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EDITORIAL

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Editorial Board Member of *World Journal of Experimental Medicine*, Mikhail Alexeyev, PhD, Associate Professor, Department of Cell Biology and Neuroscience, University of South Alabama, Mobile, AL 36688, United States

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Jin-Lei Wang, Director
World Journal of Experimental Medicine
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501, Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
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EDITORIAL

Predictive markers of endocrine response in breast cancer

Duniya Mosly, Arran Turnbull, Andrew Sims, Carol Ward, Simon Langdon

Duniya Mosly, Arran Turnbull, Andrew Sims, Applied Bioinformatics of Cancer, University of Edinburgh Cancer Research Centre, MRC Institute of Genetics and Molecular Medicine, Edinburgh EH4 2XR, United Kingdom

Duniya Mosly, Arran Turnbull, Carol Ward, Simon Langdon, Cancer Research UK Edinburgh Centre and Division of Pathology Laboratory, MRC Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh EH4 2XU, United Kingdom

Carol Ward, the Royal (Dick) School of Veterinary Studies and Roslin Institute, University of Edinburgh, Easter Bush, Roslin, Midlothian, Edinburgh EH25 9RG, United Kingdom

ORCID number: Duniya Mosly (0000-0001-8477-5871); Arran Turnbull (0000-0002-2287-0889); Andrew Sims (0000-0001-9082-3665); Carol Ward (0000-0002-9969-959X); Simon Langdon (0000-0002-7200-9237).

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Correspondence to: Simon Langdon, PhD, Reader (Associate Professor), Cancer Research UK Edinburgh Centre and Division of Pathology Laboratory, MRC Institute of Genetics and Molecular Medicine, University of Edinburgh, Crewe Road South, Edinburgh EH4 2XR, United Kingdom. simon.langdon@ed.ac.uk
Telephone: +44-131-5371763
Fax: +44-131-5373159

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Abstract

Ongoing clinical and research efforts seek to optimise the use of endocrine therapy in the treatment of breast cancer. Accurate biomarkers are needed that predict response for individual patients. The presence of the estrogen receptor (ER) as the direct (for tamoxifen and fulvestrant) or indirect (for aromatase inhibitors) target molecule for endocrine therapy remains the foremost biomarker and determinant of response. However, ER expression only poorly predicts outcome and further indicators of response or resistance are required. The development and application of molecular signature assays such as Oncotype Dx, Prosigna, Mammaprint and Endopredict have provided valuable information on prognosis and these are being used to support clinical decision making on whether endocrine therapy alone alongside surgery is sufficient for ER-positive early stage breast cancers or whether combination of endocrine with chemotherapy are also warranted. Ki67, the proliferation marker, has been widely used in the neo-adjuvant (pre-operative) setting to help predict response and long term outcome. Gene expression studies within the same setting have allowed monitoring of changes of potential predictive markers. These have identified frequent changes in estrogen-regulated and proliferation genes. Specific molecules such as mutant ER may also prove helpful biomarkers in predicting outcome and monitoring response to treatment.

Key words: Estrogen; *IL6ST*; Biomarker; Breast cancer; Predictive

Core tip: The expression level of estrogen receptor remains the major determinant of response for endocrine

therapy in breast cancer. Molecular signatures provide increasing confidence for helping identify breast cancers for which endocrine therapy alone is likely to be sufficient. Estrogen and proliferation related genes have come to the fore in many of the molecular signatures. In neo-adjuvant studies, Ki67 expression at baseline and after 2 wk treatment can provide useful prognostic and predictive information. Neo-adjuvant studies continue to seek new markers that relate to tumor response.

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INTRODUCTION

Breast cancer is the second most frequently diagnosed cancer worldwide with an estimated 1676000 new cases each year[1]. Of these cancers, approximately 70%-80% will have estrogen receptor (ER) expression and be considered candidates for endocrine therapy. Tamoxifen and the aromatase inhibitors represent the major endocrine treatments in use worldwide. Tamoxifen was first approved in 1977 for treatment of breast cancer and continues to be used in many post-menopausal women, but is primarily recommended for use in pre-menopausal women[2]. The 3rd-generation aromatase inhibitors (anastrazole, letrozole and exemestane) have demonstrated superiority over tamoxifen in post-menopausal ER-positive breast cancer and have become the preferred option for this group of cancers[3]. Fulvestrant, a "pure" anti-estrogen and ER down-regulator, is an alternative after treatment failure in post-menopausal women and being considered in other settings[4]. Meta-analyses of multiple clinical trials have demonstrated that these endocrine agents can halve the risk of breast cancer relapse and reduce the risk of breast cancer death by 40%[5].

With recognition of the molecular heterogeneity present both within and between individual breast cancers, strenuous efforts have been undertaken to optimise individual patient management. This has led to the search for predictive biomarkers that might identify ER-positive breast cancers which are sensitive to endocrine therapies and those in which endocrine therapy is likely to be insufficient, hence requiring either chemotherapy or new agents. Since prognostic molecular signatures are now also helping to stratify patient groups into those for which endocrine therapy alone is likely to be sufficient, these will be mentioned briefly as well.

ER, PROGESTERONE RECEPTOR AND HER2

Foremost and most powerful of the biomarkers identified to predict response to endocrine therapy is the ER itself, specifically ER-alpha (ESR1)[6]. The routine classification of breast cancers into ER-positive and ER-negative categories was based on the early identification of the requirement for ER expression for response to tamoxifen with 60%-70% of ER-positive patients responding to this endocrine agent compared to only 5%-10% responding with ER-negative metastatic disease[2]. Consistent with this, the likelihood of response increased with increasing ER concentration with ER-rich tumors responding better than ER-poor cancers[7]. However, even for responders, up to 50% will eventually relapse hence predictive biomarkers are required that will identify ER-positive patients most likely to respond to therapy and those for whom endocrine therapy is likely to be insufficient[2].

Other forms of ER include ER-beta, G-protein coupled ER (GPER1) (previously GPR30) and mutated versions of ER-alpha and these have all been investigated as predictive markers of response to endocrine therapy. The role of ER-beta appears complex and dependent on whether ER-alpha is present leading to a bi-faceted role[8], however several clinical studies have suggested predictive effects for specific ER-beta isoforms[8,9]. Low expression of the membrane-bound GPER1 is associated with favourable outcome to tamoxifen[10] while high expression has been associated with tamoxifen resistance[11]. The role of ER mutants is discussed below.

The ER is one of 3 markers (ER, PR and HER2) routinely measured at diagnosis to help determine potential treatment options. Expression of the progesterone receptor (PR), an estrogen-regulated protein, is highly estrogen dependent and has therefore been regarded as an indicator of estrogen-drive and signaling. It has been associated with both disease-free as well as overall survival in tamoxifen-treated breast cancers with PR-positive breast cancers responding better than PR-negative[12] cancers, but this is not a universal finding[13]. Breast cancers that are both ER-positive and PR-positive have > 70% likelihood of response to endocrine therapy and these two receptors have become the prototypic predictive markers of endocrine response in this disease[14]. The third molecule routinely assessed at diagnosis, HER2 (assessed for amplification or overexpression), while developed as a predictor for anti-HER2 targeted therapies, *e.g.*, trastuzumab or lapatinib, is generally associated with poor response to endocrine therapy[15,16]. One multi-protein assay tool using immunohistochemistry, the IHC4 score, combines information from ER, PR, HER2 and the proliferation index Ki67 into a score that helps estimates the risk of distant recurrence at 10 years in post-menopausal women with ER-positive breast cancer who have received 5 year of endocrine therapy[17]. These same 4 markers are also components of the Oncotype Dx and Prosigna assays which will be described later.

