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REVIEW

Candidate prognostic markers in breast cancer: focus on extracellular proteases and their inhibitors

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David M Roy[1]
Logan A Walsh[2]

[1]Weill Cornell/Rockefeller/Sloan-Kettering Tri-Institutional MD-PhD Program, New York, NY, USA; [2]Human Oncology and Pathogenesis Program, Memorial Sloan-Kettering Cancer Center, New York, NY, USA

Abstract: The extracellular matrix (ECM) is the complex network of proteins that surrounds cells in multicellular organisms. Due to its diverse nature and composition, the ECM has a multifaceted role in both normal tissue homeostasis and pathophysiology. It provides structural support, segregates tissues from one another, and regulates intercellular communication. Furthermore, the ECM sequesters a wide range of growth factors and cytokines that may be released upon specific and well-coordinated cues. Regulation of the ECM is performed by the extracellular proteases, which are tasked with cleaving and remodeling this intricate and diverse protein matrix. Accordingly, extracellular proteases are differentially expressed in various tissue types and in many diseases such as cancer. In fact, metastatic dissemination of tumor cells requires degradation of extracellular matrices by several families of proteases, including metalloproteinases and serine proteases, among others. Extracellular proteases are emerging as strong candidate cancer biomarkers for aiding and predicting patient outcome. Not surprisingly, inhibition of these protumorigenic enzymes in animal models of metastasis has shown impressive therapeutic effects. As such, many of these proteolytic inhibitors are currently in various phases of clinical investigation. In addition to direct approaches, aberrant expression of extracellular proteases in disease states may also facilitate the selective delivery of other therapeutic or imaging agents. Herein, we outline extracellular proteases that are either bona fide or probable prognostic markers in breast cancer. Furthermore, using existing patient data and multiple robust statistical analyses, we highlight several extracellular proteases and associated inhibitors (eg, uPA, ADAMs, MMPs, TIMPs, RECK) that hold the greatest potential as clinical biomarkers. With the recent advances in high-throughput technology and targeted therapies, the incorporation of extracellular protease status in breast cancer patient management may have a profound effect on improving outcomes in this deadly disease.

Keywords: uPA, RECK, ADAMs, MMPs, TIMPs, ECM

Introduction

Extracellular proteases are complex and heterogeneous enzymes that play a key role in many pathophysiologic processes. Included in this group are metalloproteinases such as matrix metalloproteinases (MMPs) and serine proteases such as plasmin, among others.[1] These proteins have the capacity to completely remodel the extracellular matrix (ECM) and can therefore alter a variety of biologic processes, including angiogenesis, growth factor bioavailability, cytokine modulation, receptor shedding, cell migration, proliferation, invasion, and apoptosis.[2,3] Not surprisingly, these proteases and their protein inhibitors have been implicated in many diseases, including cancer. Specifically, several extracellular proteases have been shown to alter tumor

Correspondence: Logan A Walsh
Human Oncology and Pathogenesis Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, Box 20, New York, NY 10065, USA
Email walshl@mskcc.org

aggressiveness and patient response to therapy.[4–6] Herein, we summarize the most recent and relevant literature detailing the role of extracellular proteases and their inhibitors as prognostic indicators and putative therapeutic targets in breast cancer.

Materials and methods

Tumors analyzed in this study were from a previously published dataset of over 2,000 woman diagnosed with breast cancer.[7] Expression and clinical data were downloaded from Oncomine™ (http://www.oncomine.org). Nonparametric analyses were performed since expression values were not normally distributed. Mann–Whitney U test was used to compare gene expression values with clinicopathological features ($P<0.05$= significant). For patient outcomes, data were analyzed in two ways. First, gene expression values were divided into "high" and "low" expression groups based on median values in all samples and Kaplan–Meier tests were used to determine significance (log-rank). Second, gene expression values were treated as a continuous variable and subjected to a univariate Cox regression analysis and Wald test ($P<0.01$= significant). All statistics were carried out using SPSS software (v 20; IBM Corporation, Armonk, NY, USA).

The extracellular matrix (ECM)

The ECM is the complex network of proteins that surrounds and supports cells in multicellular organisms. It is composed of three main types of proteins with distinct roles: structural proteins (eg, collagen, elastin), specialized glycoproteins (eg, fibronectin), and proteoglycans (eg, syndecans).[8] Initially, it was believed that the sole function of the ECM was to provide tissues with structural support.[9] More recently, however, it has been shown that the ECM plays a more active – and critical – role in many fundamental cellular processes such as cell growth, proliferation, migration, and differentiation.[10] In fact, it is the intrinsic diversity of the ECM that underlies its pleiotropic role as a structural scaffold, cytokine reservoir, and regulator of developmental and physiologic signaling.

