Proteases as Prognostic Markers in Cancer

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Abstract

It is the ability to invade and metastasize that ultimately determines the prognosis in cancer. Comprising one of the key groups of molecules involved in invasion and metastasis are proteases such as urokinase plasminogen activator and cathepsins B, D, and L, as well as various metalloproteases. These proteases catalyze degradation of the interstitial matrix and basement membranes, allowing cancer cells to invade locally and metastasize to distant sites. If proteases are directly and causally involved in cancer spread, they have the potential to be new prognostic markers in cancer. One of the best examples of a correlation between high levels of a protease in a primary tumor and poor prognosis is urokinase plasminogen activation in breast cancer. In this malignancy, the urokinase plasminogen activator is a strong and independent prognostic marker and may be a marker for axillary node-negative disease. The urokinase plasminogen activator may also be a prognostic marker in other cancers such as gastric, colorectal, lung, bladder, cervical, and ovarian cancers. In a number of studies, cathepsin D has been shown to be a prognostic factor in breast cancer. However, results with cathepsin D, especially when immunocytochemistry is used for its detection, are conflicting. Levels of cathepsin B, cathepsin L, and certain metalloproteases may also supply prognostic data in certain cancers, but results with these proteases are still preliminary.

Introduction

The aggressiveness of a tumor is primarily dependent on its ability to invade adjacent tissue and to metastasize to distant sites. During the process of cancer invasion and metastasis, natural barriers such as interstitial connective tissue and basement membranes have to be degraded. It is now widely believed that the breakdown of these barriers is catalyzed by proteolytic enzymes released from the primary tumor. Evidence implicating proteases in cancer spread has recently been described in detail (1–3) and is summarized in Fig. 1. A number of different proteases are involved in invasion and metastasis. These include uPA, CB, CD, CL, and various MMPs. These proteases may act in a cascade manner to mediate dissolution of the ECM.

The original evidence linking proteases to cancer invasion and metastasis was based on correlations between levels of specific proteases and metastatic potential in animal tumors (1). Approximately 10 years ago (4), we initially proposed that if a similar situation existed in human cancers, certain proteases might be markers of metastatic potential, i.e., proteases might be new prognostic markers in human malignancies. Evidence supporting this hypothesis has now been obtained for a variety of proteases in different cancers. The aim of this article was to critically review the current status of proteases as prognostic markers in human cancers.

Proteases Shown To Be Prognostic Markers

uPA. uPA is a serine protease with multiple actions that can enable it to play a role in cellular migration, tissue remodeling, and cancer spread (for review, see Ref. 5). Its best documented role is to catalyze activation of the inactive plasminogen to plasmin. Plasmin is a broad spectrum protease that can both catalyze degradation of diverse substrates in the ECM as well as the activation of certain MMPs (5). However, uPA can also directly degrade certain ECM proteins such as fibronectin (5) and activate a form of collagenase IV (5). Other actions of uPA that may be relevant to its role in metastasis include its ability to activate certain growth factors such as hepatocyte growth factor (6) and to stimulate both cellular migration (7) and mitogenesis (8). Most, if not all, of these actions of uPA are carried out while the protease is attached to a membrane-bound receptor (5).

Although uPA is the main form of plasminogen activator involved in degradation of the ECM, a different form of plasminogen activator, known as tPA, plays a role in fibrinolysis. Although uPA and tPA have different biological functions, both are nevertheless of prognostic value in human malignancy.

uPA was the first protease shown to be a prognostic marker in human malignancy. In 1988, we showed that patients with breast tumors containing high levels of uPA catalytic activity had a significantly shorter disease-free interval than patients with low levels of activity (9). These results have now been confirmed by many groups using an ELISA to measure uPA (Table 1). Furthermore, uPA whether measured by activity assays or ELISA also correlate with shortened overall survival in patients with breast cancer (Table 1).

uPA appears to be one of the strongest prognostic markers so far described for breast cancer. With both univariate and multivariate analysis, uPA is at least as strong a marker as nodal
status and stronger than other prognostic indexes for this disease such as tumor size, ER status, and CD levels (10, 11, 19, 20).

uPA is also a prognostic indicator for different subgroups of patients with breast cancer. In particular, a number of groups have shown it to be a marker of disease outcome in axillary node-negative patients (11, 15, 20–22), the group of patients with breast cancer where new prognostic markers are most urgently required. However, it is also prognostic in node-positive patients, premenopausal patients, postmenopausal patients, and the ER-positive subgroup (20, 22).

