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Expression of Sialyl Lewis X, Sialyl Lewis a, E-Cadherin and Cathepsin-D in Human Breast Cancer: Immunohistochemical Analysis in Mammary Carcinoma *In Situ*, Invasive Carcinomas and their Lymph Node Metastasis

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Abstract. *Objective: Breast cancer cells can invade and generate metastasis via either lymphatic or blood vessels. Sialyl Lewis X (SLe^X) and Sialyl Lewis a (SLe^a) are carbohydrate molecules that mediate the adhesion between tumor cells and the endothelium. These antigens are not expressed on normal breast tissue. Overexpression of SLe^X and SLe^a is combined with poor prognosis and malignant relapse. E-cadherin mediates tumor cell-tumor cell adhesion. Partial or complete loss of E-cadherin expression has also been found to correlate with poor prognosis in breast cancer patients. Another factor involved in metastasis is Cathepsin-D, a lysosomal protease. Cathepsin-D plays a role in cell proliferation and inhibition of tumor cell adhesion. In this study, we analysed the combined expression of SLe^X, SLe^a, Cathepsin-D and E-cadherin in breast carcinoma in situ, invasive carcinomas without metastasis and invasive carcinomas with lymph node metastasis. Materials and Methods: Slides of paraffin-embedded tissue of carcinoma in situ (8 DCIS, 2 CLIS), invasive carcinomas without metastasis (9 ductal, 1 lobular) and carcinomas (7 ductal, 2 lobular, 1 mucinous) with their lymph node metastasis (10 cases) were used. Breast cancer tissue was fixed and incubated with monoclonal antibodies against SLe^X (IgM) and SLe^a (IgM), Cathepsin-D (IgG) and E-cadherin (IgG). Staining reaction was performed with the ABC reagent. The intensity of immunohistochemical reaction on digital images of the slides was analyzed using a computer-aided detection system. Results: We found a weak expression of both Sialyl Lewis antigens, a strong expression of E-cadherin and a moderate expression of Cathepsin-D in carcinoma in situ. The expression of both Sialyl Lewis antigens, E-cadherin and Cathepsin-D was moderate in invasive carcinomas without metastases. However, a strong expression of both Sialyl Lewis antigens and a very weak expression of E-cadherin was detected in primary carcinoma with lymph node metastases. The expression of Cathepsin-D was moderate in this tissue. E-cadherin expression was elevated whereas SLe^X and Cathepsin-D expression was reduced in lymph node metastases compared to the primary tumor. Conclusion: Combined analysis of tumor antigens involved in adhesion of breast cancer cells showed a negative correlation between Sialyl Lewis antigens and E-cadherin as the risk of metastasis progresses. Furthermore, there were significant differences of expression of the Sialyl Lewis antigens, E-cadherin and Cathepsin-D in primary breast cancer cells and their metastases. Evaluation of a panel of markers involved in cell adhesion could be a useful method for defining the metastatic risk in breast cancer patients.*

Metastasis in breast cancer depends on the coordinated expression of adhesion molecules to remodel cell-cell and/or cell-matrix attachments and of proteolytic enzymes to degrade the surrounding extracellular matrix (1). If breast cancer is detected at an early stage, the disease may be restricted solely to the breast. Yet, it may recur elsewhere when only local treatment is applied. Due to breast

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Key Words: Sialyl Lewis X, Sialyl Lewis a, E-cadherin, cathepsin-D, breast cancer.

