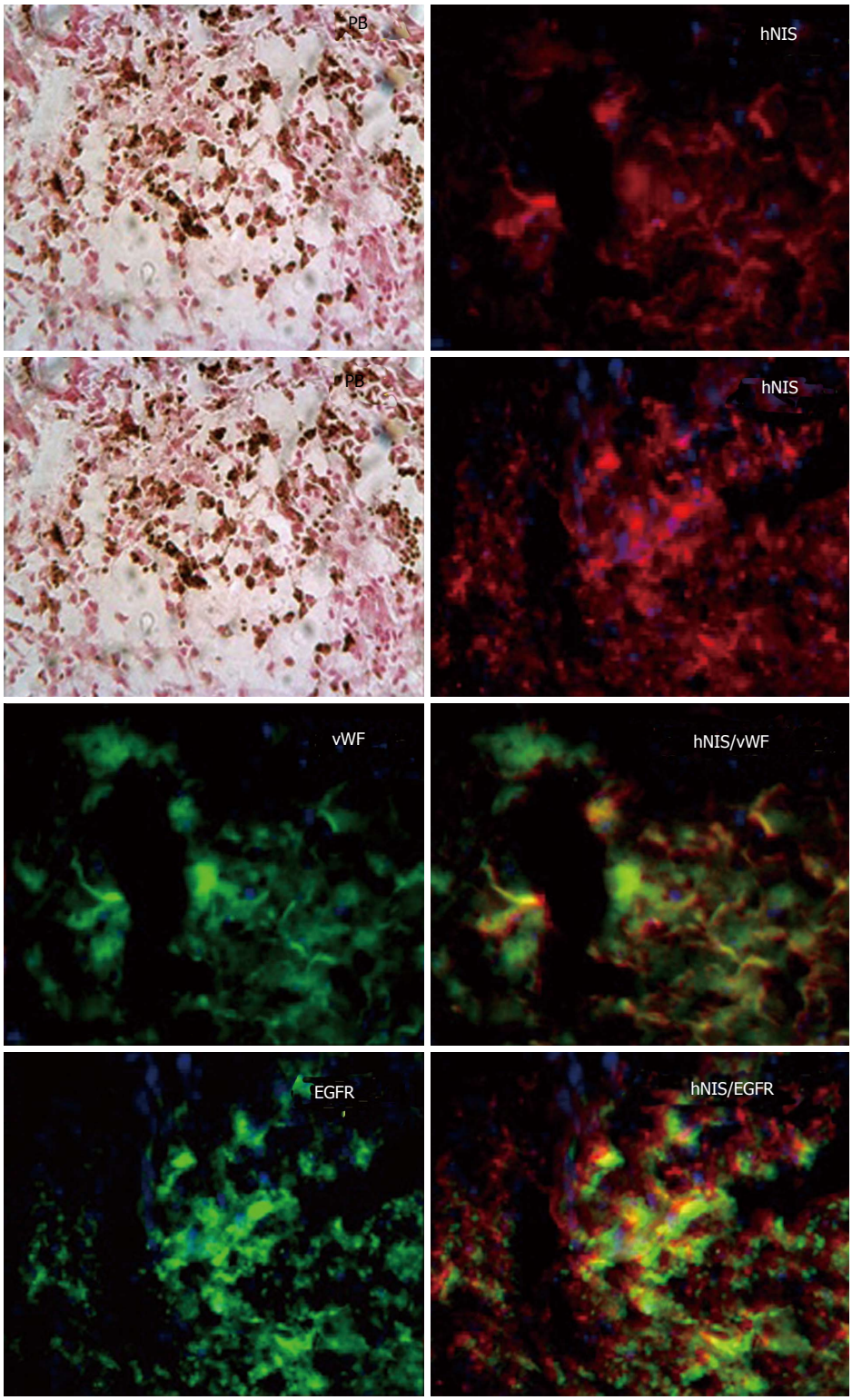

Figure 6 Analysis of transgene delivery to tumor cells by double labeling. Sections were double with hNIS/vWF or hNIS/EGFR. Sections stain with hNIS/vWF clearly shows some of the red hNIS positive cells also express green vWF positive markers [positive for endothelial progenitor cells (EPC)] indicating the administered EPCs. While hNIS/EGFR double staining clearly shows some of the red positive cells (hNIS positive cells) also express green *EGFR* positive markers indicating expression of transgenes in glioma cells. Prussian blue staining shows the iron positive cells at the corresponding sites.