Although the exact protein composition can vary considerably due to unique tissue architecture and function, the major protein component of the ECM is collagen. In fact, collagen is the most abundant protein across the animal kingdom, serving to provide tissues with strength and resilience.[11] Accordingly, there are many diseases that stem directly from defects in collagen production and homeostasis, either from underlying genetic alterations and/or abnormal collagen processing (eg, osteogenesis imperfecta, Alport syndrome, Ehlers–Danlos syndrome).[12] In addition, the integrity of collagen in the ECM plays a key role in cancer – the active degradation of type IV collagen by extracellular proteases facilitates tumor cell invasion through the basement membrane.[13] In fact, this hallmark histopathologic feature of epithelial cancers is what defines the transition from carcinoma in situ to invasive carcinoma, carrying significant prognostic significance in breast cancer.[14]

Specialized glycoproteins are important for proper cell–ECM adhesion. For example, cells can bind to fibronectin via integrin receptors to form focal adhesions, which facilitate cellular migration.[15] As with other ECM components, improper remodeling of glycoproteins is associated with pathological processes including tumor growth and metastasis.[16]

In addition to direct structural and cell adhesion roles, the ECM also sequesters and moderates the passage of many cytokines and growth factors between cells. This process is governed by proteoglycans, of which syndecans are a major constituent. Syndecans are cell surface proteoglycans that are ubiquitously expressed and contain a heparan sulfate side chain, which allows interactions with heparan-binding proteins, including cell surface receptors.[17] Cleavage of these proteoglycans and the subsequent release of cytokines can stimulate cellular signaling cascades.[18] In fact, one particularly overlooked function of the ECM is its role in facilitating intercellular communication. The extensive array of cytokines and growth factors sequestered by the ECM can powerfully regulate cell behavior through activation of signaling cascades. Accordingly, the importance of extracellular proteases, the enzymes tasked with maintaining homeostasis within the ECM microenvironment, cannot be overstated.[19] Considering the many structural and functional roles played by the ECM, it is evident that its cleavage and remodeling must be highly regulated. Not surprisingly, altered extracellular protease activity in cancer is common and is increasingly linked to significant changes in patient outcomes.[20]

The urokinase plasminogen activator (uPA) system

Several biomarkers for breast cancer have been currently validated at the highest level of evidence (LOE-1) and are already utilized in the clinical setting to guide management strategy.[21] Patient prognosis and treatment efficacy are strongly correlated to these biomarkers, which include estrogen receptor (ER), progesterone receptor (PR), and receptor tyrosine-protein kinase erbB-2 (HER2).[22] In fact, testing the status of these proteins via tissue-based assay is now the standard of care in all newly diagnosed breast cancer

patients. Despite this, there exists a continued need for new biomarkers in breast cancer in order to better stratify patients for therapeutic regimens and improve outcomes.[23,24]

Two components of the uPA system hold exciting potential as biomarkers in breast cancer: the serine protease uPA and plasminogen activator inhibitor-1 (PAI-1), a serine protease inhibitor (serpin).[25] Although they are not yet in widespread clinical use, they have already been validated as biomarkers for clinical breast cancer management at the highest level;[26,27] therefore, they could be an important addition to existing clinical prognostic tools used in breast cancer (Table 1).[28,29]

The uPA system consists of the serine protease uPA, its membrane-anchored receptor uPAR, and the serpins PAI-1 and PAI-2.[30] The uPA system is causally involved in multiple steps of breast cancer progression, including ECM remodeling, increased cell proliferation and migration, and modulating cell adhesion.[31] Therefore, it is not surprising that uPA in primary breast cancer is independently associated with adverse outcome.[32] Interestingly, and somewhat paradoxically, high levels of PAI-1 (an inhibitor of plasminogen activation) also correlate with poor prognosis in breast cancer patients.[33] Nevertheless, the prognostic value of uPA/PAI-1 in axillary node-negative breast cancer patients has been validated in two independent level 1 evidence studies – a prospective randomized trial and a pooled analysis of primary data.[34]

Less is known about the predictive clinical value of uPA system components in regard to systemic therapy for recurrent breast cancer. In a recent study by Harbeck et al, uPA/PAI-1 levels in primary breast tumors were predictive for response to adjuvant systemic chemotherapy.[33] The benefits of chemotherapy as opposed to endocrine therapy were greatly enhanced in patients with high uPA/PAI-1 levels. These data support earlier findings by Jänicke et al, who studied 556 patients with lymph node-negative breast cancer and found that uPA and PAI-1 status was sufficient to classify a subset of patients as low risk (50%), for whom adjuvant chemotherapy may be avoided.[26]

