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PRECLINICAL STUDY

Legumain expression as a prognostic factor in breast cancer patients

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Abstract Invasive tumor cells and their microenvironments are enriched with a broad spectrum of different proteases. Legumain, a novel asparaginyl endopeptidase, has been observed to be highly expressed in several types of solid tumors. However, there is no data available identifying the relationship of legumain expression and clinicopathologic or biological variables in invasive breast cancer. For the first time, the prevalence of legumain expression in invasive breast cancer (n = 432) and non-neoplastic breast tissues (n = 128) was investigated by immunohistochemistry. Three staining patterns were observed in the cytoplasm: diffuse positivity, tiny dots and vesicles. Whereas vesicular positivity in the majority of tumor cells was significantly correlated to an adverse outcome, cytoplasmic and dot-like staining showed no prognostic effect. Vesicular positivity was observed in 24% of carcinomas, but only in one case of non-neoplastic breast tissue (<1%; proliferative mastopathy). This staining pattern was found to be independent of other factors analysed as grading, nodal status or HER2 expression. Besides being of prognostic value, legumain might prove to be an important predictive factor in breast cancer, since its unique cleavage specificity is already used in prodrug activation strategies.

Keywords Legumain · Breast neoplasms · Immunohistochemistry · Prognosis

Jessica Gawenda and Frank Traub contributed equally to this study.

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Introduction

Legumain, an asparaginyl endopeptidase, is a member of the C13 family in the Merops database classification of peptidases, whereas all other lysosomal cysteine proteases identified to date, e.g. the cathepsins, are grouped in the C1 family [1]. All lysosomal endopeptidase known prior to the discovery of mammalian legumain show a broad action on proteins, so the strict specificity of legumain to asparagin bonds is striking [2].

Legumain has been proposed to activate the zymogene progelatinase A. This activated form plays an important role in the degradation of extracellular matrix [3]. Therefore, the overexpression of legumain has been shown to be combined with increased migration, invasion and metastasis in a mouse colon cancer model [4]. In human colorectal cancers, the overexpression of legumain tended to be related to a worse differentiation and was shown to be of prognostic relevance [5]. Legumain is highly expressed in macrophages, on the cell surfaces and in membraneous vesicles in the cytoplasm of solid tumors. However, in normal tissue (kidney, liver and spleen) only a limited quantity of legumain is detectable [6, 7].

Legumain specifically cleaves prothymosin alpha-1 by removing the N-terminal position [8]. In a previous study, we could demonstrate that the activated form (thymosin alpha-1) was associated with the estrogen receptor (ER) negative phenotype of breast cancer and differentiated between ER positive and negative invasive tumors [9]. Because of the importance of ER for prognostic and therapeutic issues in breast cancers, we hypothesized that the ability of legumain to activate thymosin alpha-1 may be of prognostic significance.

Consequently, we were interested in the expression of legumain in human breast cancers. Proteases are presumed to be of pivotal importance in breast cancer progression and have been demonstrated to be associated with an aggressive course of disease and metastasis.

The expression level of legumain and distribution of staining were evaluated by immunohistochemistry on a large number of primary invasive breast cancer specimens and histological normal breast epithelium. The findings were compared with the clinical outcome and other established breast cancer specific properties to reveal the prognostic significance, if any, of legumain expression.

Materials and methods

The characteristics of the carcinomas included in the study have been described before [10]. In brief, primary breast carcinomas treated at the Department of Gynecology and Obstetrics and diagnosed at the Institute of Pathology, Medizinische Hochschule Hannover, between 1978 and 1990 were retrieved from the archives for analysis. Only cases which fulfilled the following criteria were included: UICC-R0 resection, pM0 status, histologic confirmation of invasive breast carcinoma, lack of a history of radiation and/or chemotherapy or previous breast neoplasms or second malignancy, long-term follow-up data available and no perioperative lethality (survival longer than 2 months after surgery).

Thus, 432 cases qualified and were included in the study. Therapies applied depended on stage, surgery, year of treatment and individual decisions of the patients. The clinicopathological characteristics are summarized in Table 1. In addition, 128 samples of non-neoplastic breast tissues were investigated.