DEVELOPMENT OF ENDOCRINE RESISTANCE

A major limitation of endocrine therapy is the development of resistance and markers that reflect these resistance mechanisms may predict outcome[14]. Resistance may

be present at the outset (*de novo*) or develop on drug treatment (acquired) and can arise in multiple ways[18]. Two well defined mechanisms of endocrine resistance are the loss of ER function and the development of estrogen-insensitivity.

ER function can be lost as a result of decreased ER expression or ER co-activator expression or function. ER expression is lost in approximately 10% or so of breast cancers on neo-adjuvant treatment[14], and these cancers have a poorer outcome than where ER expression is maintained[19]. This would be reflected in reduced down-stream signalling such as decreased PR expression or estrogen-regulated gene expression in the absence of an inhibitor and these can be indicative of a lack or loss of estrogen signaling.

The development of estrogen-independent signaling can lead to insensitivity to estrogen. This can occur *via* ER gain-of-function mutations[19-21] or by indirect activation of ER phosphorylation or ER-coactivator phosphorylation (hence avoiding the need for estrogen activation) *via* growth factor pathways including EGF receptor, HER2 and IGFIR[18]. Gain-of-function mutations in ER may bypass inhibition produced by endocrine agents. Although these ER mutations are infrequent in initially diagnosed disease, a much higher mutation rate has been observed in metastases (up to 20%) and circulating tumor DNA (up to 40%) in metastatic breast cancers[19-21]. This may be a cause of endocrine resistance to aromatase inhibitors (since production of estrogen is no longer needed to activate the receptor) and tamoxifen or fulvestrant therapy may be more effective in these cancers[19].

Increased expression of EGFR, HER2 or IGFIR have all been associated with reduced or loss of endocrine regulation and are potential indicators of endocrine resistance[18]. Moreover, the pathways they use, *i.e.*, the PI3K/AKT and Ras/Raf/MEK/ERK pathways, may have activating mutations, *e.g.*, in components such as PI3K, which in turn may lead to ER activation[22]. To date, this information has been used to develop combination drug approaches that combine an endocrine agent with an inhibitor (*e.g.*, HER2, PI3K, mTOR, CDK inhibitor, *etc.*) that targets a component of the growth factor driven pathway. Although this has been valuable for the strategic development of inhibitory strategies in endocrine-resistant disease, it hasn't yet led to the development of specific markers to predict endocrine resistance. Even for ER-positive/HER2-positive breast cancers, wherein many cancers are responsive to endocrine treatment, it remains unclear which tumors are sensitive and which are resistant indicating the need for further markers of response.

The detection of ER mutations in circulating tumor DNA is promising and supports the use of plasma sampling to help monitor the changing status of the disease in the patient. Retrospective analyses of ER mutations in baseline plasma circulating tumor DNA from completed clinical trials suggest that these mutations are prognostic and predictive of resistance to aromatase inhibitors in metastatic disease[23] however prospective studies will be needed to validate clinical utility.

MULTIGENE SIGNATURES

It is nearly 20 years since the first detailed molecular portrait of breast cancer was published by Perou *et al*[24] that stratified breast cancers into molecular subtypes based on gene expression data. Four groups (luminal, HER2, basal and normal breast like) were identified with the luminal group describing the ERα-positive group. Further studies by the same investigators demonstrated that the ERα-positive luminal group could usefully be sub-divided into luminal A and luminal B cancers[25-27]. Luminal A cancers comprise about 40%-75% (cf. large geographical variation) of breast cancers with relatively higher levels of estrogen signalling and lower proliferation. Luminal B cancers represent approximately 10%-20% of breast cancers and tend to have lower estrogen signaling and higher proliferation or HER2 over-expression. Over time further ERα-negative subgroups such as claudin-low and molecular apocrine clusters have been suggested along with the so-called 4-6 Lehman TNBC subtypes[28-30], however luminal cancers remain the endocrine-sensitive group with luminal A in general being sensitive to endocrine therapy alone while luminal B cancers may require both endocrine therapy and chemotherapy. As further molecular portraits were characterised, a number of gene sets were developed as prognostic signatures and have been useful to help stratify groups of patients (Table 1). Several commercial assays have been developed that generate risk of recurrence scores that can be used to help determine the likely risk of relapse. These have been particularly valuable in clinical decision making to help identify which early stage ER-positive HER2-negative patients without lymph node spread (encompassing over half of all breast cancer patients) should receive endocrine therapy alone and which should receive chemotherapy or novel treatments as well in the adjuvant setting.

The multigene test most widely used in the clinic to date is the Oncotype Dx signature. Oncotype DX is a 21-gene recurrence score assay initially developed to predict likelihood of recurrence of tamoxifen-treated, node negative breast cancer[31]. This assay includes proliferation-related genes (*Ki67, STK15, Survivin, CCNB1, MYBL2*), estrogen-related genes (*ER, PGR, BCL2, SCUBE2*), HER2-related genes (*HER2, GRB7*), invasion-related genes (*MMP11, CTSL2*) and 3 others (*GSTM1, CD68, BAG1*) alongside 5 reference genes (*ACTB, GAPDH, RPLPO, GUS, TFRC*). Levels of expression of these genes are combined into an algorithm to generate a recurrence score between 0 and 100 which is predictive of overall survival[31]. If the score is high (> 31) then chemotherapy has been shown to be beneficial. If the score is low (< 10), then this is prognostic of a very low rate of recurrence (< 2%) and endocrine therapy alone is likely to be sufficient. Until recently, it was unclear whether endocrine therapy alone was adequate for patients with cancers with intermediate scores (10-25) since these can comprise 2/3 of patients, but the TAILORx trial has now demonstrated that endocrine therapy alone without added chemotherapy produces the same outcome suggesting

Table 1 Summary of the multigene tests

Test name	Samples	Key references	Method	Genes No.	Genes
Oncotype DX	FFPE tumor tissue	[31,32]	QRT-PCR	16 + 5	*MKI67, AURKA, BIRC5, CCNB1, MYBL2, ERBB2, GRB7, ESR1, PGR, BCL2, SCUBE2, MMP11, CTSL2, GSTM1, CD68, BAG1* (+ ref genes *ACTB, GAPDH, RPLPO, GUS, TFRC*)
MammaPrint	Fresh or freshly frozen breast cancer tissue or FFPE tissue	[38-40]	DNA microarray	70	*AA555029_RC, ALDH4A1, AP2B1, AYTL2, BBC3, C16orf61, C20orf46, C9orf30, CCNE2, CDC42BPA, CDCA7, CENPA, COL4A2, DCK, DIAPH3, DTL, EBF4, ECT2, EGLN1, ESM1, EXT1, FGF18, FLT1, GMPS, GNAZ, GPR126, GPR180, GSTM3, HRASLS, IGFBP5, JHDM1D, KNTC2, LGP2, LIN9, LOC100131053, LOC100288906, LOC730018, MCM6, MELK, MMP9, MS4 A7, MTDH, NMU, NUSAP1, ORC6L, OXCT1, PALM2, PECI, PITRM1, PRC1, QSCN6L1, RAB6B, RASSF7, RECQL5, RFC4, RTN4RL1, RUNDC1, SCUBE2, SERF1A, SLC2A3, STK32B, TGFB3,TSPYL5, UCHL5, WISP1, ZNF533*
Endopredict	FFPE tumor tissue	[41,42]	QRT-PCR	8 + 4	*BIRC5, UBE2C, DHCR7, RBBP8, IL6ST, AZGP1, MGP, STC2* (+ ref genes *CALM1, OAZ1, RPL37A, HBB*)
Prosigna (based on PAM50)	FFPE tumor tissue	[33-37]	Nanostring	50 + 8	*MIA, SFRP1, KRT14, KRT17, KRT5, FGFR4, GRB7, ERBB2, BAG1, MDM2, ACTR3B, BLVRA, CXXC5, TMEM45B, MMP11, FOXC1, EGFR, CDH3, PHGDH, MYC, CCNE1, CDCA1, CDC20, KIF2C, TYMS, KNTC2, UBE2T, MELK,PTTG1, CCNB1, CDC6, MYBL2, BIRC5, CENPF, EXO1, ORC6L, ANLN, UBE2C, RRM2, MKI67, CEP55, PGR, NAT1, SLC39A6, BCL2, ESR1, MAPT, GPR160, MLPH, FOXA1* (+ 8 ref genes)

FFPE: Formalin-fixed paraffin-embedded; qRT-PCR: Quantitative reverse transcriptase-PCR.

that endocrine therapy alone is sufficient for this large group of patients[32]. The trial results though did not exclude a benefit of chemotherapy for patients aged < 50 years with a high-intermediate score[32].

