Diffuse Growth Pattern Affects E-Cadherin Expression in Invasive Breast Cancer

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Abstract. We investigated the correlations between growth patterns and E-cadherin expression by immunohistochemistry and the presence of mutations of exons 6-10 of the E-cadherin gene by PCR-SSCP, in 79 cases of invasive lobular and ductal breast cancer. E-cadherin expression showed a tendency to be lower in lobular than in ductal carcinomas (p=0.064). In 60% of lobular carcinomas the diffuse growth pattern and in 72% of ductal carcinomas the compact growth pattern predominated. E-cadherin expression was significantly lower in diffuse than in compact tumor area (p<0.001) and not related to carcinoma type when it was considered in tumor areas with either diffuse (p=0.278) or compact (p=0.128) growth pattern. No mutations were detected. In conclusion, loss of E-cadherin expression is related to an increase of diffuse growth pattern in both lobular and ductal types of breast cancer, and the differential proportions of growth patterns in both tumor types cause the tendency for lower E-cadherin expression in the lobular type.

Alteration of cell-to-cell adhesion systems is a major event in tumor development and progression. The functional units of cell adhesion are typically multiprotein complexes (1). One of these is E-cadherin, which is a transmembrane protein with 5 tandemly-repeated extracellular domains and a cytoplasmic domain that connects to the actin skeleton through a complex with alpha, beta and gamma catenins (2, 3). E-cadherin has been implicated in numerous cellular functions, ranging from controlling morphogenesis to suppressing tumor invasion (4). In humans, loss of E-cadherin function is associated with increased proliferation, de-differentiation, invasion and metastasis in a wide variety of tumors (5-7).

In breast cancers, inactivation and decreased expression of E-cadherin have frequently been observed in invasive lobular carcinomas and high-grade tumors, respectively (8-13). Also correlations of reduced E-cadherin expression have been reported with presence of lymph node metastases (11) and poor outcome (11, 13, 14). Both mutations and epigenetic factors may affect E-cadherin expression in breast cancer (8, 15-20). Mutations of the E-cadherin gene in invasive breast cancer have been detected first in the lobular type (8, 18, 19). These mutations affected preferentially exons 2, 3, 6-10, 12 and 13 (8, 18). Most mutations were found in exons 6-10 (8).

The relationship of E-cadherin expression with growth patterns (diffuse versus compact) in invasive breast cancer is unknown. Invasive lobular breast carcinoma, which is characterized by an extensive diffuse invasion mode, has been found to exhibit rather low levels of E-cadherin expression when compared to invasive ductal carcinoma, which has a tendency for more extensive compact growth (10). However, it is unclear as to whether the tendency for lobular and ductal type-related differences of E-cadherin expression reflect variations of growth patterns. We investigated E-cadherin expression by immunohistochemistry and determined the extent of diffuse and compact growth pattern in 79 cases of invasive lobular and ductal breast cancer. We also studied the mutational status of the most frequently mutated exons (6-10) of the E-cadherin gene by PCR-SSCP and their relationship to E-cadherin expression.

Materials and Methods

Tissue samples. Tissue specimens from 79 primary infiltrating breast carcinomas of untreated patients were included in this study. The tumor tissues were received within 30 minutes after surgical removal, were immediately snap-frozen in liquid nitrogen and stored at -70 °C. Routine histological examination was performed.
on parallel samples by formalin fixation, paraffin embedding and staining with hematoxylin and eosin. Conventional histological classification schemes were applied (21).

Grading was performed according to Elston and Ellis (22). Histological examination also included determination of the percentage of the tumor specimen with compact and diffuse growth patterns in each specimen. A compact growth pattern was defined as the presence of coherent tumor cell complexes and diffuse growth pattern as the presence of single tumor cells surrounded by stromal tissue.

Immunohistochemistry. For monitoring E-cadherin expression in routine paraffin-embedded tissue samples, 3- to 4-μm-thick sections were mounted on glass slides coated with poly-L-lysine. After deparaffination and rehydration, the slides were immersed in 10 mM sodium citrate buffer (pH6) and heated five times for 5 minutes in a microwave oven at 600 W. Endogenous peroxidase was blocked with 0.6% H2O2 in 40% methanol-PBS for 30 minutes. After pretreatment with 10% horse serum in PBS, the tissue sections were incubated with monoclonal antibody 5H9 (Progen, Heidelberg, Germany) at a final concentration of 1 μg/ml at 37°C for 120 minutes. Bound antibody was detected using the avidin-biotin-complex (ABC) peroxidase method (ABC Elite Kit, Vector, Burlingame, CA, USA). Non-specific staining was blocked by adding 2% skim milk to the horse serum and all subsequent incubations. Immunohistochemical staining was performed with 3,3'-diaminobenzidine and 0.01% H2O2 for nuclear counterstaining hematoxylin was used. In negative controls the primary antibody was replaced by PBS.