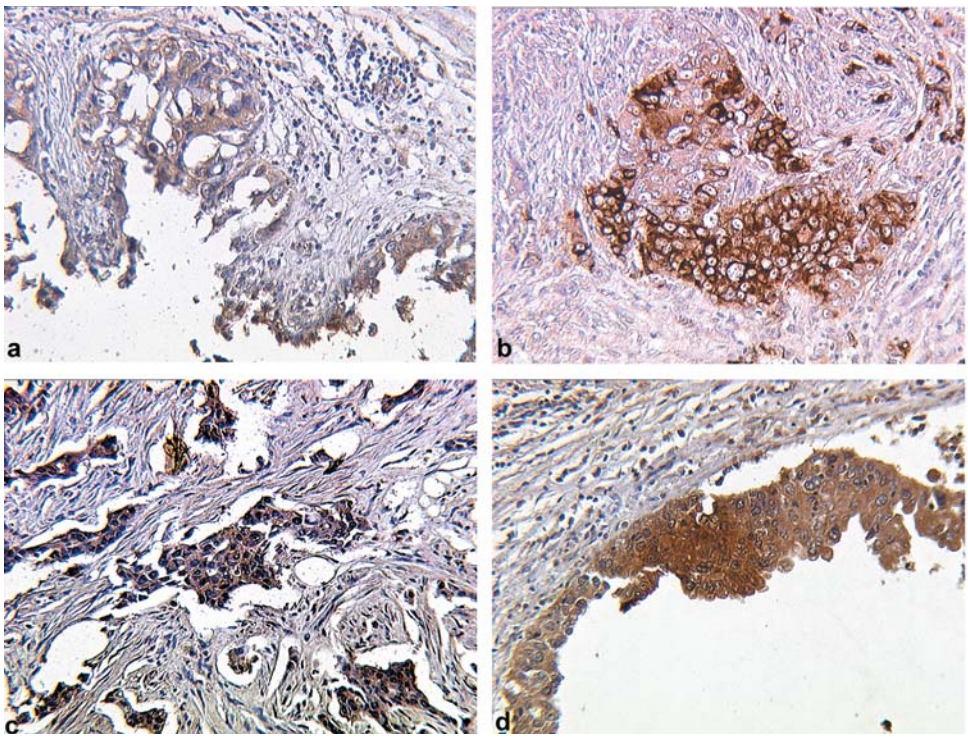

Figure 1. *Expression of Sialyl Lewis a (SLe^a) in carcinoma in situ (a), invasive carcinomas without metastasis (b) and invasive carcinomas (c) with their lymph node metastasis (d).*

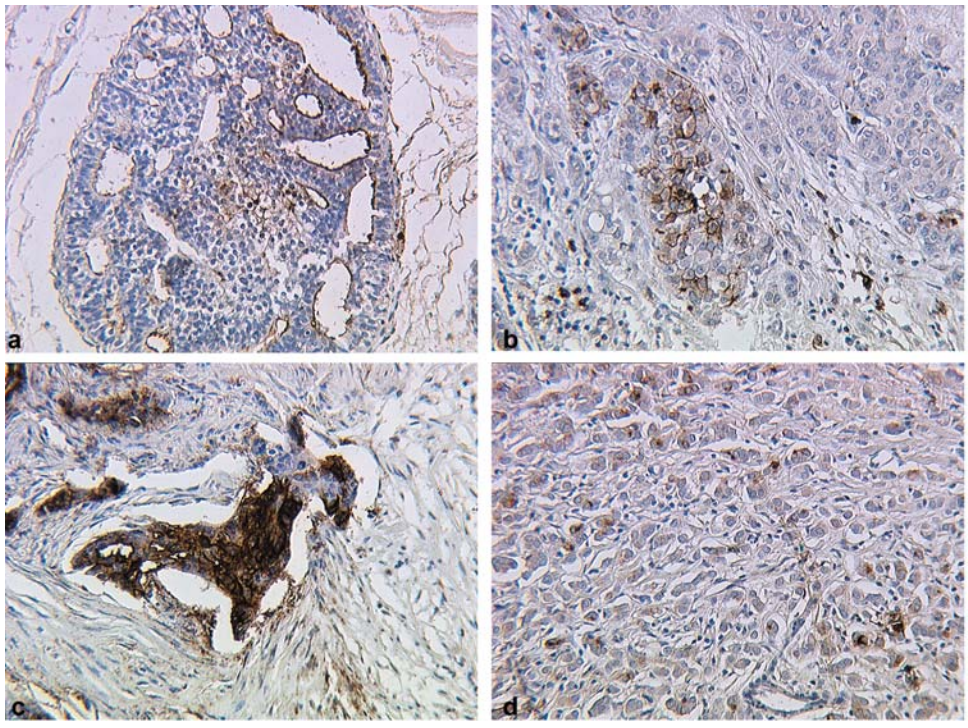

Figure 2. *Expression of Sialyl Lewis X (SLe^X) in carcinoma in situ (a), invasive carcinomas without metastasis (b) and invasive carcinomas (c) with their lymph node metastasis (d).*