Several other multigene signatures have been shown to produce similar prognostic data for this group of ER-positive, HER2-negative patient group. These include Prosigna (based on PAM50), Mammaprint and Endopredict.

The Prosigna classifier uses the PAM 50 (Prediction Analysis of Microarrays) set of 50 genes together with a set of 8 reference genes to identify the intrinsic gene expression subtype (*i.e.*, luminal A, luminal B, HER2 or basal-like)[33]. This classifier identifies the cancer subtype based on comparison of the cancer's gene expression profile to the characteristic subgroup profiles and generates a risk of recurrence score. Its prognostic value has been demonstrated in multiple cohorts of breast cancer patients including those treated with tamoxifen or anastrazole alone[34,35] and tamoxifen plus anastrazole[36]. A recently developed PAM50-based chemoendocrine score has been developed that highlights luminal to basal differences and response to treatment[37].

The Mammaprint assay is a classifier based on the 70-gene Amsterdam signature[38] developed to help identify early stage breast cancer patients most likely to develop distant metastases and therefore benefit from adjuvant chemotherapy[39]. Its value has been tested in multiple clinical trials, but the largest trial has been the 6693 patient MINDACT trial[40]. In this trial, it was demonstrated that the group of patients identified as high risk for recurrence according to clinical and pathological factors but who were classified as Low Risk by MammaPrint were unlikely to benefit from chemotherapy[40].

The Endopredict test measures 8 genes of which 3 are proliferation associated (*BIRC5, UBE2C, DHCR7*) and 5 are estrogen-related genes (*RBBP8, IL6ST, AZGP1, MGP, STC2*) by RT-PCR from fixed tissue and generates a score between 0 and 15 (< 5 is low risk; > 5 is high risk)[41]. This data is combined with nodal status and tumor size information to provide an EPclin score[41,42]. The test has been validated within a number of trials[41,42].

DYNAMIC NEO-ADJUVANT STUDIES

Neo-adjuvant (pre-operative) studies, wherein breast cancer patients are treated with endocrine therapy prior to surgery, have provided opportunities to study and identify predictive biomarkers of endocrine response. In these studies, tumors have commonly been serially sampled at diagnosis, after 14 d and at 3 mo of treatment and assayed for gene or protein expression levels[43]. These studies have demonstrated that several parameters may be informative including the expression level of a biomarker at diagnosis prior to treatment, the change in expression over time during treatment and the residual level compared to baseline value after a period of treatment.

The most extensively studied pharmacodynamics marker in neo-adjuvant endocrine trials is Ki67 (MKI67) which is a nuclear protein expressed only in proliferating cells[44]. The pre-treatment value of Ki67 reflects prognosis, while the change in Ki67 relates to response to treatment, hence is predictive[45]. The 14 d value then provides an indicator of residual risk[44]. This biomarker has already been incorporated into the IHC4, Oncotype Dx and Prosigna tests and is currently being studied in the POETIC phase III multicentre trial. The POETIC trial is the largest study to assess the validity of Ki67 as a marker of response and long-term outcome in a pre-surgical window-of-opportunity setting and has recruited 4500 women with early stage ER-positive breast cancer. The study is assessing whether time to recurrence and overall survival

are influenced by 2 wk of aromatase inhibitor therapy prior to and after surgery to improve outcome compared to standard adjuvant therapy alone[44]. To date, the trial has provided evidence that measurement of Ki67 at baseline and at 2 wk is informative. If baseline Ki67 is low (value < 10%), prognosis is good and pre-operative treatment and a second measurement aren't needed. However, if baseline Ki67 is high (value > 10%) and stays high at 2 wk, then prognosis is poorer and patients should be considered for further therapy (chemotherapy or new agents)[46].

Gene sets associated with both aromatase inhibitor sensitivity and resistance have been identified within neo-adjuvant studies and gene expression changes after 14 d and 3 mo of treatment linked to tumor growth response[47,48]. A common finding in many of the gene expression changes is that both estrogen-dependent genes and proliferation-associated genes can be down-regulated on treatment, however there can be discordant patterns of change as well. These changes can occur in resistant as well as sensitive treated cancers suggesting different mechanism of resistance[49]. Higher basal expression of certain immune-related genes such as SLAMF8 and TNF as well as lymphocytic infiltration have been associated with poor anti-proliferative response and resistance[50] while high expression of ribosomal proteins is associated with response to letrozole[48].

A four-gene classifier of clinical response to the aromatase inhibitor letrozole has recently been described with an accuracy of 96% based on the expression levels of two genes (*IL6ST* and *NGFRAP1*) at baseline and two proliferation associated genes (*ASPM* and *MCM4)* after 2 wk of therapy[51]. This gene set was then validated in an independent group of patients treated with anastrazole[51]. This is now being evaluated in prospective studies. It will be important to understand the roles and functions of these genes if they are to be used alongside more traditional markers such as the estrogen-regulated PR or proliferation associated Ki67. Measurement of proliferation after endocrine treatment is also a component of the Preoperative Endocrine Prognostic Index (PEPI), that was developed to identify patients at low risk of relapse after neoadjuvant endocrine therapy so that adjuvant chemotherapy can safely be avoided[52,53].

CONCLUSION

ER expression together with PR expression continues to be the major determinant of endocrine response in breast cancer, but further markers to more accurately guide treatment would be valuable. Markers of endocrine sensitivity are helpful to provide confidence that the use of endocrine therapy alone is sufficient treatment for a tumor and there are now multiple molecular signatures that can do this. Markers of endocrine resistance will help direct change of therapy and dependent on the marker used may provide some insight into potential inhibitory strategies that may be helpful. The use of on-treatment sampling (serial biopsy or circulating tumor cells) ideally in comparison with baseline sampling will provide the best information to aid this.

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EDITORIAL

Autonomic function and ventricular tachyarrhythmias during acute myocardial infarction

Theofilos M Kolettis

Theofilos M Kolettis, Department of Cardiology, Cardiovascular Research Institute and University of Ioannina Medical School, Ioannina 45500, Greece

ORCID number: Theofilos M Kolettis (0000-0002-6680-9131).

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Correspondence to: Theofilos M Kolettis, MD, PhD, Professor, Department of Cardiology, Cardiovascular Research Institute and University of Ioannina Medical School, 1 Stavrou Niarxou Avenue, Ioannina 45500, Greece. thkolet@cc.uoi.gr
Telephone: +30-265-1007227
Fax: +30-265-1007053

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Abstract

Most cases of sudden cardiac death are attributed to sustained ventricular tachyarrhythmias (VTs), triggered by acute coronary occlusion. Autonomic dysfunction, an important arrhythmogenic mechanism in this setting, is being actively investigated, aiming at the advent of preventive strategies. Recent experimental studies have shown vagal withdrawal after anterior myocardial infarction, coinciding with high incidence of VTs, followed by more gradual sympathetic activation coinciding with a second arrhythmia peak. This article summarizes recent knowledge on this intriguing topic, generating hypotheses that can be investigated in future experimental and clinical studies.