Janicke et al. (23) have recently shown that to obtain the optimum prognostic information from uPA in breast cancer, extraction with detergent, i.e., Triton X-100, is necessary. Extraction with detergent was particularly important in obtaining significant prognostic information in the node-negative and premenopausal subgroups of patients. The failure to extract with Triton X-100 may explain why Foekens et al. (15), using high-speed cytosols, were unable to find uPA prognostic in premenopausal patients, whereas other investigators (20, 22) who did extract with detergent found a significant relationship between uPA levels and disease outcome in this subgroup.

Why extraction with detergent enhances the prognostic strength of uPA is not clear. It is known, however, that the presence of Triton X-100 leads to approximately a 2-fold greater yield of uPA (23). This additional uPA may contain a greater proportion of receptor-bound proenzyme than is found in high-speed cytosols. As mentioned above, uPA mediates its actions while attached to a membrane-bound receptor.

Although uPA has consistently been shown to be a prognostic marker in breast cancer, different investigations use different cutoff points to discriminate high from low levels. The cutoff points vary from as low as 0.52 ng/mg protein (13) to as high as 10 ng/mg protein (10). Potential reasons for these differences include the following: (a) whether or not detergent was used to extract uPA; (b) different uPA standards used in ELISAs; and (c) different specificity of the antibodies in the different assays. This last point may be important because uPA can form complexes with multiple endogenous molecules such as its two inhibitors PAI-1 and PAI-2 and its receptor uPAR. Ternary complexes, such as uPA-PAI-1-uPAR, uPA-PAI-2-uPAR, as well as quaternary complexes involving associations between these ternary complexes and the α2-macroglobulin receptor may also exist. The α2-macroglobulin receptor is involved in the internalization of uPA-uPAR-PAI-1 complexes (24). In addition to these complexes, uPA can exit either in a precursor form, a mature form, or in degraded forms. Whether the available ELISAs are detecting all of these forms of uPA with similar efficiencies is presently unclear. Indeed, it remains to be shown which form of uPA is the most important in determining prognosis.

uPA has also been shown to be a prognostic marker in cancers other than that of the breast. In a study with 76 completely resected gastric cancers, Nekarda et al. (25), using univariate analysis, showed that high levels of uPA were significantly associated with decreased survival. However, using multivariate analysis with variables such as tumor size, nodal status, presence of distant metastases, grade, and PAI-1, uPA was not an independent prognostic marker. However, if PAI-1 was removed from the calculation, uPA became an independent factor.

Similarly in colorectal cancer, uPA has been reported to be a prognostic indicator using univariate but not with multivariate analysis (26). However, preliminary data suggest that the ratio of uPA in malignant and corresponding normal mucosa is an independent prognostic marker in colorectal cancer (27). Using immunocytochemistry, tumor epithelial staining for uPA was found to be prognostic in Duke's B colorectal cancer (28). In this study, stromal cell staining for uPA did not correlate with patient outcome. It should be stated that the Duke's B subgroup is where new prognostic markers are most needed in colorectal cancer.

Preliminary data also suggest that uPA is an indicator of disease outcome in patients with cancers of the bladder (29), lung (30), cervix (31), and ovary (32). However, in these cancers, uPA by itself has not yet been shown to act as an independent prognostic factor.

According to Clark (33), a prognostic marker should not only supply information on tumor aggressiveness but also on likely response to therapy. Preliminary data suggest that high levels of uPA correlate with lack of response to hormonal therapy in patients with advanced breast cancer (34). Clearly, confirmation of this finding would be most desirable.