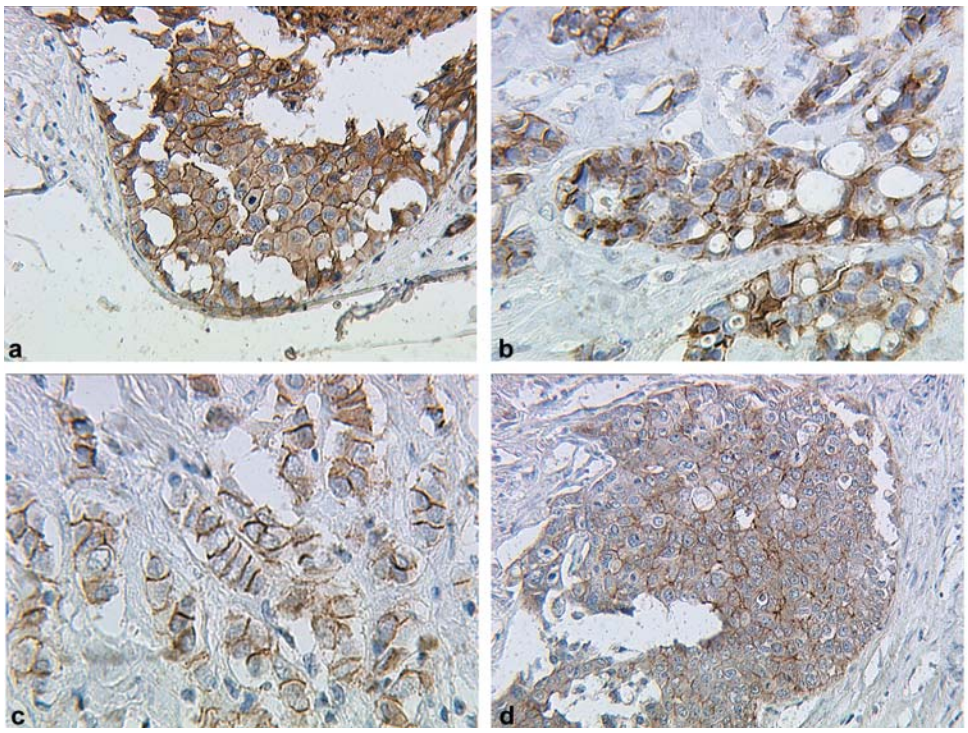

Figure 3. *Expression of E-cadherin in carcinoma in situ (a), invasive carcinomas without metastasis (b) and invasive carcinomas (c) with their lymph node metastasis (d).*