Key words: Sudden cardiac death; Acute myocardial infarction; Ventricular tachyarrhythmias; Ventricular fibrillation; Delayed arrhythmogenesis; Ventricular tachycardia; Early arrhythmogenesis; Vagal activity; Sympathetic activity; Arrhythmogenic mechanisms

Core tip: Autonomic dysfunction in response to acute myocardial infarction is subject of continuous investigation. Recent experimental data indicated vagal withdrawal, followed by more gradual sympathetic activation, coinciding with early and delayed arrhythmogenesis, respectively. These findings call for further research on the pathophysiologic role of the autonomic nervous system on the ischemic ventricular myocardium.

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INTRODUCTION

Sudden cardiac death is a major health-related problem worldwide, accounting for more than half of cardiovascular mortality[1]. It is invariably caused by sustained ventricular

tachyarrhythmias (VTs), occurring in the setting of acute myocardial infarction (MI). The high incidence and the ominous prognosis of ischemia-related VTs dictate ample research efforts toward in-depth understanding of the underlying mechanisms, aiming at the advent of preventive strategies[2].

During acute-MI, epinephrine is released in the ischemic myocardium, followed by activation of chromaffin cells in the adrenal medulla[3]; epinephrine, either locally released or circulating, alters ventricular electrophysiology and has been long known to exert a prominent role in genesis of VTs[4]. Acute-MI is also often accompanied by marked autonomic dysfunction, but its precise time-course along the acute phase of MI and the ensuing arrhythmogenic effects remain incompletely understood. This article briefly summarizes recent knowledge on this topic that may offer further insights into the complex pathophysiology of sudden cardiac death.

AUTONOMIC DYSFUNCTION DURING MI

Afferent stimuli

Although cardiogenic reflexes were first recognized in the mid-19th century, studies on the autonomic effects on the ischemic myocardium and their impact on VTs were systematically performed only a century later[5]. These led to early clinical reports introducing the role of autonomic dysfunction on ventricular electrophysiology following acute coronary occlusion[6]. The activation of ventricular afferent fibers in the ischemic myocardium was subsequently demonstrated, mediated by hemodynamic changes induced by acute-MI, as well as by the local production of chemical stimuli[7]. This process is dynamic, determined by the time-course of left ventricular hemodynamics and by the balance between the rate of production and metabolism of various mediators.

Sympathetic afferents are mainly nonmyelinated, with only occasional thinly myelinated Aδ-fibers, that form a network over the epicardium[8]. Most sympathetic afferents are activated by adenosine triphosphate and are classified as ischemia-sensitive[7], although the pathophysiologic significance of those not responding to adenosine triphosphate remains unknown. Afferent activation depends on the location of the ischemic myocardium, as shown by experimental[9] and clinical[10] data; in this regard, vagal Aδ- and nonmyelinated C-fibers, located in the inferior left and right ventricular wall, are frequently activated during ischemia involving these walls.

Efferent autonomic activation

Afferent stimuli reach the nucleus tractus solitarius, which acts as an integrative center, signaling emergency changes in the central nervous system. In this structure, a series of sensory nuclei, embedded in the medulla oblongata, form circuits with other nuclei in the brainstem and with a large number of other central regions. The medulla contains sympathetic cell bodies, with respective nerves travelling along the spinal cord; from there, sympathetic fibers synapse with sympathetic ganglia, and postganglionic fibers ultimately synapse at their target sites. The parasympathetic cell bodies exit the medulla as long preganglionic efferent fibers that form synapses with postganglionic fibers within the myocardium.

The effects of the autonomic nervous system on ventricular electrophysiology during myocardial ischemia have attracted rigorous research efforts[11-14]: Sympathetic activation shortens the ventricular action potential and the refractory period under normal conditions, but these actions vary in the ischemic ventricular myocardium. Thus, in addition to ionic imbalance, sympathetic activation enhances the dispersion of repolarization across the energy-depleted ischemic myocardium and lowers the fibrillation-threshold[11], perhaps without altering local conduction[12]. By contrast, parasympathetic stimulation prolongs the action potential duration and the effective refractory period[13]; hence, vagal activation exerts potent anti-fibrillatory actions on the ischemic myocardium, although transmural dispersion of repolarization seems unaffected[14].

Early clinical reports have underscored the involvement of both arms of the autonomic nervous system post-MI[15]; however, the precise time-course of sympathetic and vagal alterations and their contribution to arrhythmogenesis remain incompletely understood[2]. This can be explained by the marked individual variation, attributed to the size and location of MI, its hemodynamic sequelae, and to the magnitude of the accompanying symptoms of pain and anxiety. Moreover, accurate pathophysiologic conclusions are hindered by the inevitable delays in monitoring patients in coronary care units, coupled with the confounding effects of treatment.

VTs during acute MI

In response to acute coronary occlusion, two temporally distinct peaks have been described in various species, with several lines of epidemiological data pointing towards a similar curve in man[1,2]. Although this topic has been long debated, classification into VTs linked to reversible ischemia versus those occurring during evolving necrosis is based on firm pathophysiologic differences; more importantly, classification into early and delayed VTs is clinically sound, as it corresponds to the pre- and in-hospital phases, respectively, carrying profound consequences on survival rates and potential treatment strategies. As noted above, scarce data exist in humans on the incidence of early-phase VTs and concurrent autonomic responses. Therefore, the investigation on the underlying mechanisms of ischemia-induced VTs relies largely on *in vivo* animal models; indeed, these models offer clear-cut advantages in monitoring physiologic parameters during specific periods after coronary ligation, in the absence of the confounding effects of various interventions.

ANALYSIS OF RECENT EXPERIMENTAL STUDIES

Our group recently examined the autonomic responses

and the incidence of VTs in the *in vivo* rat-model, by comparing sham-operated controls with an animal-group post-ligation of the left coronary artery[16]. Continuous electrocardiographic recording was performed in conscious rats *via* implanted telemetry transmitters, and autonomic indices were derived by heart rate variability techniques; specifically, sympathetic activity was assessed by detrended fluctuation analysis, and vagal activity by time- and frequency-domain analysis. Frequent VTs were observed post-ligation, following the typical pattern of an early prominent peak and a more prolonged delayed arrhythmogenic window. Vagal activity decreased markedly immediately post-ligation and remained low throughout the 24 h-observational period. The pattern of sympathetic activation differed, showing a progressive rise; it became significant at a later stage post-MI and remained elevated until the end of the recording. Using micro-neurographic recordings, such delayed sympathetic activation post-MI was also observed by Jardine *et al*[17] in the ovine-model, in which enhanced cardiac sympathetic nerve-activity was observed only after the first hour post-ligation. These findings support the notion of attenuated parasympathetic, rather than enhanced sympathetic-inputs, contributing to early-phase VTs, given the aforementioned anti-fibrillatory vagal effects on the ischemic myocardium[14].

Two recent studies lend further support to this hypothesis: in the canine-model[18], no antiarrhythmic effect was found after suppression of the left stellate-ganglion for 60 min post-MI, except from experiments in which its action was completely abrogated. Likewise, a study from our group[19] examined the incidence of VTs post-ligation in rats pretreated with clonidine, a centrally acting inhibitor of sympathetic preganglionic-neurons; treated rats displayed a lower incidence of VTs occurring during the delayed phase post-MI, but early phase arrhythmogenesis was unaffected[19].