improved gene delivery. In this context, cord blood derived EPCs have qualities such as their unique ability to self-renew, are easy to extract, give less immune response making them as candidates for gene delivery and recently several genetic tools have been developed to manipulate them to carry transgenes further, which enhance their case[14,26,36]. Our recent studies suggest that cord blood derived EPCs can be used as therapeutic gene delivery vehicles to brain tumors in animal models[14]. In our previous report, we showed systemic injection of lentivector

transduced EPCs to carry transgene to glioma. Where we used a replication deficit lentivirus to integrate the reporter gene and showed their deliver capacity of transgenes into tumors[14]. In this present study, we have used replication competent adenovirus to transfect EPCs and deliver transgenes directly into tumors *via* intratumor injections. The advantage with this delivery system is one way it can deliver transgenes to tumors and secondly it can destroy the tumor cells by its self-replication properties[7,23]. Intratumor administration of cells helps in direct loading of therapeutic genes into tumors and avoids distribution of transfected EPCs to other organs following administration. In addition, adenovirus transfected EPCs can help self-replication of the virus, which helps release a large volume of transgenes into glioma[41,42,44,46]. To generate transgenic EPCs, we have transfected EPCs with adenovirus carrying the *hNIS* gene. We tested the transgene expression using Tc-99m uptake assay.

The route of administration of transgenic EPCs is important for the safe and optimal gene delivery. When transduced (by lentivector) EPCs were injected intravenously, most of the transgenic cells accumulate in the liver, spleen, and bone marrow[14]. These are vital organs and if it were by replication competent adenoviral vector mediated transfection, adenovirus could have infected the cells in some of these organs[47,48]. On the other hand, intratumor injection of EPCs would less likely go beyond the margin of the tumor or the primary organ that contains the tumor. In tumor conditions, tumor cells releases several factors which signals the migration of endothelial cells from bone marrow towards the glioma[49]. In this context, intratumor injected EPCs would migrate to the margin of tumors by similar signal mechanisms. In our previous studies we already showed that the locally implanted EPCs migrated towards the periphery of the tumor[50]. Several other studies indicate the success of cells delivering viral vectors to tumor, which include mouse fibroblasts, neural precursor cells, T-lymphcytes and MSCs[51-54]. To take the cell therapy to clinics it is also important to monitor the migration of the administered cells away from the site of injection using *in vivo* imaging tools. MRI is a non-invasive, high resolution imaging tool for tracking the migration of administered cells *in vivo*[55]. Tracking of viral vectors is important aspect in gene therapy to analyze their biodistribution around the glioma and to study their targeting of infiltrative tumor cells[42]. Adenoviral vectors targeting glioma has been covalently tagged with super-paramagnetic iron oxide nanoparticles and monitored using MRI[42]. We have developed efficient labeling methods to label EPCs using superparamagnetic iron oxide nanoparticles (SPION) and tracked these ferumoxides labeled EPCs *in vivo*[14,21,36]. In this study, we infected the EPCs and labeled them with SPIONs and migration and homing of labeled cells was monitored with MRI. We labeled the EPCs instead of labeling the virus, which might have an advantage to generate high signal and easy detection compared to direct labeling. In addition, adenovirus is free from the conjugation since EPC is labeled with iron particles which help the virus to stay in native form and might increase viral infection ability. High labeling efficiency can be achieved by incubation with SPIONs particles which is rather simple when compared with conjugation[36]. In addition, cell labeled with iron oxide as delivery vehicles can be translated to the clinics since SPIONs are FDA approved agents[56]. Ahmed *et al*[57] used neural stem cells to deliver oncolytic adenovirus to glioma and their results showed NSCs based delivery increased adenovirus survival rate compared with the adenovirus alone. These studies clearly indicate the advantage of using stem cells to deliver the oncolytic adenovirus to tumors[57]. To our knowledge, this is the first report to use the cord blood EPCs as adenovirus delivery vehicles to glioma. In addition, we used clinical MRI (3 T) for the monitoring of the distribution of intratumor injected transgenic EPCs which further help in direct translation into clinics. One of the major disadvantage with iron labeling is that it cannot differentiate the dead and live cells, since iron positive signal can be generated from the dead cells, thus it is important to monitor the cell fate in gene therapy approach[58]. Moreover, it is important to determine the distribution kinetics of virus at glioma and to determine therapeutic effect as well as subsequent dose calculations[58]. To facilitate these qualities, we chose hNIS as reporter gene to help in monitoring viable cells, quantitate EPCs engraftment, and to determine the viral load[58].