To date, steroid hormone receptor status is the prevailing parameter assessed in deciding whether to treat with endocrine therapy. Interestingly, both ER and PR status are inversely correlated with uPA ($P=10^{-6}$ and $P=0.05$, respectively) or uPAR ($P=10^{-6}$ and $P=0.0005$, respectively) expression in breast invasive carcinoma (Memorial Sloan-Kettering Cancer Center cBio Cancer Genomics Portal).[35,36] Selective ER modulators such as tamoxifen and raloxifene, GnRH (gonadotropin-releasing hormone) agonists, and aromatase inhibitors are the most widely used endocrine therapies.[37] The nonsteroidal estrogen antagonist tamoxifen is a type II competitive inhibitor of estradiol at its receptor and is the most popular endocrine treatment in premenopausal women. Endocrine treatment is a mainstay therapy for patients with ER-positive cancers and non-life-threatening advanced disease. Nonetheless, despite the fact that endocrine therapy has a relatively low morbidity, only one-half of patients treated with tamoxifen will receive clinical benefit.[38] Therefore, there is a strong need for more sensitive and predictive biomarkers for response to endocrine therapy. In a recent study by Meijer-van Gelder et al, uPA, uPAR, and PAI-1 were all predictive for improved efficacy of tamoxifen therapy in patients treated for recurrent breast cancer.[39] Therefore, the status of uPA system proteins in patient tumor specimens may be useful in developing individualized therapy protocols.

Table 1 Clinically utilized biomarkers in breast cancer

Biomarker	Platform	Outcome measure
ER + PR	Tissue-based assay	Response to hormone therapy
HER2	Tissue-based assay	Response to trastuzumab
uPA + PAI-1	Tissue-based assay	Prognosis in lymph node-negative tumors
21-gene signature	Oncotype DX®	Distant recurrence after treatment with tamoxifen or AIs
70-gene signature	MammaPrint™	Prognostic for 5-year recurrence
97-gene signature	Genomic grade index	Prognostic for relapse after endocrine treatment in ER+ tumors
76-gene signature	Rotterdam signature	Prognostic for development of distant metastasis

Notes: Oncotype DX® (Genomic Health Inc., Redwood City, CA, USA); MammaPrint™ (Agendia Inc., Irvine, CA, USA).
Abbreviations: AIs, aromatase inhibitors; ER, estrogen receptor; PR, progesterone receptor; HER2, receptor tyrosine-protein kinase erbB-2; PAI-1, plasminogen activator inhibitor-1; uPA, urokinase plasminogen activator.

Matrix metalloproteinases

MMPs are members of a large multigene family of zinc-dependent endopeptidases. There are more than 24 MMPs known to play a vital role in remodeling the ECM.[40] In addition to ECM proteins, MMPs target and cleave a wide range of substrates such as other proteases, growth factors, cell adhesion molecules, clotting factors, and cell surface receptors. Thus, MMPs are essential in regulating many cellular interactions under conditions that promote tissue turnover.[41] Not surprisingly, MMPs are primarily active during development, when the majority of ECM remodeling occurs. In adults, the majority of remaining MMP activity is isolated to remodeling processes such as wound repair and angiogenesis. However, in human disease, aberrant MMP hyperactivity has been observed in many pathological conditions, such as osteoarthritis, multiple sclerosis, and cancer.[2,42]

MMPs are translated as inactive pro-enzymes, secreted, and then activated through the catalytic removal of their pro-domain by other proteases in the ECM. Membrane-type MMPs (MT-MMPs), however, are a family of six proteins that contain a furin cleavage site motif that is recognized for intracellular activation prior to secretion.[43] Though most MMPs cleave a number of specific substrates, including several ECM components, MT-MMPs have an additional role in pro-MMP activation. It is important to note that MMPs have considerable structural and functional similarity. Likewise, this relative redundancy may account for the fact that most MMP knock-out mice are viable.[44] However, despite high levels of homology, MMPs still retain some substrate specificity and tissue-specific expression patterns. As a result, there are a number of MMPs that have been shown to possess great prognostic potential for clinical outcome in breast cancer.[45–47]

Currently, there exist many studies and review articles that detail the potential prognostic utility of individual MMPs in specific cancers.[48–53] However, most of the published original data are derived solely from retrospective analyses performed on patient cohorts of limited size. Recently, McGowan and Duffy analyzed a well-defined published database of 295 breast cancer patients in the most comprehensive study of MMPs in breast cancer to date.[54] Of the 17 MMPs investigated in their study, five (MMP-1, -9, -12, -14, and -15) were significantly associated with poor outcome. Additionally, MMP-14 was found to be an independent predictor of outcome, irrespective of tumor size, grade, lymph node status, and ER status. Despite these MMPs being linked to adverse outcome previously, there were several novel findings worth noting. First, MMP-1 expression was significantly higher in basal-like breast cancer compared to other subtypes. Also, although low MMP-9 expression was shown to be significantly associated with poor prognosis in the total population of breast cancer patients, it was not associated with outcome in a systemically untreated cohort.[54] This suggests that MMPs may be only predictive of outcome depending on the therapeutic regimen.