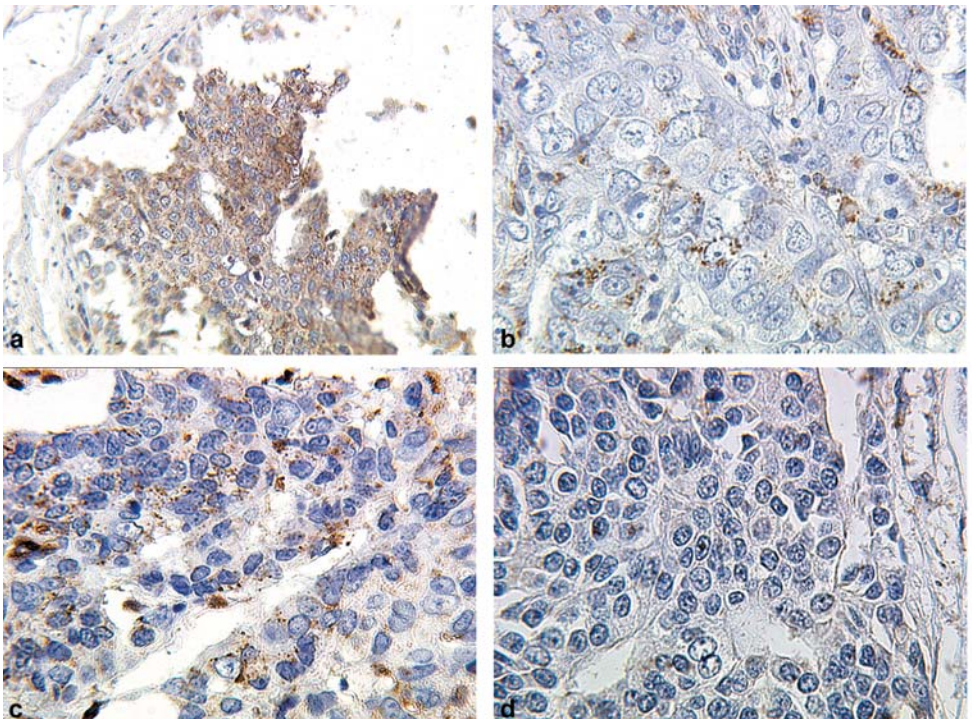

Figure 4. *Expression of Cathepsin-D in carcinoma in situ (a), invasive carcinomas without metastasis (b) and invasive carcinomas (c) with their lymph node metastasis (d).*

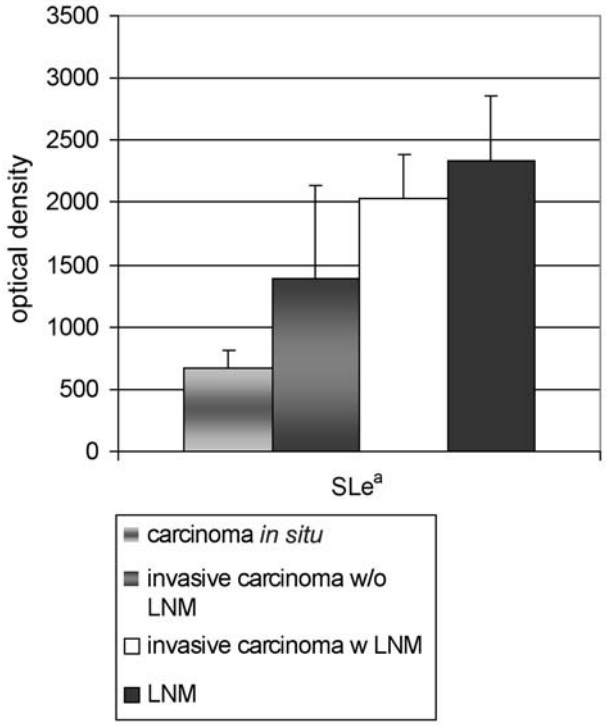

Figure 1e. *Staining intensity of SLe^a in breast cancer subtypes determined by computerised analysis of the immunohistochemical reaction on the different tissue slides.*

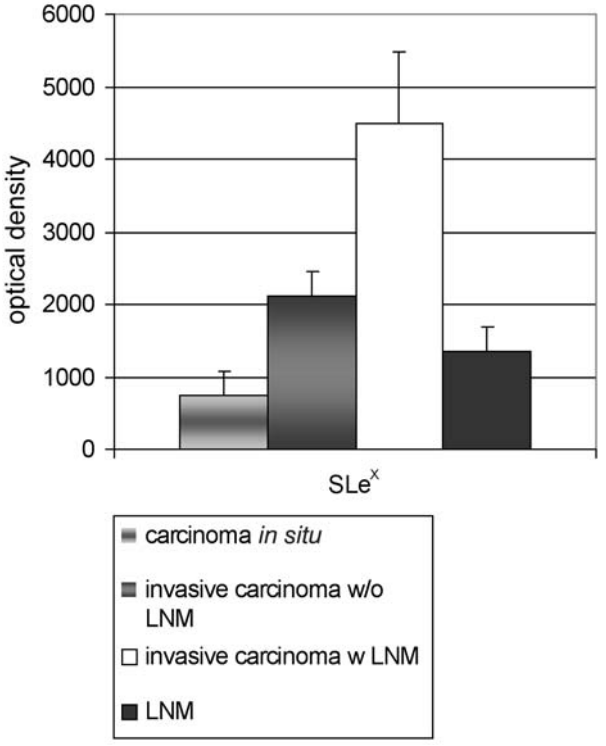

Figure 2e. *Staining intensity of SLe^X in breast cancer subtypes determined by computerised analysis of the immunohistochemical reaction on the different tissue slides.*

