Original Paper

Cathepsin-D expression in breast lesion: an immunohistochemical study

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Abstract
Cathepsin-D (CathD) is an aspartyl lysosomal protease expressed in all tissues that might play a role in antigen processing, cell proliferation and tissue renewal, and activation of different pro hormones. The aim of our study was to compare the expression of CathD in most common breast tumors and tumor-like breast lesions. The study includes 21 patients with histologically verified breast lesions (adenosis, ductal hyperplasia, fibroadenomas, and different types of invasive carcinoma). We investigated the cathepsin-D expression in these breast lesions using immunohistochemistry (IH; paraffin-embedded tissues). Cathepsin-D staining within each lesion was assessed by estimating the area of the objects and the medium pixel intensity per object, as the integrated optical density (IOD). The immunostaining was more obvious in breast invasive carcinomas and macrophages. The reaction in tumor tissue was heterogeneous with little variation of staining intensity in positive tumor cells. Adenosis had the maximum area/signal intensity from all studied breast benign lesions (p<0.001, Student t-test). The general tendency (all benign lesions, lobular carcinomas and G3 ductal invasive carcinoma) was a more prominent representation of the cellular compartment. In the G3 ductal invasive carcinoma-type, the group of patients with metastases had a stronger expression in the cellular compartment. These results suggest that CathD expression was strongest in malignant than in benign breast disease, the positivity being present in both epithelial neoplastic and stromal cells. We also conclude that our procedure in IOD measurement is prone to less subjective-related biases, and thus more accurate and constant than other methods employed by other authors.

Keywords: breast, carcinoma, cathepsin-D, immunohistochemistry.

Introduction
Breast cancer still remains a major world health problem, being the second leading cause of cancer death in women, exceeded only by lung cancer. It is estimated that breast cancer will affect five million women worldwide over the next decade, and the incidence of the disease is increasing at an average of about 1% per year in industrialized countries and at a greater rate in developing countries [1, 2].

Considerable progress has been made in understanding the mechanisms of breast tumor growth and progression. Over the years, there were studied more than thirteen categories of breast tumor markers, hoping to improve prevention, screening, treatment, and surveillance of breast cancer [3].

One of these breast cancer markers is CathD. This biological marker is a peptidase belonging to the family of aspartic peptidases. Major function of CathD is the digestion of proteins and peptides within the acidic compartment of lysosome [4]. Other physiological effect includes hormone and antigen processing, and breakdown of the extra cellular matrix. In the latest decades, there has been an increasing number of data, describing elevated levels in certain tumor tissues, associated with their progression and metastases [5–7].

The aim of the present study was to compare the expression of CathD in most common breast tumors and tumor-like breast lesions.

Materials and Methods
Tissues and histopathological processing
Twenty-one formalin-fixed, paraffin-embedded breast tissue blocks from the archive of the department of pathology (No. 1 Emergency County Hospital, Craiova) were included in the current study. All these samples originated from complete resection material. Sections from these paraffin-embedded blocks were stained with Hematoxylin and Eosin (HE). Two experienced pathologists (S.C. and F.C.) without knowledge of the clinical data performed re-evaluation of the HE stained sections. Diagnosis and tumoral grading were performed according to WHO criteria.
Immunohistochemistry

We performed immunostaining on formalin-fixed, paraffin embedded tissue sections using a labeled streptavidin-biotin immunoenzymatic antigen detection system, respectively the Ready-to-Use UltraVision Plus Detection System Anti-Polyvalent, HRP/DAB (from Lab Vision Corporation, USA). All the experimental procedures have been carried out in the facility of the Research Center for Microscopic Morphology and Immunology (University of Medicine and Pharmacy Craiova).

Five-micrometer-thick serial sections were cut from each paraffin-embedded block. The sections were deparaffinized in xylene and rehydrated through graded concentrations of alcohol. As antigen retrieval, we used heat-induced epitope retrieval (HIER), a technique of boiling tissue sections in 10 mM citrate buffer, pH 6.0 for 20 minutes followed by cooling at RT for 20 minutes. Three-percent hydrogen peroxide in PBS for 15 minutes was then applied to block endogenous peroxidase activity. For nonspecific background blocking, we used 7 minutes incubation at room temperature with Ultra V Block.