PERSPECTIVE

Autonomic dysfunction, commonly observed during acute MI, contributes to the genesis of VTs. Autonomic responses vary, depending on several modulating factors, some of which remain incompletely understood; hence, the precise nature and time-course of such responses during the acute phase of MI is subject of continuous investigation. Early-stage VTs are at the center of research-efforts, because they invariably occur prior to medical attendance and they are responsible for most cases of sudden cardiac death. Recent *in vivo* experimental studies have drawn the attention toward vagal withdrawal, associated with pro-fibrillatory effects in the ischemic ventricular myocardium. Such decreased parasympathetic inputs appear to occur swiftly in response to ischemia, whereas sympathetic activation is more gradual and coincides with a second cluster of VTs. These studies provide further insights into the pathophysiology of acute MI and sudden cardiac death. Nonetheless, these findings should be viewed as hypothesis-generating research that warrants further validation in animal models and, ultimately, in patients. The investigation of autonomic dysfunction during acute MI is an intriguing topic of high clinical importance that may unravel further aspects of the interrelation between the brain and the heart.

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EDITORIAL

Prognostic role of tumor budding in breast cancer

Ioannis A Voutsadakis

Ioannis A Voutsadakis, Algoma District Cancer Program, Sault Area Hospital, Sault Ste. Marie, Ontario, Canada; and Section of Internal Medicine, Division of Clinical Sciences, Northern Ontario School of Medicine, Sudbury, P3E 2C6, Canada

ORCID number: Ioannis A Voutsadakis (0000-0002-9301-5951).

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Correspondence to: Ioannis A Voutsadakis, MD, PhD, Doctor, Algoma District Cancer Program, Sault Area Hospital, 750 Great Northern Road, Sault Ste Marie, ON P3E 2C6, Canada. ivoutsadakis@yahoo.com
Telephone: +1-705-7593434
Fax: +1-705-7593434

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Abstract

Tumor budding, defined as a small number of cancer cells observed in pathology sections detached from the main tumor mass, is a common phenomenon in cancer. It is suggested that cells in buds are in the process of actively moving away from the primary tumor in the first step of metastasis. Tumor budding has been observed in a variety of carcinomas and is best studied in colorectal cancers where it portends poor prognosis. More recently, tumor budding was found to be of prognostic significance in other cancers including breast cancer. Tumor budding in breast cancer is associated with other adverse pathologic factors, such as larger tumor size and lymphovascular invasion, but may have additional independent prognostic value. In the future, standardization of the quantification criteria for tumor budding may further aid in its adoption as a prognostic marker.

Key words: Tumor budding; Infiltration; Metastasis; Breast cancer; Prognosis; Epithelial to mesenchymal transition

Core tip: Tumor budding, defined as scattered cells or small islands of tumor cells in the vicinity but not connected to the main tumor mass, is a common occurrence in different cancers. In breast cancer, it may portend an adverse prognosis.

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INTRODUCTION

Tumor budding is a pathologic phenomenon associated with many cancers. Although its specific definition differs from study to study, it generally consists of a small number of cells, usually up to five cells in the most commonly used definition, which have detached from the bulk of the tumor and are observed as isolated cells or small clusters of cells in histologic sections. Cancers in which tumor

budding has been observed and studied include colorectal, gastric and esophageal, lung, head and neck, and also breast cancers[1]. Tumor buds may be observed in areas near the margins of tumors at the invasive tumor front and are called peritumoral buds, or inside the tumor mass and are thus called intratumoral buds[2]. Identification of the tumor buds has been undertaken using plain eosin and hematoxylin sections or immunohistochemical methods. Although plain section staining is often sufficient in order to identify tumor budding, in some occasions involving significant inflammatory cell infiltration, immunohistochemical methods increase the confidence of the assessment and the inter-observer agreement. In addition to the area of the tumor where budding is observed (intratumoral versus peritumoral) as well as the method of staining used, studies have also used differing field examinations in quantifying budding. Some studies quantify budding in five high-power fields (HPF), while others count ten HPF. Some investigators use the areas of highest budding observed in order to classify cases, while others use mean counts of all fields examined. These methodological variations make comparisons across studies less straight-forward and hamper adoption of tumor budding as a more widely-used histologic phenomenon for clinical purposes such as prognostication.

PATHOPHYSIOLOGIC SIGNIFICANCE OF TUMOR BUDDING

Tumor budding is believed to represent cancer cells caught in the process of invasion[3]. From a pathophysiologic perspective, tumor budding has been explained as a sign of cancer cell motility and as a first step in the metastatic process[1]. The metastatic process begins with detachment of cells from the tumor bulk, infiltration through surrounding tissues into small blood vessels, and travel through the circulation to remote locations where they extravasate and may eventually establish colonies of metastatic disease. Paramount in metastasis is the process of epithelial to mesenchymal transition (EMT) and the reverse process of mesenchymal to epithelial transition (MET)[4]. These processes, sometimes collectively referred to as epithelial mesenchymal plasticity, are part of normal embryogenesis and physiologic wound healing, and have been usurped by cancer. During EMT, detached cancer cells partially or completely lose their epithelial characteristics, detach from neighboring epithelial cells and gain mesenchymal characteristics, including expression of mesenchyme-associated proteins, to become motile. In metastatic sites, the reverse process takes place when arriving cells, helped by cues in their new microenvironment, regain epithelial properties and re-establish connections with neighboring cells[5]. EMT/MET associated with cancer may be incomplete, and intermediate forms with partial epithelial or mesenchymal characteristics may be part of a continuous spectrum[6,7]. In fact, cancer-associated EMT/ MET is believed to endow cells with stem cell properties, and the plasticity associated with this stemness may help motile cells alternate along the spectrum between epithelial and mesenchymal states during their metastatic journey[8,9]. Partial EMT may be the state of cells in tumor buds with two to five cells, where connections between them are maintained and the cells of the bud are destined to remain connected and move together through the circulation to the metastatic site. Alternatively, in some instances, buds may represent an initial step of detachment and, subsequently, individual cells may further detach from the other bud cells and move individually. Both scenarios have been observed in experimental studies[10,11].

Tumor cells in buds of various epithelial cancers, including colorectal, pancreatic, lung and breast adenocarcinomas, lose the normal expression of membrane E-cadherin, which shows a modified cytoplasmic pattern of expression[12]. Subsequently, the mesenchymal transcription factor ZEB1 is upregulated in the nucleus. These changes are observed in both budding cells within protrusions still connected to the main tumor mass and in cells of tumor buds already detached from the main mass[12]. Budding cells, despite expressing the mesenchymal marker vimentin, do not completely lose cytokeratin staining, consistent with an incomplete EMT[13]. ZEB1, along with the related transcription factor ZEB2, as well as other transcription factors such as Snail, Slug, Twist1 and FOXC2 constitute the core network of EMT[14]. These core factors receive signals from a complement of signaling pathways and cooperate with additional transcription factors such as NF-κB and c-Myc to influence cell fate across the epithelial-mesenchymal continuum[5]. Interestingly, NF-κB and Twist1 have been confirmed to be expressed in the cells of tumor buds and the surrounding stroma[15,16]. Two additional observations, pertaining to the biologic implications of tumor budding as a first step of the metastatic process and its relationship to EMT and stemness properties, have been reported in studies done on colorectal cancer. First, cancer cells in tumor buds lose expression of the transcription factor CDX2, which is a marker of intestinal differentiation expressed in most colorectal cancers and associated with improved prognosis compared with colorectal cancers that do not express it[17,18]. CDX2 is usually observed to be re-expressed at metastatic sites. Second, the expression of the proliferation marker Ki67 is low in tumor buds, denoting a quiescent state[19]. These observations are consistent with the dedifferentiation of tumor cells in tumor buds and low proliferation during invasion, suggestive of their acquisition of an EMT/stemness phenotype which is reversed at the metastatic sites.

