Expression of Cathepsin-D in Primary Breast Cancer and Corresponding Local Recurrence or Metastasis: An Immunohistochemical Study

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Abstract. Background/Aim: The role of cathepsin-D is well established in breast cancer progression, being correlated with worse clinical outcomes. However, to our knowledge, no study has been performed investigating its expression in primary breast cancer tumors and their corresponding recurrences or metastasis. Materials and Methods: Tissue sections from ten breast cancer cases and their corresponding local recurrences and six breast cancer cases and their corresponding metastases were immunohistochemically assessed for cathepsin-D reactivity. Cases diagnosed as either ductal carcinoma in situ (n=7), or breast carcinoma with no evidence of local recurrence or metastasis during follow-up (n=8) served as controls. Results: Cathepsin-D was significantly up-regulated in all the study groups compared to controls. No difference was found between primary tumors and their corresponding recurrences or metastases. Conclusion: Cathepsin-D-expressing breast cancer cells seem to be involved in local recurrence or metastasis formation. Large series are needed to further verify this result with the aim of possible future molecular intervention.

Cathepsin-D is a soluble aspartic protease found in lysosomal and endosomal cellular compartments (1). The cathepsin-D gene has been reported as both a housekeeping gene and as a hormone-regulated gene. Its promoter has several regulatory sites among which are a 5'-TATAAA-3' DNA sequence (TATA-box) and two p53-binding sites (2, 3).

In breast cancer, an estrogen-dependent neoplasm, cathepsin-D expression is induced via this TATA-box, while in any case of oncosuppression – mainly via chemotherapeutic regimes – cathepsin-D is up-regulated via the p53 pathways (3). The end ‘mature’ cathepsin-D is the result of a multiple processes by which premature forms are post-translationally modified as they pass through different cytoplasmic compartments. This procedure is low pH-dependent. Cathepsin-D is finally located in lysosomes, from which it will be released into the cytoplasm in case of cell death/apoptosis (4).

In cases of disrupted cathepsin-D production, premature cathepsin-D forms can be accumulated in the cytoplasm or even in the extracellular space. Such disrupted cathepsin-D production has been reported in cancer cells where procathepsin-D is overexpressed (5).

The role of cathepsin-D in breast carcinoma is substantial. Procathespin-D, a premature cathepsin-D form reported as being abundant in breast cancer, is presented to have autocrine properties, inducing cell proliferation in MCF-7 breast cancer cells (6). This procathespin-D form is reported as being induced by estrogen in breast cancer (7). Additionally, several mechanisms involve cathepsin-D in modulation of the extracellular matrix (ECM), thus facilitating metastasis: a) the extracellular cathepsin-D can easily become active under conditions of a low ECM pH, a fact often seen in tumors due to hypoxia and lactate production (8); b) cathepsin-D can be released immediately to the ECM by necrotic cells (1); and c) as ECM is degraded, several ECM-bound growth factors are released, supporting further tumor growth, angiogenesis and thus metastasis (9, 10). These facts could explain the well-established positive correlation between cathepsin-D overexpression and increased metastatic potential (11).

Several studies, well summarized in recent reviews (1, 4, 5), have reported upon the role of cathepsin-D in breast cancer progression; most of them suggest that increased
cathepsin-D concentrations in breast cancer tissues are related to worse prognosis, and shorter disease-free and overall survival. Of note is one meta-analysis showing that in node-negative breast cancer, increased cathepsin-D concentrations are indeed correlated to a worse prognosis (12).

However, none of the studies performed so far has investigated the expression of cathepsin-D in the actual recurrence or metastasis. Herein, we present an immunohistochemical study involving both primary breast carcinomas along with their corresponding local recurrence and distant metastasis. We show that both local recurrences and metastases maintain the same cathepsin-D expression pattern seen in their corresponding primary tumors.

Materials and Methods

Population. In this study, 31 patients treated with surgery for primary breast carcinoma between 1998-2002, and followed-up for a mean period of 8 years, were enrolled according to the following criteria: i) breast carcinoma diagnosed with recurrence during follow-up (Group A, n=10) along with their corresponding local recurrence tissue sample (Group AR, n=10); ii) breast carcinoma diagnosed with distal metastasis during follow-up (Group B, n=6), along with their corresponding distal metastasis tissue sample (Group BM, n=6). Cases diagnosed as either ductal carcinoma in situ (DCIS) (Group C1, n=7), or breast carcinoma with no evidence of local recurrence or metastasis during follow-up (Group C2, n=8), were randomly selected as controls, from the pathology archive of the First Department of Obstetrics and Gynecology, Ludwig Maximilians University of Munich, Germany.