Tissue microarray (TMA) experiments were performed using a commercially available tissue microarray kit (MaxArray, Zymed, San Francisco, CA) as described before [11]. Overall, eight tumor TMA and three control TMA were constructed each containing 60 tissue cores. Sections of 2 μm were cut from the TMA in the same way as from any conventional paraffin block.

Immunohistochemistry

Sections were deparaffinized in xylene, rehydrated with a series of ethanol and epitope retrieval was carried out in a microwave oven (30 min; 100°C in 10 mM sodium citrate; pH 6.0) and transferred to Tris-buffered saline (TBS). Tissue peroxidase activity was quenched by incubation with 3% hydrogen peroxide for 10 min, followed by rinsing with Tris buffer and a repeated protein blocking. Sections were incubated with a primary antibody against legumain (goat antibody, R&D, Lot VCJ 015041) in a 1:25 dilution for 60 min. For detection, a standard ABC method was used (ZytoChem Plus HRP, Zymed, San Francisco, CA). Counterstaining was done with hemalaun.

Breast tumor sections known to stain positively for legumain were included in each run as positive control. All tumors had previously been characterized for the expression of ER, PR, p53, HER-2, and Ki-67 and these findings were included in the evaluation [10].

Evaluation

Three different staining patterns in tumor cells could be observed, either exclusively or in combination. First, a diffuse cytoplasmic positivity occurred in several carcinomas (Fig. 1). Second, tiny dots within the cytoplasm could be seen (Fig. 2), which varied in number, sometimes even forming small clusters. Third, vesicles could be detected in the cytoplasm, their size being at least 0.7 μm in diameter (Fig. 3). Nuclear or membrane-associated positivity was not observed.

Diffuse cytoplasmic staining was always homogenously detectable in the majority of tumor cells, varying only in staining intensity. Therefore, cases were considered to be either negative or positive and positivity was graded by staining intensity into weak, medium and strong.

Dot-like positivity and vesicles showed a strong staining intensity but varied in the number of positive tumor cells. Similar to a previous study [5], six different percentage groups were distinguished: 0, >0–<5, ≥5–<30, ≥30–<60, ≥60–<90 and ≥90%. Evaluation was carried out by two independent observers (JG, FT), without knowledge of any clinicopathological data.

In a preliminary statistical evaluation, the cytoplasmic staining and the dot-like staining did not exhibit any prognostic relevance or association with other clinicopathological parameters (detailed data not

Table 1 Patient stratification and legumain expression in relation to clinicopathologic and biological parameters

	Definition	*n*	Leg. low (%)	Leg. high (%)	*P*-value
Stage pT	1	172	79.1	20.9	n.s.
	2	208	74.5	25.5	
	3/4	52	71.2	28.8	
Stage pN	Negative	242	77.3	22.7	n.s.
	Positive	190	74.2	25.8	
Grade	1	49	79.6	20.4	n.s.
	2	219	75.3	24.7	
	3	128	76.6	23.4	
Menopausal	Pre	119	77.3	22.7	n.s.
	Post	313	75.4	24.6	
Ki-67	High (>25%)	146	76.0	24.0	n.s.
	Low	254	76.8	23.2	
ER	Pos. (>10%)	295	73.2	26.8	0.038
	Neg.	114	83.3	16.7	
PR	Pos. (>10%)	175	77.1	22.9	n.s.
	Neg.	233	75.5	24.5	
HER2	Pos.	91	78.0	22.0	n.s.
	Neg.	310	75.5	24.5	
P53	High (>30%)	136	77.2	22.8	n.s.
	Low	296	75.3	24.7	

shown). Therefore, all further studies focused on vesicle staining only.

Among the 432 cases evaluated for vesicles, there was a discordance rate in 5.38%. These cases were reexamined and a final score was determined by consensus on a multiheaded microscope. 55.5% were negative for the detection of vesicles in the cytoplasm. 1.6% were positive for up to 5% of tumor cells, and 8.7, 9.8, 11 and 13.3% were positive for the other groups defined above, respectively.