PROGNOSTIC IMPLICATIONS OF TUMOR BUDDING

The clinical significance of tumor budding has begun to be elucidated in recent years with studies associating the phenomenon with adverse clinical outcomes[20,21]. The cancer location where tumor budding has been initially

described and remains still more extensively studied is the colon and rectum[2]. A meta-analysis of reports of the prognostic role of tumor budding in rejected stage Ⅱ colorectal cancers observed worse survival outcomes in patients with tumor budding, with an odds ratio for death at five years of 6.25 (95% CI: 4.04-9.67) in patients with budding compared to those that had no tumor budding in their tumors[22]. In rectal cancer, the presence of tumor budding in biopsies before neo-adjuvant chemo-radiation was associated with poor response to neo-adjuvant treatment[23]. No patients among those with tumor budding had complete pathologic response rates (pCR) to neo-adjuvant treatment, whereas pCR was observed in 17% of patients without budding in their pre-treatment biopsy.

Tumor budding has also been studied in other gastrointestinal cancers. In a series of squamous esophageal cancer patients who received neoadjuvant chemotherapy with the 5-fluorouracil, cisplatin and doxorubicin regimen, tumor budding in the post-treatment surgical specimen was the most important predictive factor for overall survival (OS) and progression-free survival in multivariate analysis[8]. Patients with high-grade budding, defined as five or more scattered cell formations (buds) in a low power field of maximal budding, had a five-year OS of 17% compared with a five-year OS of 49% in patients whose tumors had low-grade budding, defined as less than five buds in the low power field of maximal budding[8].

In patients with gastric adenocarcinoma, high-grade tumor budding was a prognostic factor of worse OS[24]. High-grade tumor budding was defined in this study as five or more tumor buds on average in ten HPF (400 ×), and conferred an increased risk of death with a hazard ratio of 2.26 (95% CI: 1.61-3.15) compared with patients whose tumors had low-grade budding. The prognostic value of budding for OS remained significant after adjustment for other factors in multivariate analysis. In a series of pancreatic cancer patients, tumor budding was observed in all cases where patients with high-grade budding (defined in this study as more than ten buds per HPF) had a worse OS than patients with low-grade tumor budding[25]. Additional reports concur with a role of tumor budding as an adverse prognostic factor in pancreatic adenocarcinoma[26,27].

Beyond gastrointestinal cancers, additional reports have shown that tumor budding is a prognostic factor in other cancers such as lung cancer and head and neck carcinomas. In an extensive study of stage Ⅰ lung adenocarcinoma patients, high-grade tumor budding, defined as five or more buds in an HPF, was associated with a recurrence rate that was worse than low-grade tumor budding[21]. This was true for all histologic subtypes investigated (acinar-predominant, papillary-predominant and solid-predominant), and for stages Ⅰ A and Ⅰ B. In early-stage oral squamous cell carcinomas, the presence of high-grade tumor budding of ten or more buds per HPF was associated with a worse disease-free survival (DFS) than intermediate level budding (five to less than ten buds per HPF), and intermediate-grade budding had worse progression-free survival than low-grade budding (less than five buds per HPF)[28]. Differences remained significant in the multivariate analysis. The study used pan-cytokeratin immunostaining to ascertain the identification of tumor buds.

TUMOR BUDDING IN BREAST CANCER

The above studies suggest that tumor budding is a phenomenon observed across cancer types and has adverse prognostic significance. Based on this evidence, studies have been undertaken to investigate whether tumor budding could be of clinical importance in breast cancer. Of note, breast cancer-associated tumor budding akin to budding observed in other cancers should not be confused with the process of tumor cells of the breast duct invading the basal membrane, which has also been referred to as "budding" by some investigators[29]. In a study of 244 estrogen receptor (ER)-positive, human epidermal growth factor receptor 2 (HER2)-negative and 131 triple negative localized breast cancers, tumor budding was associated with worse OS in triple negative but not in ER-positive, HER2-negative patients[30]. Interestingly, tumor budding was not predictive of DFS in either group, but it was predictive of a poorer DFS in the sub-group of ER-positive, HER2-negative patients with an intermediate Oncotype Dx score. This study examined budding in areas of maximal presence (termed H-TB) as well as the average budding in five HPF (termed A-TB), and supports the notion that H-TB is sufficient for prediction while A-TB does not add significant information[30]. In another study that included localized breast cancers across the sub-type spectrum, higher tumor budding (> seven buds per a 200 × power field in a slide with the maximal invasive margin) was observed in about two thirds of patients, while the remaining one third displayed low tumor budding (seven or fewer buds per 200× power field in a slide with the maximal invasive margin). High tumor budding as well as tumor size, nodal status and the presence of lymphovascular invasion were independently associated with OS[31]. Immunohistochemical studies showed that tumor bud cells had increased vimentin expression and decreased E-cadherin expression compared with the center of the tumor, suggesting that they had undergone an EMT[13]. In addition, they were less positive for the proliferation marker Ki67 than the center of the tumor. Higher tumor budding (defined in this study as more than 20 buds at the field with the highest budding) was also independently associated with worse cancer-specific survival in a series of over 400 breast cancer patients with localized disease[32]. With the definition used in this series, 35% of patients had high tumor budding and 65% had low tumor budding. The hazard ratio for cancer-specific survival was 2.08 (95% CI: 1.14-3.09) in patients with high tumor budding compared with patients with low tumor budding[32]. Another series with early breast cancer patients across sub-types, but mostly consisting of luminal cancers, showed that high tumor budding was

associated with lymphatic invasion and positive lymph node disease[33].

A series of 146 ductal carcinoma patients with operable disease was evaluated for both tumor budding, defined as less than five cells per bud, as well as for the presence of buds of five or more tumor cells not forming glands, termed "poorly differentiated clusters"[34]. Both higher levels of tumor budding and poorly differentiated clusters were associated with a worse DFS and OS. In multivariate analysis, both phenomena remained significant, along with tumor size and nodal status. Authors of this study propose poorly differentiated clusters to be the preferred marker of prognosis, as they consider this easier to evaluate than tumor budding[34].

Given the suggested participation of cells of tumor buds in EMT and the associated changes in protein expression, an interesting question is whether cells in the tumor buds of breast cancers maintain the same ER, progesterone receptor and HER2 profile as the main tumor mass. A study addressing this question showed that expression of hormone receptors and of HER2 is mostly concordant between the main tumor mass and tumor buds in 96.5% of tumors examined[35]. However, another study showed that isolated tumor cells at the invasive front of ER-positive, HER2-negative luminal cancers co-expressed HER2 and aldehyde dehydrogenase, in contrast to the main tumor mass[36]. Thus, it appears that there is heterogeneity in the stability of the profile of tumor buds. It is also possible that, at least in some cases, cells in buds, despite undergoing a partial EMT, maintain their initial hormone receptor and HER2 status. This uncertainty could be elucidated by studies examining concomitant expression of hormone receptors and the HER2 receptor, along with EMT markers at tumor buds from the same cancer specimens.

PERSPECTIVES

The association of tumor budding with the pathophysiologic correlation between metastasis and EMT is an important avenue to further explore in breast cancer clinical research. EMT is also associated with stemness characteristics, and the status of tumor bud cells across the stem cell differentiation axis would thus be interesting to define[8,9]. Cancer stem cells are commonly quiescent, and this would correlate with the low Ki67 index shown in some cases[19]. Further study of stem cell markers in tumor buds is warranted.

As mentioned in a previous section, tumor budding in biopsies of rectal cancer patients was predictive of response to neo-adjuvant chemoradiation[23]. In addition, the presence of tumor budding in post-neoadjuvant chemotherapy surgical rejection specimens of esophageal carcinomas was associated with worse survival outcomes[20]. Neoadjuvant chemotherapy is increasingly used in breast and other cancers in order to down-stage locally advanced disease prior to definitive surgical rejection of the tumor. In breast cancer, specifically, it is applied when breast conserving surgery is desired but not initially technically possible due to the size and extent of the tumor. It is also used in node-positive disease, especially in tumors with aggressive biology, defined as triple negative or HER2-positive. These cancers tend to respond better to chemotherapy (or the combination of chemotherapy and HER2-targeting treatments in the case of HER2-positive cancers) than ER-positive cancers[37]. Complete pCR to neoadjuvant chemotherapy range between 30% to 40% in triple negative and HER2-positive cancers, but are observed only in about 10% of hormone receptor-positive cancers[38]. However, the majority of patients will still have residual disease after neoadjuvant chemotherapy, independent of their cancer subtype. In addition, there are no predictive markers for the response of patients to neoadjuvant treatment besides tumor subtype. Thus, in this scenario, tumor budding could be an additional predictive marker to consider in order to better predict tumor responses to treatment, should further studies confirm its predictive value.