SPECT imaging was used to track injected cells *in vivo*, and most of the works used In-111 oxine labeling to monitor the cells[59]. However, this approach has drawbacks due to radioisotope's (In-111 oxine) half-life and the signal goes down with time, which leads to short term monitoring (up to 7 d) of the injected cells. These short comings can be overcome by using reporter genes such as *hNIS*, which allows repeated detection of injected cells for long periods of time[7]. The first report on the use of hNIS as a reporter to monitor the delivery of oncolytic adenovirus was the path barker in monitoring and optimizing oncolytic viral therapy[7]. Barton *et al*[7] showed adenovirus delivery of therapeutic genes (cytosine deaminase/thymidine kinase) along with reporter genes (*NIS*) to monitor the gene therapy. These studies further indicate safety and efficacy of the adenoviral base gene delivery[5,7,23]. Most of the work on utilizing hNIS to monitor the adenovirus delivery was done on cardiac, prostate, and cystic fibrosis models[58] and not much data is available on NIS based monitoring of cell based therapies in glioma models. In this study, we transfected EPCs with adenovirus and injected them into the glioma to deliver the transgene (*hNIS*). SPECT imaging detects the uptake of Tc-99m (radioisotope) as long as the cells are alive and express hNIS[7,14,60]. We used dual imaging modalities to monitor the transgenic EPCs' ability to deliver transgenes and to determine the intratumor homing and migration of administered cells by SPECT and MRI, respectively. For transgene delivery, we observed

radiotracer (Tc-99m) uptake in the tumor site. Histological staining of brain sections revealed presence of iron labeled cells in the tumor not only at the site of injection but also away from the site of injection. We also stained consecutive sections with anti-hNIS antibody and visualized the transgene expression. Double staining of the hNIS stained sections with either vWF or EGFR showed that cells positive for hNIS expression also expressed either vWF or EGFR. These findings indicate that not only endothelial cells, (vWF positive) but also tumor cells (EGFR positive) expressed the transgenes, and this is only possible due the transfected EPCs' ability to deliver the adenoviral vectors in the surrounding tumor cells.

In a conclusion**,** this study is an exploration of EPCs' capacity to deliver transgenes in glioma upon intratumor administration. We showed transfected EPCs can be tracked once implanted in tumors using MRI imaging. We successfully monitored the transgene delivery by EPCs to tumor cells using SPECT imaging and immhunohistochemistry. This study showed the usefulness of EPCs as delivery vehicles of adenoviral vector to deliver therapeutic genes to glioma and act as imaging probe.

ACKNOWLEDGMENTS

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COMMENTS

Background

Glioblastoma is one of the aggressive primary brain tumors with survival period falls from one to three years upon treatment. Based on the characteristics of endothelial progenitor cells (EPCs), it is possible to use these cells as vehicles for the delivery of therapeutic genes to gliomas (using viral vectors). In addition, EPCs can be collected from a patient's own peripheral blood and bone marrow, which in turn eliminate the possibility of immune response.

Innovations and breakthroughs

Authors showed transfected EPCs can be tracked once implanted in tumors using magnetic resonance imaging imaging. Authors successfully monitored the transgene delivery by EPCs to tumor cells using single photon emission computed tomography (SPECT) imaging and immhunohistochemistry.

Applications

The transfected EPCs can be used to deliver the gene in tumor gene therapy. EPCs can be used as both imaging and therapeutic probes.

Peer review

In this study, authors have shown application of SPECT in monitoring EPCs transfected by recombinant adenoviral vectors harboring hNIS-reporter for the final aim of gene therapy in glioma cells. Results are clear and are informative for publication

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CASE REPORT

Pulmonary artery sarcoma successfully treated by right pneumonectomy after definitive diagnosis

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Abstract

Pulmonary artery sarcoma (PAS) is a rare and lethal neoplasm that is usually diagnosed during surgery or autopsy. Early diagnosis and radical surgical resection offer the only chance for survival. However, making a preoperative histopathological diagnosis is quite difficult. We encountered a 57-year-old woman presenting a PAS that mimicked a pulmonary thromboembolism. After confirming a definitive diagnosis using a catheter suction biopsy, we successfully performed a right pneumonectomy *via* a median sternotomy without cardiopulmonary bypass. Eighteen months after surgery, no recurrence was observed.