Here, we perform our own analysis of MMP status in breast cancer using a previously published dataset that contains microarray data in over 1,500 breast primary breast tumors, the largest dataset currently available.[7] Details of this dataset are outlined in Table 2. In our investigation, we chose to analyze patient survival by two distinct methods. First, we stratified tumor samples into "high" and "low" MMP expression groups based on the median MMP expression for each gene and performed Kaplan–Meier analysis for disease-specific survival. For our second analysis, we treated MMP expression in each patient as a continuous variable and performed a Cox regression analysis and Wald test for any significance between MMP expression and disease-specific survival (Table 3). Of the 19 MMPs tested, only MMP-9, -11, and -15 were significantly associated with worse survival in both analyses ($P<0.01$). Interestingly, MMP-11 was not previously identified by McGowan and Duffy as holding any prognostic value in breast cancer.[54] However, Cheng et al examined MMP-11 expression in paired tumor

Table 2 Characteristics of 1,545 breast cancer samples (Curtis dataset)

Characteristics	n
Tumor size (cm)	
≤2	505
2–4	815
≥4	216
No information	9
Grade	
1	128
2	607
3	770
No information	40
Number of positive lymph nodes	
0	779
1–3	508
≥4	254
No information	4
Age (years)	
20–29	12
30–39	96
40–49	281
50–59	393
60–69	438
70–79	267
≥80	58
PAM50 subtype	
Normal	159
Lum A	532
Lum B	380
HER2	190
Basal	280
No information	4
Treatment	
None	189
Chemotherapy	40
Radiotherapy	184
Hormone therapy	309
Chemotherapy + radiotherapy	163
Chemotherapy + hormone therapy	28
Hormone therapy + radiotherapy	481
Chemotherapy + hormone therapy + radiotherapy	151

Abbreviations: HER2, receptor tyrosine-protein kinase erbB-2; Lum, luminal.

Table 3 Relationship between MMP expression and disease-specific survival in 1,545 breast cancer patients

Gene	Log-rank *P*-value	Wald test *P*-value	Hazard ratio	95% CI
MMP1	**0.0023**	0.4161	1.058	0.92–1.21
MMP2	0.7380	0.1427	0.857	0.70–1.05
MMP3	0.3930	0.2413	0.941	0.85–1.04
MMP7	0.1470	0.8498	0.995	0.95–1.04
MMP8	0.9380	0.5478	0.837	0.47–1.49
MMP9	**0.0004**	**0.0002**	1.110	1.05–1.17
MMP10	0.6070	0.0922	0.897	0.79–1.02
MMP11	**0.0011**	**0.0002**	1.124	1.06–1.19
MMP12	**0.0001**	0.0186	1.088	1.01–1.17
MMP13	0.7730	0.9051	0.992	0.87–1.13
MMP14	0.2150	0.8873	1.032	0.67–1.60
MMP15	**0.0000**	**0.0000**	1.708	1.44–2.03
MMP16	0.2560	0.1007	1.520	0.92–2.50
MMP17	0.0410	0.0513	1.843	1.00–3.41
MMP19	**0.0062**	0.2969	0.756	0.45–1.28
MMP20	0.7830	0.4546	1.169	0.78–1.76
MMP24	0.0212	0.2754	0.811	0.56–1.18
MMP25	0.2590	0.4204	1.303	0.68–2.48
MMP28	0.3330	0.3034	0.744	0.42–1.31

Note: Bold indicates significance $P<0.01$.
Abbreviations: CI, confidence interval; MMP, matrix metalloproteinases.