In contrast to uPA where high levels are generally associated with poor disease outcome, high tumor levels of tPA correlate with a favorable outcome, at least in breast cancer (35). Furthermore, since tPA is induced by estrogen in some breast cancer cell lines (36) and correlates with estrogen and progesterone receptors in breast tumor biopsies (37), tPA may be an adjunct to steroid receptors in predicting hormone-dependent breast cancers. In patients with colorectal cancer, high tPA levels in morphologically normal mucosa remote from tumor also indicated a good outcome (26).
CD. CD, unlike uPA, is a lysosomal protease and thus has an acidic optimum pH. CD belongs to the aspartyl family of proteases. Interest in the relationship between CD and cancer was initially stimulated by the work of Westley and Rochefort (38), who showed that this protease was induced in ER-positive breast cancer cell lines by estradiol but was produced constitutively in ER-negative cell lines. Subsequently, CD was shown both to be a mitogen for estrogen-deprived breast cancer cell lines (39) and to catalyze degradation of the ECM (40). According to Schmitt et al. (3), CD initiates the protease cascade involved in degradation of the ECM. CD may accomplish this by activating CB and CL.

CD, as produced by breast cancer cells in vitro, can exist in multiple molecular weight forms. It is initially synthesized as a Mr 52,000 protein. This precursor protein is transported to lysosomes where it is processed to an intermediate Mr 48,000 protein. The Mr 48,000 form is later converted into mature forms with Mr 34,000 and 14,000 (41). Processing of CD appears to be slower in cancer cells than in normal cells (41). As a result, cancer cells accumulate greater proportions of the Mr 52,000 and 48,000 forms than do nonmalignant cells (41).

In 1989, Thorpe et al. (42) first showed, using an IRMA, that the Mr 52,000 form of CD was an independent prognostic marker for recurrence-free survival in patients with breast cancer. In multivariate analysis, CD was of similar prognostic value to both axillary node status and tumor size. In this study, although high CD levels tended to correlate with poor overall survival, the relationships were not statistically significant.

These results were later confirmed by Spyratos et al. (43), who also showed that CD was a prognostic marker for the disease-free interval in patients with breast cancer. In this study, CD was reported to be a more powerful marker than lymph node status and, furthermore, to be a prognostic marker in node-negative patients. Using Western blotting and a polyclonal antibody which detects only the Mr 34,000 form of CD, Tandon et al. (44) also found CD to be a prognostic marker in axillary node-negative patients. Using this assay in multivariate analysis, CD was found to be the strongest prognostic marker investigated for predicting both disease-free interval and overall survival, i.e., CD was stronger than ER, progesterone receptor, ploidy, and tumor size (44). In this study, however, CD did not correlate with a poor prognosis in axillary node-positive breast cancer patients (44).

Since these early findings, conflicting results have emerged on the value of CD as a prognostic marker in breast cancer. Using the IRMA to detect CD, most investigators find a relationship between high levels of the protease and poor prognosis when total populations are analyzed (for review, see Ref. 45). However, when subgroups are studied different results are obtained. Thus, while some find CD prognostic in node-negative patients (45, 46, 47), others find it prognostic only in node-positive patients (48, 49).

The most conflicting findings, however, are found when CD is detected using immunocytochemistry. In one of the first publications describing immunohistochemistry detection of CD in breast cancer, high levels of the protease were positively associated with a good outcome (50). More recently, high levels of CD have been found both to correlate with aggressive disease and to show no relationship with patient outcome (Table 2).