Key words: Pulmonary artery sarcoma; Preoperative diagnosis; Surgery; Catheter suction biopsy; Pneumonectomy

Core tip: Pulmonary artery sarcoma (PAS) is a rare and lethal neoplasm that is and usually diagnosed during surgery or autopsy. Early diagnosis and radical surgical resection offer the only chance for survival. However, preoperative histopathological diagnosis is quite difficult owing to the location of the tumor and its rarity. We report a 57-year-old woman patient for whom a preoperative definitive diagnosis of PAS was obtained using catheter-suction biopsy and describe how we successfully performed a curative right pneumonectomy *via* a median sternotomy without cardiopulmonary bypass. Eighteen months after surgery, no recurrence was observed.

Fukai R, Rokkaku K, Irie Y, Imazeki T, Katada Y, Watanabe H, Ueda Y, Miyamoto H, Chida M. Pulmonary artery sarcoma successfully treated by right pneumonectomy after definitive diagnosis. *World J Clin Oncol* 2013; 4(4): 102-105 Available from: URL: http://www.wjgnet.com/2218-4333/full/v4/i4/102.htm DOI: http://dx.doi.org/10.5306/wjco.v4.i4.102

INTRODUCTION

Pulmonary artery sarcoma (PAS) is a rare tumor that is generally considered fatal and is often misdiagnosed as a

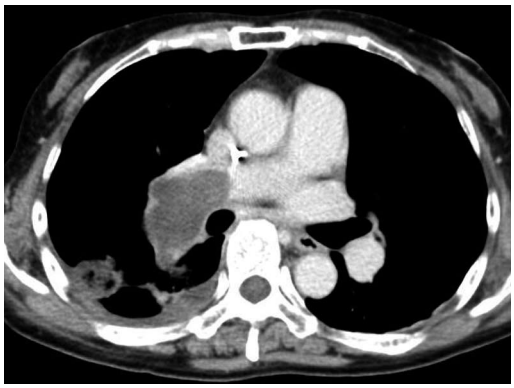

Figure 1 Axial enhanced computed tomography. At the level of the bronchus intermedius, showing a filling defect that occupies the entire lumina of the right and interlobar pulmonary arteries.

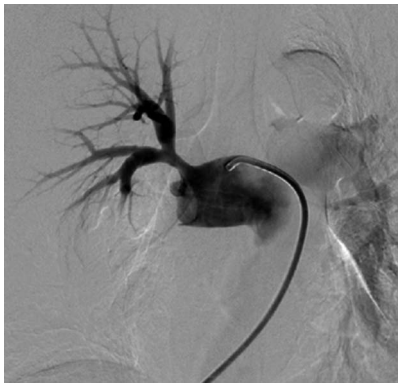

Figure 2 Pulmonary angiography. Showing the flow cutoff of the right main pulmonary artery at the base of the truncus arteriosus.

pulmonary embolism (PE)[1]. This misdiagnosis contributes to its poor prognosis, as it delays making the correct diagnosis and administering the appropriate treatment. Only a few hundred cases have been reported[2], following the first description by Mandelstamm in 1923[3]. Early diagnosis and radical surgical resection offer the only chance for survival, but owing to the location of the tumor and its rarity, preoperative histopathological PAS diagnosis has seldom been reported[4,5]. We report a patient for whom a preoperative definitive diagnosis of PAS was obtained using catheter-suction biopsy and describe how we successfully performed a curative right pneumonectomy.