From a therapeutic perspective, the associations of tumor budding with EMT and cancer stem cell characteristics may position tumor budding as a predictive marker for treatment with specific anti-metastatic treatments, and against stemness phenotypes that are investigated and may become clinically available in the future.

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MINIREVIEWS

Circulating microRNAs as biomarkers for diabetic neuropathy: A novel approach

Eleni Xourgia, Athanasia Papazafiropoulou, Andreas Melidonis

ORCID number: Eleni Xourgia (0000-0001-5766-3209); Athanasia Papazafiropoulou (0000-0002-7596-4942); Andreas Melidonis (0000-0003-0505-5708).

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Corresponding author to: Athanasia Papazafiropoulou, MD, MSc, PhD, Attending Doctor, 1st Department of Internal Medicine and Diabetes Center, Tzaneio General Hospital of Piraeus, 1 Zanni and Afentouli Street, Athens 18536, Greece. pathan@ath.forthnet.gr

Eleni Xourgia, Athanasia Papazafiropoulou, Andreas Melidonis, 1st Department of Internal Medicine and Diabetes Center, Tzaneio General Hospital of Piraeus, Athens 18536, Greece

Abstract

Oxidative stress stemming from tissue exposure to constant hyperglycemia is one of the major pathogenetic pathways of diabetic macro- and microvascular complications. Diabetic polyneuropathy, commonly manifesting as distal, symmetrical sensorimotor polyneuropathy, is characterized by progressive severity of symptoms, with rates analogous to the quality of glycemic control achieved by the patients and physicians. Palliative care with analgesics and aggressive glycemic control often improve quality of life in the absence of causative treatment. Currently, there is a growing body of evidence indicating the role of microRNAs in the pathogenesis of diabetic complications, with emphasis on diabetic nephropathy and neuropathy. Therefore, in this review, we aim to explore the role of microRNAs and their polymorphisms in the pathophysiology of diabetic polyneuropathy, as well as, the possibility of novel diagnostic and therapeutic applications by epigenetic profiling and manipulation.

Key words: Diabetic neuropathy; Type 2 diabetes mellitus; Type 1 diabetes mellitus; Epigenetic; MicroRNAs

Core tip: In this review, we aim to create a concise overview of the epigenetics underlining the pathogenesis of diabetic neuropathy with emphasis on the altered microRNA expression patterns identified on both animal and human subjects, while, exploring the manner by which they could be manipulated and utilized as novel therapeutic targets.

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INTRODUCTION

Diabetic neuropathy (DN), a common microvascular complication in type 1 (T1DM) and type 2 diabetes mellitus (T2DM) and is defined as the presence of signs and/or symptoms of peripheral nerve dysfunction in patients with diabetes after the exclusion of other causes[1,2]. Population and clinical-based studies suggest DN prevalence rates of 20% in T1DM following 20 years of disease duration and approximately 10%-15% at T2DM diagnosis increasing to as high as 50% at 10 years of disease[3]. Despite the research conducted on the topic, the pathophysiology underlying the process has not been clearly defined, on account of both the numerous intertwining causative mechanisms and the difficulty in establishing a definite diagnosis[4]. Specifically, the diagnostic approach of DN is complicated rather than standardized, commonly comprising of a combination of various qualitative and quantitative methods in order to increase the sensitivity and specificity of the results.

Additionally, effective screening for early abnormalities preceding the appearance of overt clinical manifestations, patients with asymptomatic disease course, or identification of candidates for the development of DN has not been achieved, to a satisfactory degree, by use of current methods[5]. In correlation to the lack of a highly reliable diagnostic method for DN, there is a similar degree of complexity concerning the treatment regimens currently in use. In the absence of causative treatment for the development of DN, current therapeutic approach is often comprised of a combination of glycemic control and pain management[3]. Both the need for development of a high repeatability, non-invasive, diagnostic method and the identification of a possible causative therapeutic approach for DN, allow for consideration the possible use of microRNAs (miRNAs), molecules that have been utilized as biomarkers and focus points of targeted therapy in numerous pathophysiological processes[6].

Therefore, in the present review, we attempt to summarize the existing literature data on the role of miRNAs and their polymorphisms in the pathophysiology of DN, as well as the possibility of utilizing the aforementioned research for novel diagnostic and therapeutic applications by means of epigenetic profiling and manipulation.

CIRCULATING MIRNAs AS BIOMARKERS AND THERAPEUTIC TARGETS

MiRNAs are small, non-coding RNA sequences with a regulatory role in post-transcriptional modification of gene products. Structurally, they comprise of 18-24 nucleotides in length that are organized in a partially complementary manner to cellular mRNA molecules. MiRNAs bind to mRNAs *via* base pairing and induce various changes to the latter, ranging from destabilization to cleavage of the molecule. Alternatively, the mRNA-ribosome complex formation is disrupted by miRNA interference, resulting into similar deregulation of the normal protein formation sequence[7]. Various cellular and tissue types have been shown to express miRNAs as part of their metabolic, developmental and homeostatic processes[8].

It has long been established that the prospect of utilizing circulating miRNAs as diagnostic and therapeutic tools could provide substantial insight into the mechanisms underlining numerous disease processes, as well as become the substrate for therapeutic advances in multifactorial disease states[6]. Currently, novel prospects are being explored concerning the utility of miRNA measurement in the clinical setting, such as assessing response to treatment or disease activity[7].

Researchers have isolated stable miRNA molecules from various tissue types including human plasma, indicating the ability for genetic profiling by use of blood samples, a process far more accessible and easily conducted on both in- and outpatient setting than tissue biopsy[9]. The possible value of miRNA profiling in DN is supported by the fact that mapping of aberrant miRNA expression has been performed in both nervous system and metabolic disorders, along with the observation that most of the currently discovered miRNAs are located in the brain and peripheral neural tissue[9].

Simultaneously to new details for miRNA aberrant expression patterns in disease constantly being unveiled, current methods for epigenetic manipulation of target genes are being ameliorated. The two main approaches to miRNA centered therapy are substitution of under-expressed or otherwise modified miRNAs with functional copies designed *ex vivo*, termed miRNA mimics, or delivery of small molecules, in vivo, that disrupt the pathophysiological cellular pathways in which the target genes participate. Both miRNA mimics and inhibitors have been delivered at the target tissues by use of numerous conjugate molecules in the experimental setting[9]. The

main delivery platforms are subdivided into two categories, non-viral and viral, with the latter harboring many safety concerns. Some of the non-viral molecules are cationic polymers, various conjugates and liposomes, with neutral lipid particles having a more balanced organ-wide distribution than cationic complexes, resulting in less unwanted accumulation in certain tissues. Exosomes and bacteriophages, while being efficient delivery platforms, have the possibility of triggering adverse events such as immune dysregulation and are, therefore, not the vectors of choice[10].

The integration of miRNA-centered treatments in real-world conditions is progressing rapidly, with several clinical trials currently underway[11].

MIRNAS IN DIABETES MELLITUS AND METABOLIC DYSREGULATION

Aberrant expression of miRNAs in tissue and plasma samples has been linked to the pathogenesis of several metabolic diseases, mainly because of their role in the development and homeostasis of metabolically active tissues.

