Differential Expression of E-Cadherin in Primary Breast Cancer and Corresponding Lymph Node Metastases

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Abstract. *Background/Aim: E-Cadherin is a marker with a controversial function. Its role is often interpreted in the context of the epithelial-mesenchymal transition. In ambiguous cases, it is used as a phenotypic marker of lobular subtype of breast carcinoma. It has been well-studied in primary cancer, but its expression after metastasis is not well-described. The aim of this study was to determine the evolution of E-cadherin expression in no special type (NST) primary breast carcinoma and to correlate this with that in distant, paired nodal metastases (LNM) and molecular classification. Material and Methods: We processed 88 invasive breast carcinomas of NST type and their paired LNM. The specimens were formalin-fixed and paraffin embedded. Sections were immunostained for estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (Her2), basal cytokeratin CK5, nuclear protein Ki67 and E-cadherin with a Leica Bond-Max autostainer. Results: The results obtained were grouped into four molecular subtypes: Luminal A, luminal B, HER2-overexpressing, and triple-negative/basal-like. We found that the frequency of E-cadherin expression was higher (95.45%) in primary sites than in LNM (72.73%). E-Cadherin from primary breast cancer correlated positively only with E-cadherin in LNM ($p \leq 0.003$). A single positive correlation of E-cadherin with ER ($p \leq 0.007$) LNM was found. Conclusion: E-Cadherin expression is not stable during the metastatic process. Its expression in LNM is lower than in primary sites. E-Cadherin expression in primary sites positively correlates with E-cadherin from LNM.*

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Despite the fact that histological types of breast cancer are very well described and a lot of markers are used, this type of cancer remains the main cause of death among women. The scientific world is continuously searching for new markers which can help predict the outcome of this disease.

One such marker is E-cadherin. It was characterized as a potent suppressor of invasion and metastasis in several studies dating back to the 1990s and reviewed by van Roy and Berx (1). It is a calcium-regulated adhesion molecule expressed in most normal epithelial tissues (2). It helps in gland formation, stratification and epithelial polarization (3). Knockout of E-cadherin has been associated with non-viability and abnormal epithelial morphogenesis (4). Selective loss of E-cadherin can cause de-differentiation and invasiveness in human carcinomas, leading this marker to be classified as a tumor suppressor (5).

In practical applications, E-cadherin is used as a phenotypic marker of the lobular subtype of cancer, where loss of heterozygosity in chromosome region 16q22.1, the gene region encoding E-cadherin (*CDH1*) is frequent (6).

Although the role of E-cadherin as a tumor suppressor has been established, in recent years, alternative roles for E-cadherin in tumor progression have become apparent. The experimental and physiological observations regarding the tumor-suppressor role of E-cadherin are often interpreted in the context of the epithelial-mesenchymal transition. This important process includes changes that lead to the transient down-regulation of epithelial cell characteristics such as apical-basal polarization and organized cell-cell adhesion and the acquisition of a mesenchymal cellular phenotype,

which is less adherent and more migratory (7). From this position, the presence of E-cadherin prevents cell motility, invasion, and metastasis (8). Some results also support an important role for E-cadherin in tumor intravasation, which itself is a critical step required for metastasis. Kowalski *et al*. hold that most breast ductal carcinomas, both primary and metastatic, consistently express E-cadherin and that it plays an important role in maintaining microemboli in inflammatory carcinoma models (9).

In order to establish better treatment, the molecular profile of breast cancer is frequently determined; molecular subclasses increase day after day (10). E-Cadherin is very well described in correlation with histological types of breast carcinoma, but its expression in combination with molecular types is not so well known. Chekhun *et al*. showed that the aggressiveness of clinical course and occurrence of metastasis in basal or luminal subtypes is strongly dependent on expression of this marker (11). It was demonstrated that low colony-forming activity of human breast carcinoma cells of the luminal subtype is accompanied by increased adhesive properties of these cells due to high-level E-cadherin expression in correlation with a low level of CD44 expression and absence of CD24 expression. High tumorigenicity of cells of the basal subtype is related to weakening of adhesive contacts that are caused by abnormalities of E-cadherin expression. According to Tang *et al*., E-cadherin is as an independent marker of the prognosis of the triple-negative subtype, which is contradictory to other recent data (12, 13).

In fact, some results revealed an unusual meaning for this marker by which E-cadherin plays a role in preventing invasion and metastasis, which is in contrast with the classical point of view. Chu *et al*. found that patients with basal subtype breast cancer and high E-cadherin expression had a poor clinical outcome (14). Such data suggests a new function for E-cadherin as a signaling molecule required for in vivo growth of aggressive breast cancer tumor cells that retain E-cadherin expression. It seems that because of its multiple roles in tumorigenesis and wide range of available assays, the results estimating the value of E-cadherin in patients with breast cancer remain contradictory.