The sections were incubated overnight at 4°C with anti-human cathepsin-D Ab-1 (Clone C5) Mouse Monoclonal antibody (diluted 1:50; Lab Vision Corporation, USA). Antibody dilution was made in 1% bovine serum albumin and 0.05% Tween. Next day, the sections were washed thoroughly, incubated with the Biotinylated Goat Anti-Polyvalent Plus for 15 minutes at room temperature. After washing, Streptavidin Peroxidase Plus was applying for 15 minutes at room temperature. Next step was thoroughly washing and the signal was developed with DAB Plus Chromogen under Peroxidase Plus was applying for 15 minutes at room temperature with Ultra V Block.

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All sections were finally counterstained with Hematoxylin dehydrated with xylene, and raised using Entelan raising medium. Internal negative controls were obtained by omitting the primary antibodies and external positive controls consisting in breast carcinomas specimens with known strong cathepsin-D expression. The normal controls were obtained from the resection edges of these breast tumors.

Image acquisition and analysis

The sections were imaged with a Nikon Eclipse 90i microscope equipped with a 5-megapixel CCD camera. 20× and 40× images were acquired utilizing a Nikon frame grabber and the Nikon NIS-Elements software. All images were acquired and processed in TIFF format.

For the interpretation of the CathD stained sections, two observers (MC and BI), without the knowledge of the clinical data, evaluated independently the slides.

Cathepsin-D staining within each lesion was assessed by estimating the area of the objects and the medium pixel intensity per object, as the integrated optical density (IOD). Briefly, in an initial calibration step, we recorded first an image without any section mounted on the microscope, one with no transmitted light, and then we utilized these two images as calibration standards for maximum and minimum values in our densitometric study. The same light level as for incidental light without a slide was kept for each image acquired.

Utilizing the hand lasso tool in Photoshop (Photoshop 7, Adobe Systems Inc.), each image was devised in a layer containing only the cells and another layer containing only the stroma. The layers were imported in NIS-Elements and processed for IOD measurements. An RGB profile of the immunohistochemical signal was created and then applied for each layer to create signal masks.

All these operations were recorded and run in a batch processing flow that needed operator input only when encircling the areas of the cells. This greatly increased the speed of analysis and ensured reproducible data. All the values were exported and analyzed in Excel.

The reactive stromal cells analyzed included those with morphologic features of fibroblasts, myofibroblasts and macrophages. We could not exclude from counting macrophage-like cells that surrounded and infiltrated clumps of tumor cells in breast invasive carcinomas.

Statistical analysis

All recorded values were exported and analyzed in Excel (Microsoft Corporation). Differences in CathD expression between different pathological types were assessed utilizing Student t-test.

Results

Histopathological data

The histopathological re-evaluation of the section was summarized in Table 1. The most numerous studied lesion was malignant (14) with ductal invasive carcinoma best represented (eight). The most encountered benign lesion was breast adenosis (three cases), especially of sclerosing type (two cases).

<table>
<thead>
<tr>
<th>Table 1 – Histopathological distribution of the casuistry</th>
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<tr>
<td>Histopathological breast lesion type</td>
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<tr>
<td>Sclerosing adenosis</td>
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<tr>
<td>Microglandular adenosis</td>
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<tr>
<td>Ductal hyperplasia</td>
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<td>Fibroadenomas</td>
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<td>G3 ductal invasive carcinoma</td>
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<td>G1 ductal invasive carcinoma</td>
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<td>Lobular invasive carcinoma</td>
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<td>Mucinous invasive carcinoma</td>
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CathD expression

Normal lobular or ductal epithelia both from non-tumoral and tumoral lesions showed no specific staining. In both, epithelia and stromal cells of studied breast lesions, cathepsin-D immunostaining was seen as brown, coarse, or tiny granules dispersed in the cytoplasm of the cells. In general, benign epithelium showed a low-intensity small granular staining, whereas breast invasive carcinomas and macrophages displayed a more consistent, diffuse, and higher-intensity staining of larger granules (Figures 1 and 2).
Figure 1 – Cathepsin-D expression in breast adenosis:
(a) Sclerosing adenosis (HE staining, ob. ×40);
(b) CathD expression in sclerosing adenosis (ob. ×40);
(c) Microglandular adenosis (HE staining, ob. ×40);
(d, e) CathD expression in microglandular adenosis (ob. ×20 and ×40).