Ample evidence has suggested the involvement of disrupted miRNA expression patterns in metabolic dysregulation. While the spectrum of metabolic disease is wide and includes many, often overlapping, syndromes and pathological states, some prime examples of aberrant tissue miRNA expression include those of miRNA-15b in non-alcoholic fatty liver disease, miRNA-744 in non-alcoholic steatohepatitis, miRNA-132-3p in obesity and miRNAs -30b, -455, -491 and -365-3p in pathological adipose tissue differentiation[12].

MiRNAs from many tissue types involved in diabetes have been linked to many components of the disease state. Several pancreatic, cardiac, liver, kidney, skeletal, endothelial and adipose tissue miRNAs interact directly or indirectly with β-cell pathophysiology, inducing or suppressing pathways involved in lipid metabolism and adipocyte differentiation (miRNA-181d, -27a/b, -103, - 107, -143), insulin resistance, glucose-mediated insulin secretion and exocytosis (miRNA-29a/b/c, -375, -9, -124a, -96, -34a, -30d, -223, -320, -21), β-cell development, apoptosis and function (miRNA-375, -9, -195, -126, -296, -34a, -146b, -21) cardiomyocyte apoptosis and cardiac arrythmiogenesis (miRNA-206, -1, -133a), endothelial dysfunction and angiogenesis (miRNA-93, -320, -125a/b) and glomerular activity (miRNA-192, -216a, -217)[13].

Further, disease-specific research on diabetics and more specifically, profiling of circulating miRNAs as predictors for T2DM or prediabetes in healthy subjects indicated the existence of a link between higher plasma levels of miRNA-150 and miRNA-30a-5p, and lower levels of miRNA-375 and miRNA-15a at baseline and disease development after a median follow-up of 60 mo[14].

Similarly, to miRNA-15a, the expression of several other circulating molecules has been found to be down- or upregulated in plasma samples of T2DM subjects. La Sala *et al*[15] note that miRNAs -20b, -21, -24, -126, -191, -197, -223, -320 and -486 in T2DM plasma samples are present in lower concentrations when compared to healthy controls, while miRNA-28-3p in upregulated. Among the aforementioned miRNAs, -15a, -28-3p, -126, -223 and -320 have been proposed for possible use as biomarkers of T2DM.

Additionally, recent data acquired from analysis of various expression patterns of quantitative trait loci in mouse inbred strains with varying susceptibility to metabolic dysregulation and T2DM development has indicated an upregulation of miRNA-31 in adipose tissue of obese and type 2 diabetic subjects. MiRNA-31 interacts with genes of the insulin signaling pathway and adipose tissue proliferation[12].

MIRNAS AS BIOMARKERS IN DIABETIC NEUROPATHY: WHY, WHICH AND WHEN?

The most important problem posed when discussing a novel treatment possibility in the clinical setting is the clarification of the circumstances under which a new interventional approach should be applied, or, in simpler terms, the evaluation of the cost-effectiveness of the method. In the setting of DN, the two pivotal arguments in favor of epigenetic modification are the lack of causative treatment for a major complication of a chronic disease with a high prevalence and the enormous impact of DN on the patients' quality of life, an indisputable fact that has been repeatedly documented[3,16].

Researchers on the field of epigenetics have provided insight on some miRNA molecules that could be evaluated as possible biomarkers or therapeutic targets in diabetes-induced neuropathy. A study including 60 diabetic subjects revealed a

correlation between miRNA-199a-3p and reduced expression of extracellular serine protease inhibitor E2 resulting in DN manifestation and accelerated progression[17]. A similar pathway has been described, involving miRNA-190a-5p downregulation and resultant impaired solute carrier family 17 member 6 gene inhibition in painful DN[18]. Conversely, medically induced downregulation of miRNA-25 exacerbated the development of DN *via* an increase of advanced end glycation products and their receptors in peripheral neural tissue, indicating the neuroprotective attributes of miRNA-25 molecules[19]. In an experimental model on diabetic rodents, miRNA-9 and its interaction with calcium homeostasis modulator 1 were suggested to be involved in the pathophysiological pathway of painful DN[20]. MiRNA-146 located in circulating mononuclear cells modulates inflammatory response in diabetic peripheral neuropathy[21,22]. DN painful manifestation and inflammatory response are also affected by miRNA-23a *via* chemokine CXC receptor 4-related signaling[23]. Recent data indicate that a certain genotype termed "GG" in miRNA499A has been linked to cardiovascular autonomic neuropathy as well as diabetic polyneuropathy[24]. Finally, substantial upregulation of miRNA-29c and subsequent protein kinase C iota gene under-expression have been associated with distal neural damage in a model of diabetic rodents[25]. A brief comparison of all relevant to DN miRNAs discussed above is presented in Table 1. Circulating molecules can be used more readily as biomarkers when compared to tissue-derived molecules.

Even with the existence of an abundance of possible biomarkers and therapeutic targets, as indicated by the research described above, the timeframe in which predisposition for DN development can be detected or DN can be treated is yet to be defined. While it is expected that miRNA aberrant expression patterns precede the clinical manifestation of DN, the elucidation of the exact timeline describing when these changes occur and can be detected in the research setting in advance is of uttermost importance in the design of effective intervention algorithms for complication prevention. When miRNA manipulation is examined in the scope of neuropathy treatment, it should be studied whether miRNA expression normalization can reverse damage already done to the neural tissue or impede the progression of sensory and motor deterioration. Furthermore, the particular points in disease progression appropriate for treatment initiation or termination can be defined, based on treatment efficacy at different stages and forms of disease.

CONCLUSION

MiRNA-based diagnosis and therapy are highly likely to be the future of treatment and prevention, especially in multifactorial disease processes. The transition from theory to practice, while an ongoing process, is rapidly progressing, with several molecules being used in clinical trials and many more currently on the preclinical-stage. While DN is the result of several complex pathophysiological processes, several miRNA molecules involved and their role have been described, setting the stage for the practical application of the aforementioned information. A next step in the development of miRNA-based diagnosis, staging and therapy is examining the miRNAs so far associated with neuropathy and diabetes in a prospective cohort study including diabetic subjects free of complications, with the goal of evaluating miRNA epigenetic changes accumulating over time in correlation with the appearance and severity of DN. Current available research data indicate that miRNAs -199a-3p, -146 and -499a can be used as circulating biomarkers, while the aforementioned along with miRNAs -190a-50, -25, -9, -23a and -29c have potential as therapeutic targets in DN.

Table 1 Possible biomarkers and therapeutic targets in diabetic neuropathy

MiRNA molecule	Mechanism	Expression in DN	Circulating	Citation
miRNA-199a-3p	Inhibition of SerpinE2 expression - coagulation in peripheral circulation	Upregulated	Yes	[18]
miRNA-190a-5p	Inhibition of SLC17A6 gene	Downregulated	No	[19]
miRNA-25	Inhibition of oxidative stress, decreases AGEs and RAGE production	Downregulated	No	[20]
miRNA-9	Interacts with CALHM1 in neuron-glial signalling	Upregulated	No	[21]
miRNA-146	Interacts with NFκB and inflammatory cytokines production	Downregulated	Yes	[22,23]
miRNA-23a	Targets CXCR4 - regulates neuropathic pain	Downregulated	No	[24]
miRNA499A	Prevents cardiomyocyte apoptosis and mitochondrial fission (impaired in cardiac autonomic neuropathy); regulates insulin resistance	Upregulated (GG genotype with rs3746444)	Yes	[25]
miRNA-29c	Inhibits neural axonal growth *via* inhibiting PRKCI gene expression	Upregulated	No	[26]

MiRNAs: microRNAs; CALHM1: Calcium homeostasis modulator 1; AGEs: Advanced end glycation products; RAGE: Receptor of advanced end glycation product; SerpinE2: Serine protease inhibitor E2; SLC17A6: Solute carrier family 17 member 6; PRKCI: Protein kinase C iota.

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