Table 4 Relationship between MMP-9, -11, and -15 expression and tumor characteristics

Characteristics	MMP-9	MMP-11	MMP-15
ER status			
Positive	3.343	5.036	0.588
Negative	4.362	4.795	0.851
P-value	<**0.0001**	**0.0322**	<**0.0001**
Tumor size (cm)			
≤2	3.613	5.046	0.609
>2	3.654	4.907	0.650
P-value	NS	NS	**0.032**
Tumor grade			
1–2	3.217	4.919	0.571
3	4.081	5.024	0.703
P-value	<**0.0001**	NS	<**0.0001**
Nodal status			
Negative	3.583	4.898	0.624
Positive	3.682	5.038	0.647
P-value	NS	NS	NS
PAM50 subtype			
Basal	4.674	4.375	0.655
Other	3.375	5.066	0.631
P-value	<**0.0001**	<**0.0001**	**0.044**

Note: Bold indicates significance $P<0.05$.
Abbreviations: NS, not significant; MMP, matrix metalloproteinases; ER, estrogen receptor.

and adjacent normal tissue and discovered that increased MMP-11 expression correlated with more aggressive clinical features.[55] Furthermore, Tan et al found that MMP-11 has a necessary paracrine function during mammary gland development that may later be co-opted to promote breast cancer progression.[56] Additionally, another putative role of MMP-11 in breast cancer was described by Takeuchi et al, who demonstrated that MMP-11 overexpression significantly increased resistance to anoikis, favoring anchorage-independent growth over programmed cell death in the setting of reduced cell–ECM contact.[57] To identify any additional associations, we explored whether MMP-9, -11, and -15 expression was linked with several common clinical and/or pathological features of breast cancer (Table 4). Both MMP-9 and MMP-15 were significantly associated with higher tumor grade, ER-negative status, and PAM50 basal subtype. This result is consistent with a wide body of literature implicating MMP-9 in breast cancer progression in both clinical and preclinical investigations.[58] It is important to note that our analysis is limited to gene expression. MMPs are also regulated at the posttranslational level, which may account for discrepancies between our analysis and other studies investigating the expression of MMPs in breast cancer at the protein level. For example, multiple studies have shown that MMP-2 is associated with poor outcome in breast cancer by assessing MMP-2 levels via immunohistochemistry.[59,60]

A disintegrin and metalloproteases (ADAMs)

ADAM proteins are zinc-dependent transmembrane metalloproteases responsible for proteolytic cleavage of extracellular domains of membrane-bound growth factors, cytokines, and receptors.[61] Accordingly, they are known to have myriad diverse effects on cellular behavior. In total, there are 21 ADAMs that are believed to be functional in humans. Together they cleave a diverse array of ECM constituents and alter extracellular protein localization and bioavailability.[62] Altered ADAM expression has been associated with several pathologies, though none as convincingly and well-demonstrated as in cancer.[63,64] There are several excellent reviews that highlight the mechanism by which ADAMs are involved in tumorigenesis, but none have focused exclusively on ADAMs in breast cancer.[65]

For years, both clinical and preclinical studies have detailed the important role that individual ADAMs play in breast cancer. The most extensively characterized of all ADAMs is ADAM-17.[66] ADAM-17 has been demonstrated to act via several oncogenic mechanisms, though one of the most important and relevant may be that it actively releases HER2/EGFR ligands through proteolytic cleavage, thereby increasing proliferation, invasion, and angiogenesis.[67] In fact, forced overexpression of ADAM-17 in breast cancer cells increases invasion and proliferation in vitro, and the

opposite effect is seen following ADAM-17 inhibition and knockdown.[68] At both mRNA and protein levels, ADAM-17 expression is significantly upregulated in tumor samples compared with normal breast tissue.[69,70]

The prognostic potential of ADAMs in breast cancer has been highlighted on a case-by-case basis. Collectively, ADAM-9, ADAM-12, ADAM-15, ADAM-17, ADAM-22, and ADAM-28 have been implicated in the occurrence of breast cancer.[71] Here, we analyze the prognostic potential of 15 ADAMs genes in the Curtis breast cancer dataset (Table 1). The results are summarized in Table 5. Only ADAM-8 and ADAM-17 were significantly associated with worse disease-specific survival in both of our analyses. ADAM-8 was the focus of a recent study that found that ADAM-8 was abundantly expressed in breast tumors and metastases compared to normal tissue, especially in triple-negative breast cancers.[72] Further, elevated ADAM-8 expression predicted poor patient outcome. Additionally, in their breast cancer xenograft mouse model, treatment of tumors with an anti-ADAM-8 antibody reduced primary tumor burden and the number of metastases.[72] Together, our analysis and these data demonstrate that ADAM-8 is a promising novel biomarker and a therapeutic candidate in breast cancer.

We also analyzed the association between ADAM-8 and ADAM-17 and common clinical and pathologic features of breast cancer (Table 6). ADAM-17 is most significantly associated with the presence of lymph node metastases. Both ADAM-17 and ADAM-8 expression are significantly associated with ER-negative status, increased tumor grade, and basal tumor subtype (Table 6). Therefore, these data reveal that both ADAM-17 and ADAM-8 are putative biomarkers in breast cancer and promising candidates for new targeted therapies.