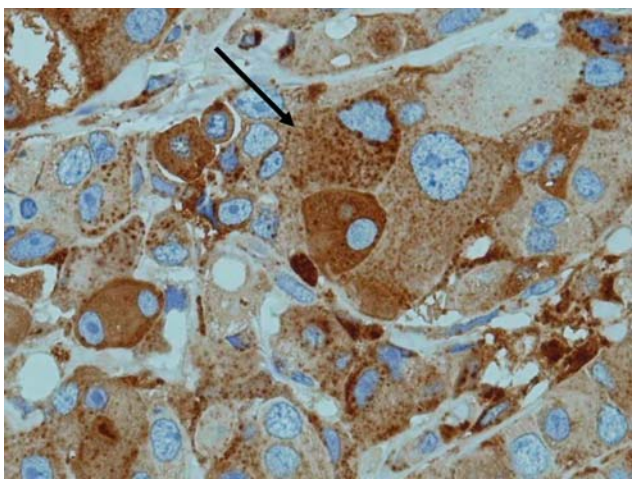

Fig. 1 Invasive ductal carcinoma expressing strongly legumain exhibited either a predominant diffuse cytoplasmatic pattern of expression as in this case or a vesicular pattern of decoration as shown in Fig. 3 and in a minority of tumor cells in this sample (arrow). The predominant diffuse pattern of immunolabelling was of no prognostic effect

For 378 (87.5%) cases long-term follow-up was available. A Kaplan–Meier analysis was performed and the graphic inspection of the results showed a dichotomous distribution of the groups: cases with less than 60% of tumor cells showing vesicles positive for legumain had a better clinical outcome and those with 60% or more a worse clinical outcome. According to this definition, two groups were discriminated: a low-risk group ($<60\%$) and a high-risk group ($\geq 60\%$) based on the legumain expression. Intra-observer agreement was 97.6% when 10% of cases were reviewed 6 months later, using the dichotomous distribution of the markers as described.

Statistical analysis

To investigate the association between the expression of legumain and the clinicopathological characteristics including oncogene and steroid receptors, data were cross-tabulated and Fisher's exact test was performed. The association of staining for legumain with patient outcome was evaluated using life tables constructed from survival data with Kaplan–Meier plots. Comparisons between groups were performed using the log-rank test. The analyses were carried out for all cases and also separately for nodal-negative (pN0) and nodal-positive cases (pN1 or higher). All statistical tests were performed using SPSS version 12.0 (SPSS Inc., Chicago, IL).

Results

Non-neoplastic breast tissue samples were either negative or showed a low-risk staining pattern for

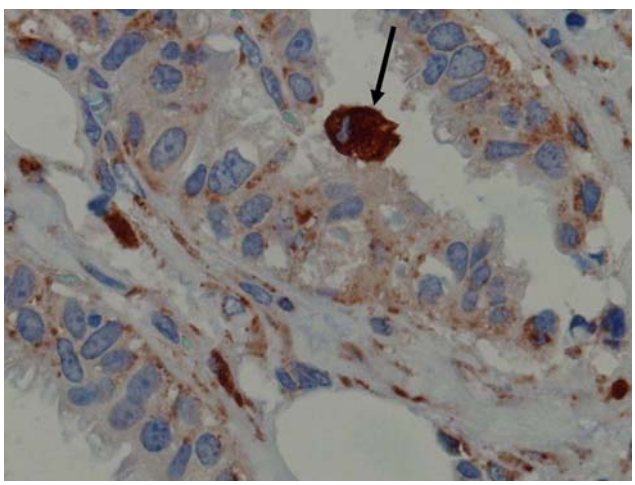

Fig. 2 Breast carcinoma with a few legumain-positive dots in the cytoplasm (low risk). Note the legumain-positive macrophage in the ductal lumen (arrow)

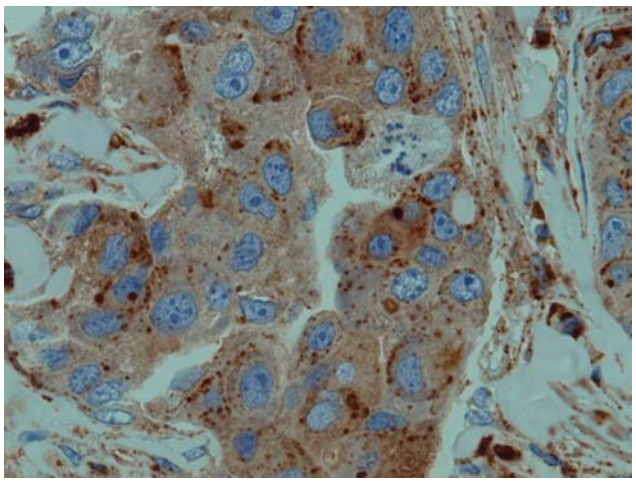

Fig. 3 Invasive breast cancer with legumain-positive vesicles in the cytoplasm of nearly all cells, indicating a worse clinical outcome (high risk)