Figure 2 – Cathepsin-D expression in breast ductal hyperplasia and fibroadenomas:
(a) Ductal hyperplasia (HE staining, ob. ×40); (b) CathD expression in ductal hyperplasia (ob. ×40).
The distribution of positive cells within tumor tissue was heterogeneous and we did not observe any difference on location of CathD-positive cells between central parts and tumors periphery (Figures 3 and 4). There was little intratumoral variation of staining intensity in positive tumor cells, but reaction seems to be more intense towards the cell membranes in those cells (Figures 3f and 4f). Many of the positive stromal cells were macrophage-like cells, and they contained numerous coarse, strongly CathD-positive granules (Figures 1e, 2e, 4b, 4e, and 4f). These sometimes surrounded and infiltrated clumps of tumor cells and varied considerably in intensity within the tumors (Figure 4, d–f).

Evaluation and analysis of cathepsin-D expression

In the group of benign lesions we observed a maximum area/signal intensity for the adenosis-type of lesions ($p<0.001$, Student $t$-test). Although the number of sections assessed did not deem significant the difference between cellular and stromal compartments in hyperplasia-type of lesions ($p=0.13$, Student $t$-test), the general tendency observed for benign lesions was a more prominent representation of the cellular compartment (Figure 5).

Figure 2 – Cathepsin-D expression in breast ductal hyperplasia and fibroadenomas: (c) Breast fibroadenoma (HE staining, ob. ×10); (d, e) CathD expression in breast fibroadenoma (ob. ×10 and ×40).

Figure 3 – Cathepsin-D expression in breast lobular and mucinous carcinomas: (a) Lobular carcinoma (HE staining, ob. ×10); (b) CathD expression in lobular carcinoma (ob. ×20).
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Figure 3 – Cathepsin-D expression in breast lobular and mucinous carcinomas: (c) Mucinous carcinoma (HE staining, ob. ×4); (d–f) CathD expression in mucinous carcinoma (ob. ×10, ×20 and ×40).

Figure 4 – Cathepsin-D expression in breast ductal invasive carcinoma: (a) G1 ductal invasive carcinoma (HE staining, ob. ×10); (b) CathD expression in G1 ductal invasive carcinoma (ob. ×20); (c) G3 ductal invasive carcinoma (HE staining, ob. ×10).
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Figure 4 – Cathepsin-D expression in breast ductal invasive carcinoma: (d–f) CathD expression in G3 ductal invasive carcinoma (ob. ×20 and ×40).

Figure 5 – Staining grading in benign lesions. Adenosis-type of lesions presented with the highest intensity-area of the signal for both cellular and stromal compartments. The cellular compartment was predominant for adenosis and fibroadenoma-type of lesions. Scale bars represent standard deviation, * – difference deemed highly significant, p<0.001, Student t-test.

The same type of analysis on the malignant lesions revealed that the cellular compartment predominated in lobular carcinomas (p<0.001, Student t-test) and G3 ductal invasive carcinoma (p<0.05, Student t-test) (Figure 6). In mucinous carcinoma we observed an inverse trend of predominance of the stroma over the cellular compartment (p<0.05, Student t-test). Probably again due to the small sample size, we could not conclude any differences between the two compartments in the G1 ductal invasive carcinoma (p=0.6, Student t-test).