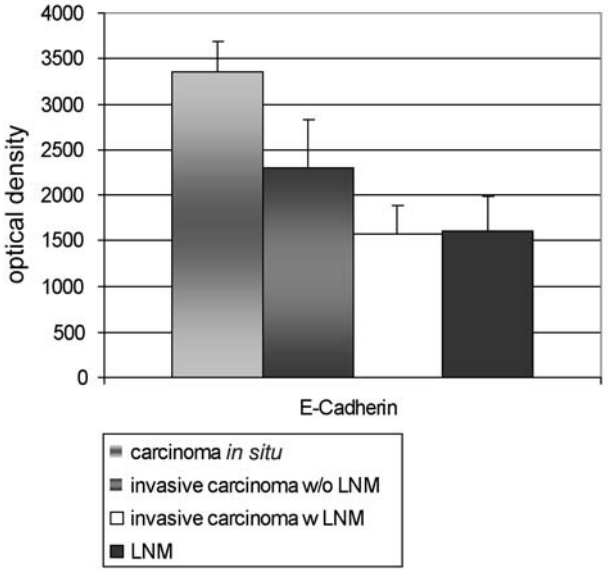

Figure 3e. *Staining intensity of E-cadherin in breast cancer subtypes determined by computerised analysis of the immunohistochemical reaction on the different tissue slides.*

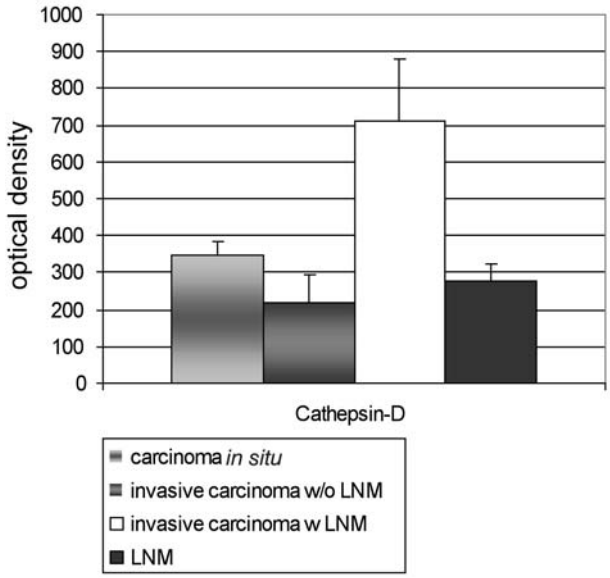

Figure 4e. *Staining intensity of Cathepsin D in breast cancer subtypes determined by computerised analysis of the immunohistochemical reaction on the different tissue slides.*

Table I. *Antibodies used in the study.*

Antigen	Antibody	Isotype	Concentration/ dilution	Source
Sialyl Lewis a	KM231	Mouse IgM	1 μg/ml	Calbiochem
Sialyl Lewis x	KM93	Mouse IgM	1 μg/ml	Calbiochem
E-cadherin	HECD-1	Mouse IgG	1 μg/ml	Calbiochem
Cathepsin-D	C 5	Mouse IgG	1 μg/ml	Dianova

screening programs and greater public awareness, 60% to 70% of all new cases of breast cancer appear without axillary lymph node involvement (2). The recurrence rate in node-negative breast cancer patients was reported to be 25% to 30% (3-8). It is possible to further reduce it through the use of systemic adjuvant therapy (9, 10). However, such therapy is usually associated with attendant side-effects such as reduced quality of life and increased morbidity and mortality. To date, few valid parameters have been established to identify node-negative patients who may benefit from systemic adjuvant therapy. For this reason, current recommendations for adjuvant treatment include all patients except those with a tumor size ≤1.0 cm, grade 1 tumor, positive hormone receptor status and age of more than 35 years (11).