Table 5 Relationship between ADAMs expression and disease-specific survival in 1,545 breast cancer patients

Gene	Log-rank *P*-value	Wald test *P*-value	Hazard ratio	95% CI
ADAM2	0.9050	0.2979	1.179	0.86–1.61
ADAM7	0.5940	0.3963	0.785	0.45–1.37
ADAM8	**0.0000**	**0.0000**	1.515	1.30–1.76
ADAM9	0.1120	0.0990	1.134	0.98–1.31
ADAM10	0.8210	0.6193	0.878	0.52–1.47
ADAM11	0.0870	0.0160	1.651	1.10–2.48
ADAM12	0.0140	0.0280	0.607	0.39–0.95
ADAM15	0.0470	0.0112	1.180	1.04–1.34
ADAM17	**0.0000**	**0.0000**	1.569	1.35–1.82
ADAM18	0.5490	0.9882	1.005	0.55–1.85
ADAM19	0.2750	0.5536	1.212	0.64–2.29
ADAM22	0.5200	0.7431	0.888	0.44–1.81
ADAM23	0.8010	0.9831	0.995	0.60–1.64
ADAM28	0.9540	0.4527	0.879	0.63–1.23
ADAM33	0.4050	0.3003	0.730	0.40–1.32

Note: Bold indicates significance $P<0.01$.
Abbreviation: CI, confidence interval.

Table 6 Relationship between ADAM-8 and -17 expression and tumor characteristics

Characteristics	ADAM-8	ADAM-17
ER status		
Positive	0.942	1.815
Negative	1.225	2.165
P-value	**<0.0001**	**<0.0001**
Tumor size (cm)		
≤2	0.999	1.860
>2	1.060	1.921
P-value	**0.045**	NS
Tumor grade		
1–2	0.906	1.818
3	1.147	2.007
P-value	**<0.0001**	**<0.0001**
Nodal status		
Negative	1.011	1.856
Positive	1.051	1.936
P-value	NS	**0.019**
PAM50 subtype		
Basal	1.304	2.205
Other	0.952	1.825
P-value	**<0.0001**	**<0.0001**

Note: Bold indicates significance $P<0.05$.
Abbreviations: NS, not significant; ER, estrogen receptor.

Tissue inhibitor of metalloproteinases (TIMPs)

The TIMPs comprise a highly conserved and homologous set of proteins. To date, four TIMPs have been identified (TIMP1–4). TIMPs are small, secreted proteins consisting of structurally and functionally distinct N- and C-terminal domains.[73] Originally, TIMPs were characterized based on their ability to inhibit MMP activity, though, more recently, TIMPs have been shown to possess a number of MMP-independent functions.[74] For example, TIMPs can bind directly to cell surface receptors to stimulate cell-signaling pathways, thereby leading to changes in cell growth, proliferation, and apoptosis. In fact, this activity requires the C-terminus of TIMP proteins and is therefore independent of their MMP-inhibitory activity, which occurs exclusively at the N-terminus.[75] Notably, TIMPs play a fundamental role in controlling cell–ECM interactions, specifically under conditions that promote tissue turnover. Considering the profound effects that ECM remodeling can have on pathophysiology, it is not surprising that TIMPs play an important role in tumor behavior.

A number of promising prognostic candidates exist among TIMPs. In fact, TIMP-1 holds potential utility in

breast cancer, as a number of studies have demonstrated an association between both high serum and tumor levels of TIMP-1 and lower overall survival.[76,77] These findings are somewhat paradoxical as one might predict that elevated levels of TIMPs would result in decreased MMP proteolytic activity and therefore suppression of breast cancer invasion and metastasis. However, these data are more consistent with the MMP-independent role of TIMPs in breast cancer progression, thereby reconciling this apparent conundrum while simultaneously highlighting the dual and opposing roles that TIMPs may have. Recently, CD63 and integrin β1 have been identified as cell surface binding partners for TIMP-1 which may modulate its antiapoptotic signaling activity, though the specific details of this pathway still remain poorly understood.[78] Similarly, TIMP-2 has been shown to bind to α3β1 integrin. This association modulates the MMP regulator reversion-inducing cysteine-rich protein with kazal motifs (RECK).[79] RECK is another MMP inhibitor and important metastasis suppressor, and is discussed in the next section of this review. However, again somewhat paradoxically, elevated TIMP-2 levels have been correlated with poor prognosis in breast cancer.[80] Conversely, low levels of TIMP-3 have been associated with poor prognosis.[81] Additionally, TIMP-3 has been associated as a biomarker for successful endocrine therapy.[82]