The current study was approved by the Research Ethics Committee of the Ludwig Maximilians University of Munich.

Immunohistochemistry. Formalin-fixed paraffin-embedded tissue sections (3 μm thick) were de-paraffinized, rehydrated in a descending series of alcohol and subjected to epitope retrieval in a pressure cooker using sodium citrate buffer (pH 6.0). After returning to room temperature, sections were washed twice in phosphate-buffered saline (PBS) and blocked with 3% hydrogen peroxide (Merck, Darmstadt, Germany) in methanol for endogenous peroxidase activity (30 min). Non-specific binding of the primary antibodies was inhibited by incubating the sections with diluted normal serum (10 ml PBS containing 150 μl horse serum; Vector Laboratories, Burlingame, CA, USA). The sections were then incubated with anti-cathepsin D (clone C5, mouse monoclonal; Dianova GmbH, Hamburg, Germany) at a concentration of 1 μg/ml overnight at 4°C. Reactivity was revealed by using Vectastain Elite ABC kit (Vector Laboratories), according to the manufacturer’s protocol. Substrate and chromogen (3,3’-diaminobenzidine DAB; Dako, Glostrup, Denmark) were finally added to the slides, which were then counterstained with Mayer’s acidic hematoxylin and covered. Incubation with mouse IgG served as negative control.

The intensity and distribution patterns of the specific immunocytochemical staining were evaluated using a semi-quantitative method (immunoreactivity score, IRS) as previously described (13). Briefly, the IRS was calculated as the product of the optical staining intensity (0: no staining; 1: weak staining; 2: moderate staining and 3: strong staining) and the graded staining extent (0: no staining; 1: <10% staining; 2: 11-50% staining; 3: 51-80% staining and 4: >80% staining). All cases were evaluated by two observers; the IRS was extracted by the two observers by consensus. Statistical analysis. Differences of the mean IRS between the main groups of the study were evaluated by applying the Mann-Whitney test. Differences in cathepsin-D expression among paired samples were assessed by performing the Wilcoxon test. Every comparison with p<0.05 was considered significant.

Results

Cathepsin-D was found to be located in the cytoplasm of breast cancer cells in all cases studied (Figure 1). Cathepsin-D was found to be moderately expressed in cases of primary breast carcinomas that presented with local recurrence during follow-up (mean IRS=5.30±0.83), while cases presenting as either DCIS or as breast carcinomas with no evidence of recurrence during follow-up revealed only a weak cathepsin-D positivity (mean IRS=2.28±0.18 and 2.50±0.37, respectively). These observations were significant (Group A vs. Group C1, p=0.04 and Group A vs. Group C2, p=0.03) (Figure 2). The same pattern of positivity was also identified in cases of primary breast tumors that presented with distal metastases during the follow-up (mean IRS=5.66±0.80), compared to cases with DCIS (mean IRS=2.28±0.18), or with breast carcinoma with no evidence of metastasis (mean IRS=2.50±0.37). These observations were also significant (Group B vs. Group C1, p=0.01 and Group B vs. Group C2, p=0.01) (Figure 2).

Interestingly, cathepsin-D expression was significantly up-regulated in both local recurrences (mean IRS=4.30±0.59) and metastases (mean IRS=6.16±1.51) when compared to DCIS (mean IRS=2.28±0.18) and breast carcinomas with no evidence of recurrence or metastasis (mean IRS=2.50±0.37), (Group AR vs. Group C1, p=0.01; Group AR vs. Group C2, p=0.03; Group BM vs. Group C1, p=0.04; and Group BM vs. Group C2, p=0.04), revealing that both carcinomas with recurrence and their corresponding recurrences, as well as both carcinomas with metastases and their corresponding metastases, maintain a moderate cathepsin-D expression.

This observation was also verified statistically by comparing cathepsin-D expression between the main two groups of study. Cathepsin-D expression did not differ significantly between primary breast carcinoma cases that finally recurred and primary breast carcinoma cases with a positive follow-up for distal metastasis (Group C vs. Group D, p=0.78). Additionally, no significant difference in cathepsin-D expression was revealed between primary breast carcinomas and their corresponding recurrences or distal metastases (Group A vs. Group AR, p=0.207; and Group B vs. Group BM, p=0.78) (Figure 2).