Lymph node involvement or confirmation of distant metastases in breast cancer always necessitates systemic treatment. Because the expression of adhesion-related antigens on tumor cells is a strong indicator of the tumor's ability to metastasize, many authors have tried to determine these antigens in lymph nodes. However, simultaneous examination of a panel of adhesion molecules on carcinoma *in situ*, invasive carcinomas without metastasis and tumors with lymph node metastasis has not yet been reported.

The present study was designed to address the role of the adhesion-involved antigens Sialyl Lewis X (SLe^X) and Sialyl Lewis a (SLe^a), E-cadherin and Cathepsin-D in breast carcinoma *in situ*, invasive carcinomas without metastasis and carcinomas with lymph node metastasis.

Materials and Methods

Tissue samples. Tumor tissues were obtained from 30 patients during surgery for breast cancer. Pathological classification: 10 patients with carcinoma *in situ* (8 DCIS, 2 CLIS), invasive carcinomas without lymph node metastasis (9 ductal, 1 lobular) and invasive carcinomas (7 ductal, 2 lobular, 1 mucinous) with their lymph node metastasis (10 cases).

Immunohistochemistry. Immunohistochemistry was performed on paraffin sections (7 μm) of the breast cancer tissue specimens, as described previously (12). Briefly, sections were incubated in methanol/H_2O_2 (30 min) to inhibit endogenous peroxidase activity, washed in phosphate-buffered saline (PBS) (5 min) and treated with goat serum (20 min, 22˚C) to reduce non-specific background staining. Incubation with the primary antibody (Table I) was done overnight at 4˚C. Sections were then thoroughly incubated with the biotinylated secondary anti-mouse antibody (1h, 22˚C) and avidin-biotinylated peroxidase (45 min, room temperature). Between each step, sections were washed with PBS (pH 7.4). Peroxidase staining reaction was done with diaminobenzidine/H_2O_2 (1 mg/ml; 5 min) and stopped in tap water (10 min). Sections were counterstained in hemalaun (1 min) and then cover-slipped. In controls, the primary antibody was replaced with pre-immune mouse serum with positive and negative controls being included. All specimens were evaluated by a pathologist with experience in immunohistochemistry. The classification of the stained cells as either micrometastases or tumor cells also required histomorphological characterisation. Unspecific reactions or staining of endothelial cells in the marginal sinus of lymph nodes were not considered to be related to tumor cells.

Computerised analysis of antigen expression. The level of antigen expression was determined in a blinded fashion in one run with identical staff, equipment and chemicals. From each section, 5 digital pictures were taken at random of different places of the tumor tissue (200-fold magnification; 3CCD color camera; Hitachi HV-C20M; Hitachi Denshi Ltd, Japan, and Axiolab, Carl Zeiss, Germany). For standardization of the measurement in each picture, the optical density of the white background color was attuned to 250. For all sections, we assessed the mean optical density and the quantity of pixels there had a positive reaction for Gd using the KSRun software (imaging system KS400, release 3.0; Zeiss, Vision GmbH, Germany).

Statistical methods. The SPSS/PC software package, version 6.01 (SPSS Inc., Chicago, IL, USA), was used for collection, processing and statistical analysis of all data. All p values resulted from two-sided statistical tests and $p \leq 0.05$ was considered to be significant.

Results

Expression of SLe^a in breast cancer tissue. We found a weak expression of SLe^a in tissue slides of carcinoma *in situ* with 90% of the tumor cells being positive for this antigen (Figure 1a). Invasive carcinoma cells without metastasis showed an elevated expression of SLe^a compared to carcinoma *in situ* (Figure 1b). We identified expression of this antigen in 80% of the tumor cells investigated. The highest expression of SLe^a was identified in tumor cells with lymph node metastasis (LNM) (Figure 1c). We found an expression of this antigen in all cases investigated. The expression of SLe^a in LNM (Figure 1d) was even higher than in the primary tumor. Computerised analysis of the staining intensity (optical density) is summarized in Figure 1e. We identified significant differences of SLe^a staining between carcinoma *in situ* and invasive carcinoma with LNM ($p=0.018$) and carcinoma *in situ* and LNM ($p=0.017$).