A delicate balance exists between MMPs and TIMPs in the ECM. This dynamic relationship is tightly and coordinately regulated, and any alterations may carry profound effects on breast cancer tumorigenesis and progression. As a result, we considered it would be of interest to determine which TIMPs were predictive of survival in the Curtis dataset. Interestingly, the only TIMP that was significantly associated with prognosis was TIMP-4 (Table 7). More specifically, decreased levels of TIMP-4 are significantly associated with poor disease-specific survival, ER-negative status, tumor size >2 cm, higher grade, and basal PAM50 subtype (all: $P<0.0001$). Decreased TIMP-4 is also associated with the presence of positive lymph node metastases ($P=0.011$). Of note, TIMP-4 is the least studied of all the TIMPs, particularly in breast cancer. Nonetheless, in an early study by Wang et al, forced overexpression of TIMP-4 was shown to significantly inhibit breast cancer growth both in vitro and in vivo.[83] In addition, Liss et al investigated TIMP-4 as a potential breast cancer biomarker via immunohistochemical staining of TIMP-4 in 314 tumors from patients with early-stage disease (defined as tumors smaller than 2 cm and no positive lymph nodes).[84] They found that tumors with elevated TIMP-4 were correlated with a reduced probability of long-term disease-free survival, especially in patients with ER-negative tumors.

Table 7 Relationship between TIMP expression and disease-specific survival in 1,545 breast cancer patients

Gene	Log-rank *P*-value	Wald test *P*-value	Hazard ratio	95% CI
TIMP1	0.4920	0.6019	1.042	0.89–1.22
TIMP2	0.3580	0.3157	0.948	0.85–1.05
TIMP3	0.0740	0.0319	0.911	0.84–0.99
TIMP4	**0.0000**	**0.0000**	0.687	0.59–0.80

Note: Bold indicates significance $P<0.01$.
Abbreviation: CI, confidence interval.

Despite the fact that our analysis only identified TIMP-4 as a prognostic marker for breast cancer outcome, we again must highlight that our analysis is restricted to gene expression. Many of the aforementioned studies clearly identify a strong relationship between other TIMPs and breast cancer. TIMP secretion and localization is vital for TIMP-mediated effects on cancer cell behavior, which cannot be assessed by gene expression analysis alone. Nonetheless, TIMP-4 represents an intriguing new prognostic candidate.

Reversion-inducing cysteine-rich protein with Kazal Motifs

RECK is a membrane-anchored glycoprotein that negatively regulates MMPs and potently inhibits tumor angiogenesis.[85] RECK is suppressed across many cancer types and transformed cell line models. Functional studies in non-transformed cells have shown that RECK serves as a negative regulator of MMP-9 and is a target for repression itself by the oncomiR miR-21.[86,87] Over a decade ago, Span et al assessed the prognostic value of RECK expression in tumor tissue specimens from 278 breast carcinoma patients via quantitative reverse transcription polymerase chain reaction.[88] Multivariate Cox regression analysis showed that RECK expression held significant independent prognostic value for improved recurrence-free survival. More recently, Zhang et al performed a retrospective analysis of 119 patients with invasive breast cancer and analyzed RECK expression by immunohistochemical staining of tumor specimens.[89] They found a significant positive correlation between RECK and 5-year overall survival. Additional multivariate analyses confirmed that reduced RECK expression was an independent and significant factor in predicting a poor prognosis. In an interesting study, Hill et al profiled DNA methylation patterns genome-wide in sporadic breast tumors using the HumanMethylation27 BeadChip® (Illumina, San Diego, CA, USA) to assess relationships between epigenetic regulation

and tumor features.[90] They identified several individually methylated genes, including RECK, and discovered a significant inverse correlation between promoter hypermethylation and relapse-free survival.

Likewise, we recently demonstrated a RECK-associated disease-specific survival advantage in several independent breast cancer datasets.[91] Our group also performed a comprehensive functional characterization of RECK using multiple in vitro and in vivo model systems. In this study, we demonstrated that RECK is a bona fide metastasis suppressor gene in breast cancer. In addition to known mechanisms, we revealed that RECK can also regulate metastasis and neoangiogenesis via suppression of uPA, VEGF, and STAT3 signaling.[91] These data all support RECK as a strong biomarker for breast cancer prognosis and a putative target for future therapy.