The results of immunohistochemical reactions were assessed for each of the 79 carcinomas separately in areas with compact (c) and diffuse (d) growth patterns. The E-cadherin expression was evaluated first on the basis of intensity of immunostaining of tumor cells (0=no, 1=weak, 2=medium or 3=strong) and second on the evaluated first on the basis of intensity of immunostaining of tumor diffuse (d) growth patterns. The E-cadherin expression was first separately calculated for compact and diffuse patterns (denoted as IHc and IHd) according to the following formula:

\[ IHc = 1*p_{c}(1) + 2*p_{c}(2) + 3*p_{c}(3) \]
\[ IHd = 1*p_{d}(1) + 2*p_{d}(2) + 3*p_{d}(3) \]

The overall E-cadherin expression for each tumor was calculated as a weighted-sum of immunostaining of both growth patterns according to the following formula:

\[ IH = IHc \times pc + IHd \times pd \in [0,300] \]

The E-cadherin expression for each growth pattern was categorized as weak (IHc,d-score=1), when IH was 0-100, as medium (IHc,d-score=2), when IH was 101-200 and as strong (IHc,d-score=3) when IH ranged between 201 and 300.

The mean percental areas of tumor cells with weak, medium and strong staining intensity were calculated separately for ductal and lobular breast carcinomas, all studied cases of breast carcinomas, areas of ductal and lobular breast carcinomas with compact and diffuse growth patterns and areas with compact and diffuse growth patterns of all studied breast carcinomas. Differences of E-cadherin expression (expressed as the IH-score) between ductal and lobular carcinomas were compared by using the IHc,d-scores. The statistical method therefore was the rank-version of the t-test for dependent samples. Areas of ductal and lobular breast carcinomas within compact and diffuse growth patterns were also compared by taking the IHc and IHd-scores, again using the Wilcoxon-Mann-Whitney test.

DNA extraction and PCR-SSCP analysis. Breast tumor DNA was isolated from frozen tissue samples with the QIAmp DNA Kit (Qiagen, Valencia, CA, USA) according to the recommendations of the manufacturer. The DNA concentration was determined using a gel electrophoresis with 1% agarose and ethidium bromide.

Primers used for PCR amplification were the same as applied previously (8). Genomic DNA was used at 80 ng per 25 μl reaction mixture containing 2 mM (exon 7, 8, 9, 10) or 3 mM (exon 6) MgCl2, 0.5 mM dNTP, 1 mMol of each primer and 1.25 U Taq polymerase. Each PCR was overlaid with mineral oil and, after an initial incubation at 95°C for 2 minutes, PCR was performed for 35 cycles. Annealing temperatures were 55°C (exons 5, 6 and 9) and 60°C (exons 7 and 10), respectively. For detection of sequence variations in the genomic E-cadherin amplicons, we performed single-strand conformation polymorphism (SSCP) and Southern blot analysis and sequenced the PCR products which showed abnormal mobility in the SSCP/Southern blot analysis as described previously (8).

Results

Regardless of histological subtype, the mean age of all 79 female patients with invasive breast cancer was 62 (range 26-92) years. The diameter of all tumors investigated

Figure 1. Median percentage of compact and diffuse growth patterns in 10 lobular and 69 ductal carcinomas.
averaged 2.8 cm. Nineteen tumors were classified as pT1, 51 as pT2, 1 as pT3 and 8 as pT4. The histological grade of malignancy was G1 in 7 tumors, G2 in 40 and G3 in 32 cases. The present series consisted of 10 invasive lobular carcinomas and 69 invasive ductal carcinomas including 2 medullary cancers, 3 tubular, 1 mucinous and 63 cases that were attributed to the category not otherwise specified.

Thereafter, the distribution of compact and diffuse growth pattern in both tumor types was evaluated (Figure 1). The median for the percentage of the compact growth pattern was 35% in lobular carcinomas and 80% in ductal carcinomas. In 6 out of 10 (60%) lobular carcinomas the diffuse growth pattern and in 50 out of 69 (72%) ductal carcinomas the compact growth pattern predominated.

**Immunohistochemistry.** First, overall E-cadherin expression was compared in both tumor types (Figure 2). Figure 3 shows the lobular and ductal tumor type for each IH-score. Half of the lobular carcinomas showed weak E-cadherin expression, while over half of the ductal carcinomas displayed strong E-cadherin expression. This disequilibrium was proved by the statistical comparison of the overall IH-score in both tumor types. The reduction of overall E-cadherin expression in lobular carcinomas when compared to ductal carcinomas, however, was not significant \((p=0.064)\). Even if there was no significance, the trend could be identified.

The main question which thus arose was: Is the differential distribution of compact and diffuse growth pattern in both tumor types the cause for the differences of the E-cadherin expression as seen in Figure 3? To answer this, we compared the E-cadherin expression in both growth patterns.