Expression of SLe^X in breast cancer tissue. A similar expression pattern of SLe^X could be identified on the tumor panel compared to the SLe^a expression. Carcinoma *in situ* showed a weak and focal expression of SLe^X in all cases investigated (Figure 2a). Invasive carcinoma cells without metastasis showed an elevated expression of SLe^X compared to carcinoma *in situ* (Figure 2b) in 80% of all cases. The expression of SLe^X was highest in invasive carcinoma that had already metastasized to the axillary lymph nodes (Figure 2c). In this type of breast cancer, 90% of all cases showed SLe^X expression. However, expression of SLe^X was reduced in LNM compared to the primary tumor. Computerised analysis of the staining intensity is

summarized in Figure 2e. We identified significant differences of SLe^X staining between carcinoma *in situ* and invasive carcinoma without LNM ($p=0.043$), carcinoma *in situ* and invasive carcinoma with LNM ($p=0.018$). Differences of SLe^X expression were also significant between LNM and its primary tumor ($p=0.047$).

Expression of E-cadherin in breast cancer tissue. We found a strong expression of E-cadherin in carcinoma *in situ* in all cases investigated (Figure 3a). A median expression was detected in invasive tumor cells without metastasis in 80% of all cases investigated (Figure 3b). Expression of E-cadherin was down-regulated in invasive tumor cells with LNM in 90% of all cases (Figure 3c) and in the LNM itself (Figure 3d). Computerised analysis of staining intensity is summarized in Figure 3e. We identified significant differences of E-cadherin staining between carcinoma *in situ* and invasive carcinoma with LNM ($p=0.043$), and carcinoma *in situ* and LNM ($p=0.018$).

Expression of Cathepsin-D in breast cancer tissue. A median expression of Cathepsin-D was investigated in carcinoma *in situ* cells in all cases investigated (Figure 4a). We found a decreased expression of Cathepsin-D in invasive tumor cells without metastasis in 90% of all cases (Figure 4b). Most intense but focal expression of Cathepsin-D was found in primary tumor tissue with LNM (Figure 4c), whereas the corresponding LNM showed only a weak expression of Cathepsin-D (Figure 4d). Computerised analysis of staining intensity is summarized in Figure 4e. We identified significant differences of Cathepsin-D staining between invasive carcinoma without and invasive carcinoma with LNM ($p=0.017$), and invasive carcinoma and its LNM ($p=0.008$).