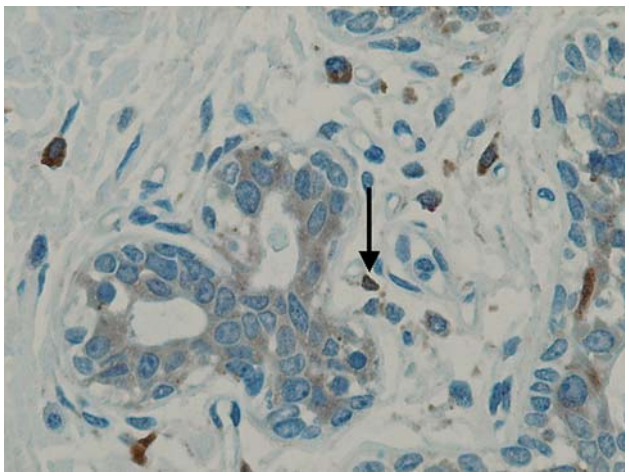

Fig. 4 Normal breast tissue did not reveal detectable legumain staining. Expression of legumain was restricted to macrophages (arrow)

legumain (127/128; 99.2%) (Fig. 4). Only one case (proliferative mastopathy) revealed a vesicular positivity in the majority of cells (high-risk profile).

In 24.3% of the carcinomas, legumain was detected in more than 60% of the tumor cells (Figs. 1 and 3). There was neither an association with pT- or pN-stage ($P > 0.05$) nor with other clinicopathological factors and markers for PR, Ki-67, p53 and HER-2. Only with ER status, there was a weak association, showing that cases with vesicular pattern of staining were more frequently positive for ER (two-sided: $P = 0.032$).

Other relevant prognostic factors in this series were provided by stage of the primary carcinoma, nodal status and histomorphological grading. The percentage of high and low expressing tumors and the association with established prognostic markers is summarized in Table 1.

Univariate Kaplan–Meier analyses

The observation of vesicular positivity for legumain in the majority of tumor cells (Figs. 3 and 4) was associated with an adverse outcome. Significant differences were observed for disease-free survival (DSF) when all patients were analysed ($P = 0.011$) (Fig. 5). A further subanalysis demonstrated that the difference was predominantly relevant in the subgroup of nodal-negative patients ($P = 0.003$). In contrast, cases positive for lymph-node metastasis revealed no significant prognostic effect of legumain overexpression ($P = 0.461$).

Cox regression analysis

Four clinicopathological factors (pT, pN, grading, menopausal status), in addition to six immunohistochemical marker proteins were included in a multivariate regression analysis (ER, PR, Ki-67, p53, HER-2, legumain). In the analysis of all cases, four factors were found to be of prognostic significance: tumor stage (pT; $P < 0.0001$; relative risk (RR) 1.797), nodal stage (pN; $P = 0.001$; RR 2.04), grading ($P = 0.013$; RR 1.532) and PR ($P = 0.006$; RR 0.551).

If only nodal-negative cases were selected for the Cox regression analyses, tumor stage (pT; $P < 0.0001$; RR 2.177), grading ($P = 0.003$; RR 2.478), Ki-67 ($P = 0.037$; RR 2.096) and vesicular positivity of legumain ($P = 0.027$; RR 2.197) were shown to be of prognostic significance.

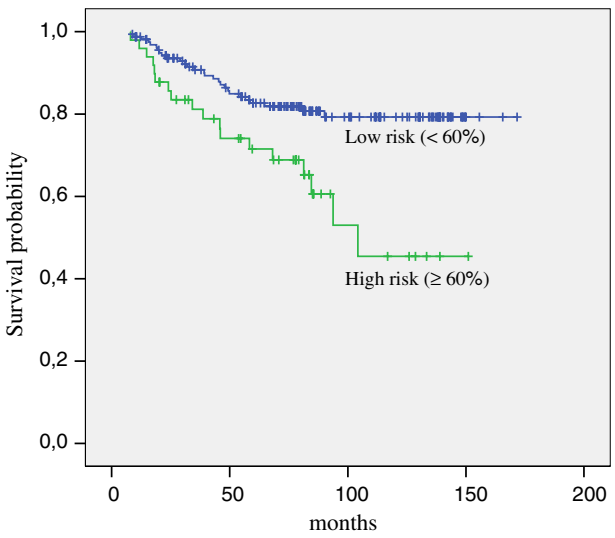

Fig. 5 Kaplan–Meier curve combining the staining intensity with the percentage of legumain expression in nodal-negative breast cancer patients in relation to disease-free survival