Discussion

Breast cancer is the most common form of cancer among women and one of the deadliest, second only to lung cancer. Each year, over 230,000 new cases of breast cancer are diagnosed and nearly 40,000 deaths occur in the United States.[92] Despite increasing incidence of breast cancer, mortality rates have been declining in recent decades, largely due to increased screening and early detection.[93] Classically, clinicopathologic features (eg, tumor size, histological grade) helped guide decision-making in the clinic, enabling physicians to tailor treatment regimens according to well-established probabilities of risk versus response. However, these prognostic markers alone have not been able to completely predict treatment efficacy and survival outcomes. Fortunately, several biomarkers have been validated and are now in routine clinical use, such as ER and HER2 status, allowing further treatment stratification following diagnosis. In addition, new technologies have given rise to entire gene panel sets as prognostic tools (eg, Oncotype DX®, Genomic Health Inc., Redwood City, CA, USA; MammaPrint™, Agendia Inc., Irvine, CA, USA). It goes without question that a clinician's ever-growing repertoire of prognostic tools has improved therapeutic management and patient outcomes in breast cancer. Regardless, there are many potential biomarkers that remain undiscovered or not validated and therefore warrant further investigation.

There has been renewed interest in recent years in the use of extracellular proteases as biomarkers in breast cancer. For example, both uPA and PAI-1 have now been validated at the highest level of evidence, though their use in the clinic is not yet widespread. In fact, studies have shown that uPA, uPAR, and PAI-1 status can be used to predict efficacy of tamoxifen therapy or when patients may avoid chemotherapy altogether. Other extracellular proteases have been significantly associated with patient outcomes, notably MMP-9, -11, and -15 and ADAM-8 and -17. Interestingly, several inhibitors of extracellular proteases have also been identified as potential breast cancer biomarkers. In particular, TIMP-4 may represent an understudied and potentially powerful new biomarker. Perhaps most interesting is the identification of RECK – a negative regulator of MMPs – as a robust biomarker for improved prognosis (overall survival and relapse-free survival) in breast cancer. Due to the plethora of evidence implicating altered ECM remodeling in cancer progression and metastasis, it is not surprising that alterations in several of these proteases correlate to patient outcomes.

The greatest unknown about the utility of extracellular protease status in breast cancer may be whether this knowledge can extend beyond tailoring existing treatment regimens. Targeting proteases, such as MMPs, has long been the subject of preclinical and clinical trials, though the early data were mostly disappointing.[94,95] Several reasons for these failures have been proposed, including the focus on treating metastases, lack of specificity among inhibitors, and broad action of MMPs on both pro- and antiangiogenic proteins (eg, ADAMTSs).[94,95] Since MMP activation occurs early in tumor progression, it is reasonable that trials on early-stage

Table 8 Select candidate inhibitors of extracellular proteases in cancer

Drug	Specificity	Highest phase completed	Reference
Rebimastat	MMP-2, -9	Phase III	97
SB-3CT	MMP-2, -9	Preclinical	98
CGS27023A	MMP-1, -2, -3	Phase I	99
Minocycline	MMP-1, -2, -3	Preclinical	100
Tanomastat	MMP-2, -3, -9	Phase III	101
Batimastat	MMP-1, -2, -3, -7, -9	Phase II	102
Neovastat	MMP-1, -2, -7, -9, -13	Phase III	103
Metastat (COL-3)	MMP-1, -2, -8, -9, -13	Phase II	104
Prinomastat	MMP-2, -3, -7, -9, -13	Phase III	105
Genistein	MMP-2, -9, MT1-, MT2-, MT3-MMP	Phase II	106
Marimastat	MMP broad spectrum	Approved	107
GI254023X	ADAM-10	Preclinical	108
PF-5480090	ADAM-17	Preclinical	109
KB-R7785	ADAM-10, -12	Preclinical	110
GW280264X	ADAM-10, -17	Preclinical	108
INCB3619	ADAM-10, -17	Preclinical	111
INCB7839	ADAM-10, -17	Terminated	112
Upamostat (WX-671)	uPA	Phase II	113
Aprotinin	uPA	Terminated	114

Abbreviations: MMP, matrix metalloproteinases; uPA, urokinase plasminogen activator; MT, membrane type.

cancers using novel, narrow-specificity protease inhibitors may attain greater success. In this vein, several exciting inhibitors of extracellular proteases are in various stages of development for use in cancer (Table 8).

Conclusion

Exploiting proteolytic activity for the selective delivery of other therapeutics is a promising new avenue and may depend on the localization and specificity of extracellular proteases.[96] Regardless, although both direct and indirect therapeutic approaches represent a more optimistic and challenging goal, the use of extracellular proteases as biomarkers in breast cancer presents an exciting new frontier in cancer management.

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Disclosure

The authors report no conflicts of interest in this work.

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