Discussion

In this study, we analysed the combined expression of SLe^a, SLe^X, E-cadherin and Cathepsin-D in carcinoma *in situ*, invasive carcinomas without metastasis and invasive carcinomas with their corresponding lymph node metastasis in breast cancer tissue. Tumor cell dissemination and development of metastases is an active biological process involving complex interaction between cancer cells, extracellular matrix, the vascular system, the immune system and the target organ (13). Adhesion molecules play a major role in this process. Adhesion can be divided into reduced adhesion of the tumor cells itself and increased adhesion of floating tumor cells on the vascular endothelial tissue. The Sialyl Lewis antigens play a major role as ligands on tumor cells that are involved in interactions with E-selectin on endothelial cells. This interaction is the first step of building metastases by floating tumor cells sticking to the endothelium (14). Our results show that increasing expression of SLe^a is correlated with higher tumor stage. Carcinoma *in situ* showed only a weak expression of SLe^a. The highest expression was observed in carcinoma cells that were identified in the lymph node itself. A similar pattern was observed for the SLe^X expression. Carcinoma *in situ* showed only a weak expression of SLe^X. The highest expression was observed in metastasized carcinoma cells. A striking difference to the SLe^a expression was the decrease of SLe^X expression in LNM compared to the primary tumor. A reason for the decreased expression especially of SLe^X in LNM could be that natural killer (NK) cells attack tumor cells expressing high levels of SLe^X (15). Although it has been shown that NK cells can mediate cytolysis of tumor cells (16), Ohyama *et al.* (15) demonstrated that a particular carbohydrate structure is recognized by NK cells as a cytolytic signal. The CD94 receptor complex on NK cells recognizes SLe^X overexpression on tumor cells. The reduced expression of SLe^X especially in the lymph nodes could be an escape of the tumor cells from the NK cell attack because the concentration of NK cells in the lymph nodes is higher compared to the primary tumor. E-cadherin is a glycoprotein with an extracellular domain that interacts with E-cadherin molecules on adjacent cells, thereby establishing adhesion between the epithelium. The intracellular domain is associated with a complex of proteins called catenins, which anchor E-cadherin to the actin cytoskeleton (17). In various carcinomas, plasma membrane-associated E-cadherin protein expression is decreased or even absent. E-cadherin is a potent invasion/tumor suppressor of breast cancer. Consistent with this role in breast cancer progression, partial or complete loss of E-cadherin expression has been found to correlate with poor prognosis in breast cancer patients (18). Our results support these findings. We found a strong expression of E-cadherin in carcinoma *in situ* in all cases investigated and a median expression in invasive tumor cells without metastasis in 80% of all cases investigated. The expression of E-cadherin was down-regulated in invasive tumor cells with LNM in 90% of all cases, while in 10% of all cases, E-cadherin expression was negative. In the LNM, we also identified a down-regulated E-cadherin expression which was increased not significantly compared to the primary tumor. Compared to the Sialyl Lewis antigens, there seems to be a negative correlation. Alpaugh *et al.* (14, 19) have described a cooperative role of E-cadherin and Sialyl Lewis a and X in tumor metastasis. Both types of adhesion molecules seem to be involved in metastasis but in an opposing role. Enhanced Sialyl Lewis a and X expression promotes adhesion of metastasis and low E-cadherin expression reduces adhesion of the primary tumor and enhances formation of disseminated tumor emboli cells.

We also analysed the expression of Cathepsin-D. Cathepsin-D is a prognostic marker in breast cancer.

Cathepsin-D increases tumor cell proliferation *in vitro* and the metastatic potential of the tumor. The mechanism by which Cathepsin-D increases the incidence of clinical metastasis involves increased cell growth and decreased contact inhibition rather than escape of cancer cells through the basement membrane. Cathepsin-D could act as a protease following its activation at an acidic pH, or as a ligand of different membrane receptors at a neutral pH. The nature of the mechanisms involved *in vivo* may depend on the microenvironment of the tumor cells (20). Overexpression of Cathepsin-D is associated with increased risk of relapse and metastasis (21). Our previous investigations showed that Cathepsin-D is expressed in normal and hyperplastic endometrial tissue. A continuous increase in Cathepsin-D expression was observed in adenomatous hyperplasia (AH), with a significant difference between AH grade I and III. Since AH grade III can be considered as a precursor of endometrial cancer, Cathepsin-D could be a possible parameter for assessing malignant transformation (22). In this study, we showed that the most intense but focal expression of Cathepsin-D was identified in primary tumor cell tissue with LNM. Surprisingly, expression of Cathepsin-D was significantly reduced in the LNM compared to the primary tumor, indicating a different cancer cell population in the lymph nodes compared to the primary tumor. This can be explained by our previous findings (12) in which we describe changes of tumor cell characteristics during the process of metastases. This is reflected by the heterogeneity of the expressed antigens and enzymes.

In addition to adhesion molecules, metastasis of breast cancer is also dependent on the action of proteolytic enzymes to degrade the surrounding extracellular matrix. The tumor cell-associated urokinase-type plasminogen activator system, consisting of the serine protease uPA, its substrate plasminogen, its membrane-bound receptor uPAR, as well as its inhibitors PAI-1 and PAI-2, play an important role in these pericellular process (23).

In summary, we performed an analysis of tumor antigens involved in adhesion of breast cancer cells. We identified a negative correlation between Sialyl Lewis antigens and E-cadherin as the risk of metastasis progresses. Furthermore, there were significant differences between the expression of the Sialyl Lewis antigens, E-cadherin and Cathepsin-D in primary breast cancer cells and their metastases. Evaluation of a panel of tumor markers involved in cell adhesion could be a useful method for defining the metastatic risk in breast cancer patients.

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