Figure 6 – Staining grading in malignant lesions. Adenosis-type of lesions presented with the highest intensity-area of the signal for both cellular and stromal compartments. The cellular compartment was predominant for lobular carcinoma, when compared to all the other types, except the G3 ductal invasive carcinoma. In mucinous carcinoma, the stromal compartment was significantly more represented compared to the cellular compartment. No difference could be noted in the G1 ductal invasive carcinoma type. Scale bars represent standard deviation, * – difference deemed highly significant, p<0.001, Student t-test, ** – difference deemed significant, p<0.05, Student t-test.

The rather large variability in the G3 ductal invasive carcinoma-type is also due to the presence of two subcategories here: patients presenting without identifiable metastases and patients with metastases. The group of patients without metastases consisted of three cases.
Discussion

CathD has long been thought to be ubiquitously distributed in lysosomes of all cells in order to degrade proteins at an acidic pH in synergy with other cathepsins [8]. Having a crucial role in activating proteins in prelysosomal compartments, it has been proposed that CathD might play a role in antigen processing [9, 10], cell proliferation and tissue renewal [11] and activation of different prohormones [12–14].

Studies on some solid tumors, inclusively on breast cancer have revealed that overexpression of CathD can be correlated with the metastatic potential of these tumors [15–18]. It has generally been proposed that CathD is involved in the degradation of the basement membrane surrounding the primary tumor, and moreover the major action may be via substrates leading to increased cell proliferation of tumor cells at distant sites. The overexpression of CathD in breast cancer was proved both at the mRNA and protein levels [15, 17–19]. Several approaches, such as immunohistochemistry, in situ hybridization, cytosolic immunoassay and Northern and Western blot analyses have indicated that in most breast cancer tumors CathD is over expressed 2- to 50-fold compared to its concentration in other cells such as fibroblasts or normal mammary glands [19].

Schultz DC et al. [20] used for the first time Western blotting analysis to determine the relative amounts of precursor and processed forms of CathD in sera and breast tissue of patients with breast cancer, benign breast disease, and normal controls. Authors noticed that malignant breast tissue contained the two forms of CathD found in sera (M, 52 000 and 27 000), and an additional M, 31 000 form which was found in significantly increased (p<0.001) relative amounts in breast tissue from 43 breast cancer patients [24 ± 12% (SD)] when compared to 51 benign breast disease patients (13 ± 8.9%) and 23 normal controls (1.8 ± 4.4%). Also, there was found that there was no significant difference (p = 0.41) in relative amounts of the M, 31 000 form of CathD between proliferative-type and nonproliferative-type fibrocystic breast disease.

Stomper PC et al. [21] studied the immunohistochemical expression of CathD on forty-five patients with 48 breast lesions, of which 25 were malignant (invasive ductal or invasive lobular carcinoma and DCIS) and 23 benign (fibro adenomas and other benign lesions). The authors showed a positive CathD staining in both benign and malignant epithelial cells and macrophages. Generally, benign epithelium showed a low-intensity small granular staining, whereas in situ invasive carcinomas and macrophages displayed a more consistent, diffuse, and higher-intensity staining of larger granules. Also, quantitatively seventeen of 25 (68%) malignant and 17 of 23 (74%) benign lesions showed more than 50% positively staining cells for CathD.

According to Stomper PC et al. our results prove that in benign lesion the epithelium had a low reactivity to CathD, whereas tumor cells from malignant breast lesion and macrophages displayed a more consistent, diffuse, and higher-intensity staining. Regarding CathD distribution within breast lesion, we conclude that the general tendency for benign lesion was a more prominent immunoreactivity in the cellular compartment. The same tendency was observed in lobular carcinomas (p<0.001, Student t-test) and in G3 ductal invasive carcinoma (p<0.05, Student t-test).

Zheng WQ et al. [22] had compared CathD expression in a range of benign and malignant breast lesions. In benign lesions, neither epithelial nor stromal cells in fibrocystic lesions and fibroadenomas were CathD positive, but a weakness in moderating positivity was observed within myoepithelial cells in mammary ducts. Out of the invasive ductal carcinomas, 61.5% showed stromal cell CathD-positivity, whereas 48.9% expressed CathD-positivity in neoplastic cells. Only 15% of intraductal carcinomas were CathD positive and expression was limited to neoplastic cells.