Table 2  Relationship between CD as determined by immunocytochemistry and patient outcome

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>Type of tissue</th>
<th>Type of antibody</th>
<th>Prognostic value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
<td>Paraffin</td>
<td>Polyclonal</td>
<td>Negative</td>
<td>50</td>
</tr>
<tr>
<td>262</td>
<td>Paraffin</td>
<td>Monoclonal</td>
<td>Positive*</td>
<td>51</td>
</tr>
<tr>
<td>136</td>
<td>Paraffin</td>
<td>Polyclonal</td>
<td>None</td>
<td>52</td>
</tr>
<tr>
<td>159</td>
<td>Paraffin</td>
<td>Polyclonal</td>
<td>None*</td>
<td>53</td>
</tr>
<tr>
<td>165</td>
<td>Frozen</td>
<td>Monoclonal</td>
<td>Positive</td>
<td>54</td>
</tr>
<tr>
<td>562</td>
<td>Frozen</td>
<td>Monoclonal</td>
<td>None*</td>
<td>55</td>
</tr>
</tbody>
</table>

* These studies used only node-negative patients; all others used both node-positive and node-negative patients.

Similarly, disagreement exists within the node-negative subgroup of patients. Thus, while Isola et al. (51) found a strong correlation between high levels of CD and both a shorter disease-free interval and overall survival in this subset of patients, others were unable to confirm this finding (53, 55).

As mentioned above, one of the earliest reports describing a relationship between CD and patient outcome in node-negative patients used Western blotting and a polyclonal antibody (44). Recently, the same authors using a monoclonal antibody in Western blotting were unable to confirm the original finding (55). This was despite the ability of both antibodies to detect the Mr 34,000 forms of CD and yield results that were highly correlated (55). In an attempt to explain the different findings with the polyclonal and monoclonal antibodies, the authors speculated that the two antibodies may be detecting different glycosylated forms of CD (55).

Differences in the specificity of antibodies may also explain some of the conflicting results obtained with immunocytochemistry. In addition, different scoring systems to quantify CD levels, different cutoff points for discriminating high from low values, and different types of tissue, i.e., fresh versus formalin fixed and paraffin embedded, may also be responsible for the conflicting findings.

It is also unclear whether stromal cell staining, tumor cell staining, or both should be assessed for correlating CD with patient outcome. In one study, staining in only the cancer cells was found to correlate with patient prognosis (51). In this investigation, although malignant cell staining correlated significantly with macrophage staining, CD levels in the latter mentioned cells did not relate to either patient disease-free interval or survival. In another study using the same monoclonal antibody for staining CD, only stromal cell staining was significantly associated with a bad prognosis (56).

Finally, the ability of CD to act as a prognostic marker may depend on whether or not adjuvant therapy was administered. Thus, Ferno et al. (57) recently showed that CD was of prognostic importance only in breast cancer patients with lymph node metastases not treated with tamoxifen. Furthermore, in this study, adjuvant tamoxifen was found to have a beneficial effect only in patients with nodal metastases and progesterone receptor-positive tumors containing high levels of CD.

It is less easy to explain the divergent results obtained using IRMA since all investigators used the same assay. Furthermore, this IRMA has been found to yield acceptable be-
between laboratory precision in an External Quality Trial performed by the EORTC Receptor Study Group (58). Possible explanations for the varying results here may relate more to differences in the patient populations used than to the CD assays. These factors may include different patient populations, different follow-up periods, different numbers of patients, whether or not adjuvant therapy was used, and different cutoff points for CD.

**CB and CL.** Like CD, both CB and CL are lysosomal proteases, but in contrast to CD are thiol proteases. Both of these thiol proteases can catalyze degradation of various components in the ECM, but CL is more effective against intact basement membranes (for review, see Ref. 59). Both CB and CL can also activate the precursor form of uPA leading to active uPA (3).

Both CB and CL are synthesized as prepro forms. Thus, at least two activation steps are apparently necessary to produce active enzyme. CD and specific metalloproteases may play a role in the activation of these thiol proteases (60, 61). However, both thiol proteases can also undergo autoactivation (59).