Discussion

The role of cathepsin-D in breast cancer prognosis although well studied is not yet established. Many research teams have assessed the impact of cathepsin-D expression on several
clinical outcomes, such as overall and disease-free survival, as well as relative risks of recurrence and metastasis (1, 5). These approaches however, have been performed using different methodologies, with the most common being protein immunoassays and immunohistochemistry. As far as protein immunoassays are concerned, there has been no consensus regarding the cut-off value above which cathepsin-D is considered as being overexpressed. Most of the reported studies have set their own cut-off value according to a statistical dichotomy that allows their sample to be clearly divided into samples of low and high cathepsin-D expression. More importantly, in several studies, the cut-off value was set on a retrospective basis to optimize the results. This is definitely a weak approach for supporting the use of cathepsin-D assessment in routine practice.

Few studies are of note regarding cathepsin-D involvement in breast cancer prognosis. In a radiometric immunoassay study of 2810 lymph node-negative and -positive breast cancer patients using a cut-off of 45.2 fmol/g, it was shown that cathepsin-D was moderately prognostic for both lymph node-negative and -positive patients (14). In another ELISA study of 1851 breast cancer patients, using a cut-off of 10 fmol/g, cathepsin-D was considered an independent prognosticator (15). At the same time, another research group presented an immunohistochemical assessment of 1348 breast cancer patients holding that it is stromal cell expression of cathepsin-D, which holds prognostic value rather than breast cancer-associated cathepsin-D; this prognostic value was however restricted to a subgroup of patients (16). These diagnostic inconsistencies led the

Figure 1. Representative microphotographs of immunohistochemical detection of cathepsin-D. A: Ductal carcinoma in situ; B: Breast carcinoma with no evidence of recurrence or metastasis during follow-up; C: Primary breast carcinoma with evidence of recurrence during follow-up; D: Recurrence of breast carcinoma; E: Primary breast carcinoma with evidence of distal metastasis during follow-up; F: Distal metastasis of breast carcinoma. Bar=100 μm.
American Society of Clinical Oncology not to recommend cathepsin-D as a tumor marker for breast cancer patient management (17), due to insufficient evidence and inconsistencies of the literature.

Despite the above recommendation, cathepsin-D is widely accepted as being involved in metastasis pathophysiology (18) and thus has already been a target of experimental interventions (19, 20). It could, thus, be accepted as a factor affecting disease-free and overall survival, either being a dependent or an independent prognosticator. Our results are in line with this hypothesis, since it is currently shown that higher cathepsin-D expression in the primary tumor is related to an increased possibility of local recurrence or distant metastasis. Of course our sample is considered rather small and for this reason we decided not to further proceed to logistic regression analysis.

Moreover, this study has revealed that the cathepsin-D expression pattern is maintained from the primary tumor in the local recurrence or metastasis. This could imply that there is a biological selection of the cells that will be involved both in local recurrence and metastasis: the cells being potent to disrupt the ECM are those likely to end up in metastatic sites and in local recurrence. An interesting question, taking as a fact that cathepsin-D expression and release could be triggered by chemotherapeutic drugs via p53 pathways (21), would be why metastases and local recurrences are considered rather refractory to treatment. Perhaps this could be explained by the fact that tumor cell populations are heterogeneous, possibly expressing not only cathepsin-D but also other proteins that could counteract this p53-induced cathepsin-D expression, not to mention mutations that could also knockout p53 expression.

These findings can also be considered as the sequel of a previous study of our group showing that cathepsin-D is down-regulated in lymph nodes compared to their corresponding primary tumors (22). Such a finding, taken together with the present results, reveals that possibly different pathophysiological schemes are involved in lymph node metastasis, local recurrence and distal metastasis; it is possible that the lymph node microenvironment may, for an unknown reason, be more receptive to low cathepsin-D-expressing cells, while breast and distal organs are more receptive to high cathepsin-D-expressing cells. This receptivity could also be attributed to the fact that in order for a cell to establish a metastasis, ECM proteolytic modification is needed (23).

In conclusion, we have demonstrated that an increased cathepsin-D expression in primary breast carcinomas is associated with an increased possibility of local recurrence and metastasis, this being in line with previous reports in the field. This increased cathepsin-D expression is maintained in the corresponding local recurrences or metastases, implying a mechanism of tumor cell selection upon recurrence or metastasis formation. Further studies are certainly needed in order for this finding to be verified in a large series and to further clarify the role of cathepsin-D in breast cancer progression.
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References


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