Over expressed CathD areas are mostly located in breast cancer tissue and not in tumor fibroblasts, as shown by immunohistochemistry [18] and RNA in situ hybridization [16]. Zheng WQ et al. [22] observed a significant correlation between neoplastic cell and stromal CathD positivity. The prevalence of CathD positivity in both neoplastic and stromal cell components were significantly higher (p<0.05 and p<0.01, respectively) in histological grade III tumors compared to grades I and II carcinomas. CathD expression by either neoplastic or stromal cells did not show significant correlation with patient’s age and tumor size. Although macrophages present in the surrounding tumor tissue also produce this enzyme, they do not seem to explain such a high CathD concentration [18]. Wolf M et al. [23] observed de novo synthesis of CathD in macrophages and possibly fibroblasts within the stroma of breast cancers, and occasionally in breast cancer cells. It is suggested that the release of CathD by tumor stromal cells, such as macrophages and fibroblasts, represents a significant autocrine and paracrine regulatory loop able to attenuate cell migration events through the disruption of a chemokine gradient in the extra cellular matrix.

According to the degree of invasive breast carcinomas, Fernandez-Aguilar S and Noël JC [24] showed that CathD expression at both stromal and epithelial level was similar in tubular carcinoma and G1 breast cancers, but lower than in G2 and G3 patients at a stromal level.

Quantification of IOD integrates better both the area and the average pixel intensity of the signal, being currently one of the most utilized parameter in the characterization of the signal intensities in image analysis. Therefore, using a common RGB signal masking procedure, IOD as the measure of the
area/intensity of the signal, and a batch-type of image processing, we consider that our method is prone to less subjective-related biases, and thus more accurate and constant than other methods employed by other authors.

According to *American Society of Clinical Oncology*, the present data are insufficient to recommend use of CathD measurements for management of patients with breast cancer [3]. Generally, the Committee has found that studies of CathD measured by IHC are variable, with no assay standardization and inconsistent associations with outcome, and, again, with little regard to the confounding effects of systemic therapy.

Nevertheless, a Dutch study on 2810 patients between 1978 and 1992 provides the result that over expression of CathD in primary breast cancer is an independent prognostic parameter correlated with the incidence of clinical metastases and shorter survival times, thus confirming it as a marker of aggressiveness [25]. In this large data set, Foeckens JA *et al.* [25] showed that CathD expression was not associated with tumor grade, but was correlated with ER, PR, menopausal, node status as well as age and tumor size. Some of these associations were confirmed by other investigators reporting that CathD levels were considerably higher in large tumors (pT2-4) than in smaller ones (pT1) as well as in node-positive than in node-negative breast tumors [26]. According to these results, we found that the group of patients with metastases from G3 ductal invasive carcinoma-type had a stronger expression in the cellular compartment than the group of patients without identifiable metastases.

In addition, Aziz S *et al.* [27] ascertained that there are no associations between axillary lymph node metastasis and CathD-positivity. On the other hand, some studies have indicated that increased levels of CathD in node-negative breast cancer patients were able to predict a shorter disease-free interval and overall survival, independently of steroid receptors’ status, tumor size and histological grade [25, 28]. On the contrary, Fernö M *et al.* [29] reported prognostic importance of CathD only for node-positive breast cancer patients. In addition, Rodriguez J *et al.* [26] reported that CathD positivity showed no significant correlation with shorter disease-free interval and overall survival when determined by immunohistochemically means.

**Conclusions**

Our results proved that CathD expression was strongest in malignant than in benign breast disease, the positivity being present in both epithelial neoplastic and stromal cells. Our method of CathD quantification in breast lesions seems to be less subjective-related biases, and thus more accurate and constant than other methods employed. All together, although further studies will be needed to include CathD in the list of prognostic markers for breast cancers, we conclude that immunohistochemical detection of CathD could be a useful reporter in evaluating of breast cancer patients.

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**References**


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