Among the nodal-positive cases, only two of the factors tested added significant prognostic information to the model: tumor stage (pT; $P = 0.001$; RR 1.612) and HER-2 ($P = 0.004$; RR 2.001).

Discussion

The identification of reliable prognostic and predictive factors of breast carcinoma beyond histomorphological staging and grading still remains of utmost importance. So far, the option to administer adjuvant therapy is predominantly based on traditional parameters such as the lymph node status and the hormone receptor status of the tumor. Many molecular markers with prognostic value have been proposed, most of them being surrogate markers of tumor differentiation or proliferation. Few predictive markers have been evaluated, offering the opportunity to apply specific molecular targeted therapies. Presently, the data available so far seem to be inconclusive or at least in part controversial (for review see: [12]).

To the best of our knowledge, this is the first study investigating the prevalence of legumain expression in breast carcinomas. Legumain expression in tumor cells was found to be enhanced when compared to non-neoplastic tissue and benign lesions. Furthermore, vesicular positivity of legumain was independent of all factors tested, except a weak association with ER expression. These observations are in agreement with findings reported from mouse models [4]. It is noteworthy that only the vesicular staining pattern was shown to carry prognostic information. In a recent study of colon cancer only the percentage of positive tumor cells were evaluated, but no information about the staining pattern was included [5]. A detailed analysis of our series demonstrated that the prognostic value of legumain is restricted to the node-negative subgroup, whereas in nodal-positive cases no prognostic differences were discernible.

So far, little is known about the pathways in which legumain is involved. A few studies have suggested that it might play a role in apoptosis or cell cycle regulation [4, 5, 13, 14]. In a mouse model, legumain overexpression was correlated to a more increased invasive growth and earlier metastasis [4]. Therefore, it has been hypothesized that legumain might play a role in tumor cell progression through processing of cysteine protease zymogens such as cathepsin B, D, H and L. Such effects could explain a diminished apoptosis rate, thereby enhancing tumor growth [15, 16]. In our previous study, we found a legumain activated substrate (thymosin alpha-1) and its occurrence could differentiate between ER positive and negative invasive breast cancers [9]. Based on these data, we hypothesized that the detection of thymosin alpha-1 could be a marker of the ER functionality. In our present series, however, ER-positive cases were positively associated with vesicular legumain positivity. To date, no data about the expression of legumain, prothymosin-alpha and thymosin alpha-1 are available. The unexpected association between steroid hormone receptor positivity and legumain expression may be due to yet unknown patterns of subcellular localization and activation of the enzyme. In addition, it cannot be excluded that other factors besides legumain play a role in the cleavage of prothymosin alpha-1. It seems interesting, therefore, to further study the correlations between thymosin alpha-1 levels and the activity of legumain in breast neoplasms.

Interestingly, legumain also turned out as a potential predictive marker. First reports have shown that a prodrug activation by legumain is highly effective. The functional capacity of tumor cell-associated legumain was explored based on the asparaginyl-specific endopeptidase activity of the enzyme [4, 17]. Therefore, modified doxorubicin (legubicin) was synthesized by adding an asparaginyl endopeptidase substrate peptide. Legubicin was very well tolerated in an in vivo model with much reduced toxicity compared with doxorubicin. Legubicin administration produced profound tumor cell apoptosis on the one hand and, on the other hand, in cells with a physiological legumain concentration such as kidney and liver, no injury was evident. Furthermore, growth arrest occurred after legubicin administration in a variety of neoplasms, including multidrug-resistant tumors in vivo [17]. Immunohistochemistry could be used as a screening method to identify those breast cancer patients who are most likely to benefit from this new therapeutic approach. Drugs like legubicin using the unique capability and the accumulation of legumain may provide a highly specific target-oriented therapeutic approach and may improve cancer therapy.

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