CASE REPORT

A 57-year-old female was referred to our hospital with an abnormal shadow on chest radiography and a history of chest pain, dyspnea, malaise, and fever. Her medical history included osteoporosis and scoliosis. Laboratory test results revealed a mild impairment of liver function, elevation of biliary enzymes and moderate increase of the erythrocyte sedimentation rate. A remarkable prolongation of the activated partial thromboplastin time (180/30.5 s) and a slight acceleration of the fibrinogen (618 mg/dL) and fibrin degradation products (6.4 μg/mL), as well as D-dimer (2.50 μg/mL), were also detected. She was initially diagnosed with a pulmonary embolism according to the enhanced computed tomography (CT) findings and underwent thrombolytic therapy with the placement of an inferior vena cava (IVC) filter. However, her symptoms and CT findings did not improve with treatment (Figure 1), and the IVC filter was retrieved. Moreover, positron emission tomography-computed tomography (PET/CT) revealed moderate ^{18}F-fluorodeoxyglucose uptake in the pulmonary embolism. We then considered the possibility of PAS and performed a pulmonary angiography, which revealed a filling defect occupying the entire luminal diameter of the right main pulmonary artery at the base of the truncus anterior (Figure 2). We performed intravenous catheter-suction biopsy using a 9 F multipurpose-guiding catheter (Vista Brite Tip®, Cordis Corporation, East Bridgewater, NJ) and a 50 mL syringe at the right peripheral pulmonary artery. The proximal portion of the lesion, which mostly consisted of a blood clot, was too soft to perform a traditional biopsy. Pathology revealed a few atypical spindle cells in a large volume of clotted blood; according to these results, the lesion was definitively diagnosed as PAS. While awaiting surgery, the patient had a recurrence of chest pain and fever; we suspected that these symptoms were due to a repeat pulmonary infarction and that her initial symptoms indicated a prior infarction. We therefore performed lung perfusion scintigraphy. Compared with a previous examination performed 1.5 mo prior, the findings were obviously worsened: the right lung was not visualized on the scan (Figure 3).

Figure 3 Lung perfusion scintigraphy. Just prior to the operation (right side) had worsened relative to that of a previous examination (left side).

We performed a right pneumonectomy through a median sternotomy, which is suitable for exposing the right main pulmonary artery between the ascending aorta and the superior vena cava without using an artificial cardiopulmonary machine. We transected the pulmonary artery after double-stapling at its origin, and the right main bronchus was also stapled at this location. The pulmonary veins in the right thoracic cavity were stapled. We did not carry out mediastinal lymph node dissection.

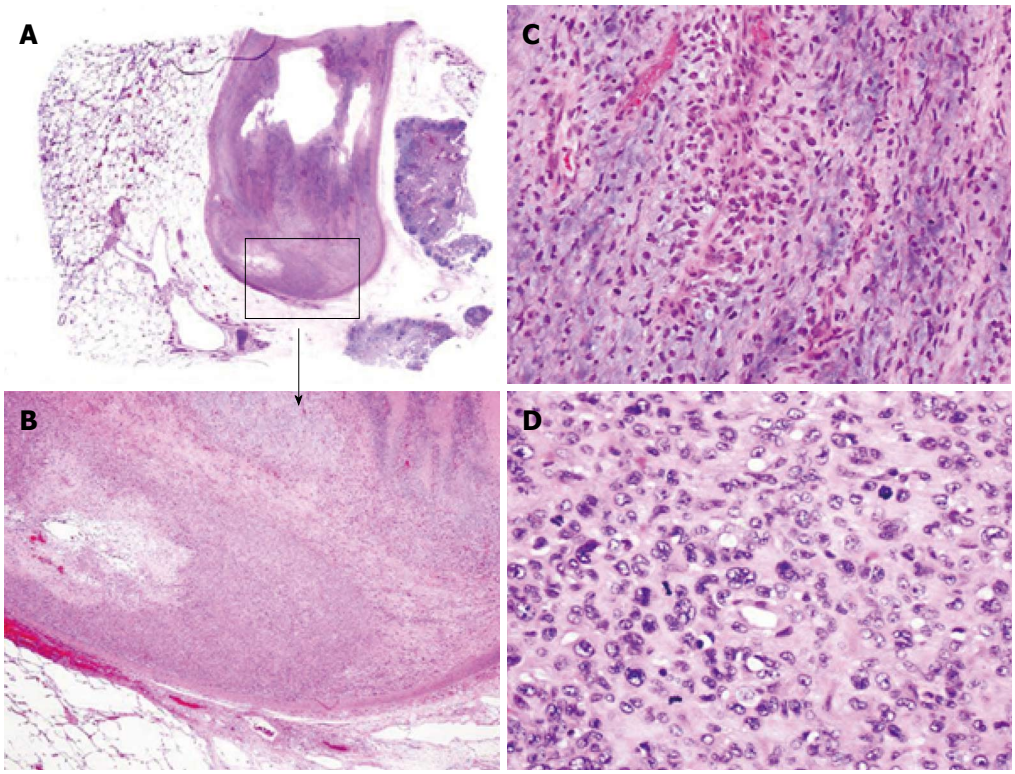

Figure 4 Hematoxylin and eosin microscopy. A, B: The tumor occupies the lumen of the pulmonary artery (A: loupe image, B: × 20 magnification); C: Tumor cells consisting of atypical spindle cells (× 200 magnification); D: Tumor cells accompanying scattered mitotic figures (× 400 magnification).