Early studies using catalytic activity assays found no relationship between high levels of CB and poor prognosis (62, 63). These activity assays, however, had the disadvantage that the substrates used were not specific for CB. Furthermore, endogenous inhibitors such as the stefins and cystatins (59) may have interfered in these assays. Recently, however, using an ELISA, Thomssen et al. (64) found a significant association between high levels of CB and a shorter disease-free interval in patients with breast cancer. No significant relationship was found between CB levels and overall survival in this study. Preliminary findings using immunocytochemistry have also demonstrated a similar relationship between high levels of CB and poor prognosis in patients with bladder (65), colorectal (66), and lung cancer (67, 68).

In breast cancer, CL appears to be a stronger prognostic marker than CB (64). In this malignancy, CL was reported to be an independent prognostic factor comparable in strength to nodal status and tumor grade (64). Furthermore, CL was of prognostic value in both node-negative and node-positive patients (64). In comparison with CL, CB was neither an independent prognostic variable or prognostic in node-negative patients. CB, however, was prognostic in axillary node-positive patients (64).

**MMPs.** The MMPs are so named because they require calcium and zinc ions as cofactors. The MMPs can be divided into three main groups: interstitial collagenases, type IV collagenases, or gelatinases and the stromelysins (for review, see Ref. 2). There are two interstitial collagenases known as fibroblast collagenase (MMP-1) and neutrophil collagenase (MMP-8). These homologous proteases are encoded by different genes (69). Although both catalyze the cleavage of types I, II, and III collagen, MMP-1 shows a preference for type III and MMP-8 for type I collagen (69).

Type IV collagenases degrade the helical regions of type IV collagen, the main form of collagen in basement membranes. In addition, these MMPs catalyze degradation of type V, VII, IX, and X collagens as well as fibronectin and elastin (2). Again, there are two main forms of collagenase IV (2), i.e., gelatinase A (MMP-2) and gelatinase B (MMP-9; Ref. 2). Gelatinase B has a similar domain structure to that of gelatinase A but in addition contains a type V collagen-like insert (69). Recently, a new MMP known as membrane type-MMP was described (70). At least four different forms of stromelysin have been described: types 1, 2, and 3 and matrilysin. The stromelysins have broad substrate specificity, catalyzing degradation of various components of the ECM (1, 2).

In one of the first studies to evaluate MMPs as prognostic markers in cancer, Daidone et al. (71) reported that high levels of collagenase IV detected using immunocytochemistry correlated with local-regional recurrence in node-negative breast cancer patients. In contrast, no relationship was found between levels of collagenase IV and either relapse-free or overall survival. In this study it was not clear which form of the collagenase IV was being detected. However, using immunocytochemistry with specific antibodies against MMP-8 and MMP-9, Visscher et al. (72) found no relationship between levels of these MMPs and disease outcome in breast cancer. However, stromelysin-3 mRNA as detected by *in situ* hybridization (73) has been found to correlate with poor outcome in breast cancer.

**Conclusion**

Proteases are among the first group of molecules causally involved in metastasis which act as prognostic markers in cancer. Although a number of different proteolytic enzymes have been evaluated as potential prognostic factors, perhaps the best documented and most consistent data have been obtained with uPA. For breast cancer, uPA is a strong and independent prognostic marker and, furthermore, appears to be a marker for node-negative disease. Presently, no reliable prognostic indicator is available for this subgroup of patients with breast cancer. If these early findings on uPA are confirmed, assay of this protease should be considered for routinely assessing prognosis in breast cancer. However, before then, methods for the extraction and assay of uPA will have to be standardized. Furthermore, it would be desirable to have an immunocytochemistry assay for uPA in breast cancer. uPA may also be a prognostic marker in other adenocarcinomas. However, results with cancers other than breast are still preliminary and need to be confirmed.

Although the early results with CD as a prognostic factor in breast cancer were promising, subsequent results, especially with the use of immunocytochemistry, have led to conflicting data. Future work on CD should attempt to explain the reasons for these discrepant findings.

At this stage, data on CB, CL, and MMPs as prognostic indicators is preliminary. However, the recent availability of ELISAs (64, 69) for these proteases should stimulate their evaluation as prognostic factors. If multiple proteases are involved in metastasis, the most useful prognostic information may be obtained by the combined measurement.

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