Taking the different growth patterns into consideration, in both ductal and lobular carcinoma, respectively, the mean
percentages of the areas corresponding to the different intensities are shown in Table I. In areas with compact growth pattern the E-cadherin expression was significantly increased when compared to the expression in tumor areas with diffuse growth pattern \((p<0.001)\). Nevertheless, within both the diffuse and the compact growth pattern, there was no significant difference of IH-score in lobular and ductal carcinomas (for diffuse \(p=0.278\), for compact \(p=0.127\)). That result underlines the fact that the different trends seen in Figure 3 were just caused by the different distribution of growth patterns in both tumor types (Figure 1), because between these growth patterns there was a highly significant difference of E-cadherin expression.

**PCR-SSCP and DNA sequencing.** SSCP analysis revealed, in two lobular and one ductal carcinomas, distinct additional bands within exon 6, 8 and 10, respectively. The affected ductal carcinoma exhibited an additional band within exon 6 between the two normal bands. One of the affected lobular carcinomas demonstrated an additional band within exon 8 closely under the two normal bands and the other lobular carcinoma closely above the two normal bands. DNA sequencing demonstrated a normal sequence of DNA bases within these exons.

**Discussion**

The pathways of reduced or lost E-cadherin expression in breast cancer are poorly understood (19). With respect to breast cancer, it is unclear as to whether E-cadherin expression or the mutational status of E-cadherin correspond to the mode of invasion. Therefore, the main result of the present study is that not the tumor type but the growth pattern is responsible for the reduced E-cadherin expression. The diffuse growth pattern displayed, irrespectively from lobular or ductal differentiation, a significantly reduced E-cadherin expression when compared to the compact growth pattern. Our finding is in accordance with previous studies on gastric cancer which yielded a correlation between diffuse growth pattern and reduction of E-cadherin expression (23). Similarly, immunohistochemical examinations have revealed reduced or heterogeneous expression of E-cadherin in poorly-differentiated carcinomas of the lung (24), liver (25), esophagus (26) and prostate (7), which frequently show weak intercellular adhesiveness. With respect to invasive ductal breast carcinoma, some relationship between reduced E-cadherin expression and low histological grade was also assessed (10, 27) but no association with growth pattern or morphology has been reported. The close correlation between loss of E-cadherin expression and diffuse growth pattern in breast cancer, assessed in our study, suggests a pivotal role of E-cadherin for adherence of the tumor cells to each other. Reduced adhesion of tumor cells characterizes the invasive phenotype (28). Interestingly, loss of E-cadherin expression has been found to be a strong prognostic indicator independent of progesterone receptor status in invasive breast cancer (11), and independent of tumor size and grade in nodal-negative breast cancer (14).

Previous studies reported a relationship between loss of E-cadherin expression and lobular differentiation that is associated with a prominent, but variable, extent of diffuse invasion mode (29). Therefore, the present study was designed to assess the differential relationship of E-cadherin to histological type (lobular versus ductal) and invasion mode as reflected by the histological growth pattern (diffuse versus compact).

Half of the lobular carcinomas show weak E-cadherin expression, while over half of the ductal carcinomas show strong E-cadherin expression. These findings are in agreement with previous studies (9-12, 30), which reported a relationship between loss of E-cadherin expression and lobular differentiation in breast cancer. Since loss of E-cadherin function has been claimed to play a key role in the diffuse mode of invasion, we established the hypothesis that differential proportions of growth patterns (diffuse, compact) in lobular and ductal types of breast cancer cause the differential expression of E-cadherin. The validity of this hypothesis was ensured in the present study on the basis of the coincidence of two findings: first, the highly significant reduction of E-cadherin expression in tumor tissue with diffuse growth.
pattern in comparison to the compact pattern and, second, the absence of any statistical difference between E-cadherin expression and the histological type within the same growth pattern.

To explain the differential expression of E-cadherin in breast cancer with dominant diffuse and compact growth pattern, the molecular genetic analysis of this gene was performed. Our molecular genetic analysis, however, did not reveal mutations of exons 6-10 in any of the 69 ductal and 10 lobular breast carcinomas. With respect to the ductal carcinomas, these results correspond to previous reports (17, 31). In contrast to our findings in lobular carcinomas, Berx et al. (8) detected protein truncation mutations involving exons 6, 9 and 10 in more than 50% (four out of seven) of the lobular carcinomas studied. A relatively low frequency of E-cadherin mutations was confirmed by a recent study on a large sample of lobular breast carcinomas, demonstrating mutations in 22% of cases (19).

In conclusion, reduction or loss of E-cadherin expression, present in more than 80% of invasive lobular breast carcinomas according to the present study, contrast with a low incidence of mutations of the gene.

References


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