Macroscopically, a yellowish-white tumor was observed within the lumen of the right pulmonary artery, and diffuse lung congestion was noted. Microscopic examination revealed many atypical polymorphic spindle cells in the lumen of the pulmonary artery; the pathological diagnosis was intimal sarcoma of the pulmonary artery (Figure 4). The postoperative course of the patient was uneventful, and she was discharged 10 d after surgery. Eighteen months later, no evidence of recurrence was observed on CT angiography (Figure 5).

DISCUSSION

PAS is an extremely rare and usually lethal neoplasm that is most commonly diagnosed during surgery or autopsy. It is often misdiagnosed as PE[1], and its prognosis is very poor, partially due to this misdiagnosis precluding more rapid treatment. Because the tumor always arises from the central pulmonary arteries, preoperative histopathological diagnosis is quite difficult and has only rarely been reported[4,5]. We used transvenous catheter-suction biopsy that required repeated suction attempts using a syringe during pulmonary angiography because few tumor cells were present within an area of extensive coagulation. With a definitive diagnosis, we successfully performed a curative right pneumonectomy, and the patient has been in good health without recurrence for 1 year and 6 mo after surgery.

There are a few reasons why we chose right pneumonectomy as the curative operation for this patient.

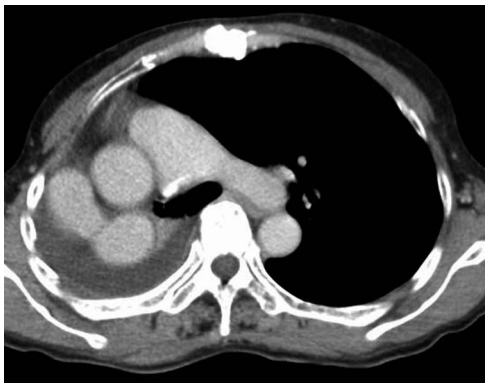

Figure 5 Axial enhanced computed tomography. Eighteen months after surgery, no recurrence is apparent.

The first was the deterioration of her right lung, with a diffuse right lung infarction that had been ongoing for almost 1 year. The patient had gradually become accustomed to this cardiopulmonary status, with a narrowing of the right thorax already having occurred by the time she was referred to our hospital. We therefore decided that right pneumonectomy would be reasonable for this patient. We also thought that right pneumonectomy was necessary to achieve complete tumor resection, as tumor cells may have existed in the distal pulmonary artery-lumen coagulation. Gan *et al*[6] reported that patients with PAS who undergo distal embolectomy live longer than patients who do not, suggesting that PAS tumor cells exist in the distal pulmonary artery thrombi.

Differentiating between PAS and PE is extremely difficult. Our strongest reason for suspecting PAS was the enlargement of the pulmonary artery diameter on CT, which increased despite anticoagulant therapy. Yi *et al*[7] evaluated 7 patients with PAS and reported that CT can help differentiate PAS from PE by indicating a low-attenuation filling defect that occupies the entire luminal diameter of the proximal or main pulmonary artery in PAS. This finding was observed in all 7 patients (100%), and expansion of any segment of the pulmonary artery, with an extensive intraluminal filling defect, was observed in 6 of the 7 patients (86%). Moreover, Cox *et al*[2] described that the presence of a hilar mass causing unilateral enlargement of the pulmonary artery and proximal branches is specific to pulmonary artery sarcoma. Unilateral central embolus is uncommon. In patients diagnosed with PE, the possibility of PAS should be considered if a unilateral widened diameter of the pulmonary artery and/or a low-attenuation filling defect occupying the entire luminal diameter at the level of the main or proximal pulmonary artery is present.

In our patient, the 2 bouts of back pain and high fever were thought to be due to a major pulmonary infarction. The visible progression of her disease on lung perfusion scintigraphy over a 1.5-mo period was valuable in the decision of the appropriate pulmonary-artery transection site through a median sternotomy. If a patient presents suspicious symptoms that might indicate advancing PAS, clinicians should not hesitate to carry out additional examinations.

In a conclusion, PAS is a rare, life-threatening tumor that arises from the pulmonary artery and its proximal branches. The most effective treatment (and the best chance for survival) is radical surgery. We treated a patient with PAS using a right pneumonectomy after making a definitive diagnosis using catheter suction biopsy. The patient is alive and well, 18 mo after surgery.

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