The aim of this study was to determine the evolution of E-cadherin expression in no special type (NST) primary breast carcinoma and to correlate this with that of distant, paired nodal metastases in accordance with molecular classification (10).

Materials and Methods

Patients. Specimens were obtained [primary breast carcinomas and their corresponding lymph node metastases (LNM)] from 88 patients from the Oncological Institute, Republic of Moldova during 2012-2013 years. No drug therapy preceded surgery and all patients underwent radical mastectomy and lymph node dissection.

Tissue processing and immunohistochemistry.The specimens were fixed in 10% phosphate-buffered formalin for 24-48 h and paraffin embedded as standard practice. For routine histopathological assessment, 4-6 μm sections were cut and stained with hematoxylin and eosin. The sections of the primary tumors and their metastases were processed together on the same slides. Two independent histologists reviewed the cases. Discrepancies in diagnoses were solved by consensus with simultaneous viewing.

Immunohistochemical assays included the use of six monoclonal antibodies: Er/6F11, Pr16, E-cadherin /36B5, Her2 /polyclonal, Ki67/K2, CK5/ XM26 (Table I). All stages of immunohistochemistry were performed automatically using a Leica Bond-Max autostainer (Leica Microsystems GmbH, Wetzlar, Germany). Hematoxylin Mayer's, Lillie's modification (HMM500, ScyTek Laboratories, Inc.) was used for counterstaining.

Microscopic evaluation. The markers used were interpreted only in invasive areas. The ER and PR markers were scored as the percentage of nuclear positively-stained cells from at least 1,000 cells assessed. The tumor was considered ER^+, or PR^+, if at least 30% of tumor cells in a section exhibited nuclear staining. The mean level of each marker from LNM was compared against that of the primary tumor.

The HER2 status was interpreted in accordance with American Society of Clinical Oncology recommendations (15): 0 if no staining was observed or weak, barely perceptible membrane staining up to 10% of cells; +1 in cases of weak membranous staining of >10%; +2 in cases of incomplete, weak/moderate circumferential membranous staining >10% of tumor cells or complete circumferential intense staining <10% of cells; +3 in cases of intense, circumferential staining of >10% of tumor cells. Cases with HER2/neu of +2 and +3 were considered as positive. The positively stained cells of normal ducts served as an internal control.

The CK5 expression was interpreted as Azoulay *et al*. previously defined: 0: no tumor cells stained; +1: fewer than 10% of tumor cells stained; +2: 10-50% positive tumor cells; +3: >50% of tumor cells stained (16). Expression was scored as positive (>0) if any cytoplasmic or membranous staining of tumor cells were observed.

For Ki67 marker, we used a 14% threshold as a limit to define positive/negative cases. The results were then grouped into four subgroups: 1: ER^+ and PR^+, $HER2^-$, $CK5^-$, Ki67<14% as luminal A; 2: ER^+ with/without PR^+, $HER2^+$, $CK5^-$ or ER^+ with/without PR^+, $HER2^-$, $CK5^-$, Ki67>14% as luminal B; 3:ER^-, PR^-, $HER2^+$, $CK5^-$ as $HER2^-$ overexpressing; 4:ER^-, PR^-, $HER2^-$ and $CK5^+$ as triple-negative/basal-like.

E-Cadherin reaction was evaluated using a scoring system purposed by Qureshi *et al*. (17) for the correlation of the percentage of positively stained cells and their staining intensity: 0: lack of staining or membrane positivity in <10% of tumor cells; 1: incomplete and weak membranous staining in >10% of tumor cells; 2: complete membranous staining, with weak or moderate intensity in >10% of tumor cells; 3: strong membranous staining in >10% of tumor cells. According to this score, the reaction was considered as negative for scores of 0 and 1, weakly positive for score 2 and strongly positive for score 3. Cytoplasmic staining was considered nonspecific and not included in the assessment. The presence of E-cadherin staining in epithelial cells of the normal ducts and acini served as an internal positive control.

Table I. *Antibodies used; source, dilution, systems of detection and retrieval, and time of incubation.*

Receptor	Clone	Source	Incubation time	Dilution	Detection	Time	Retrieval system	Time
ER PR	6F11 16	Leica Biosystem Newcastle Ltd, Newcastle Upon Tyne, UK	15 min	RTU	Bond Polymer Refine Detection System (Leica Biosystems, Newcastle Upon Tyne, UK	15 min	Bond Epitope Retrieval Solution 1 (Leica Biosystems, Newcastle Upon Tyne, UK)	20 min
E-cadherin	36B5	Novocastra, Newcastle Upon Tyne, UK	15 min	RTU				
Her2/neu	Policlonal	Dako Glostrup Denmark	30 min	RTU	EnVision-HER	30 min	Dako Target Retrieval Solution, pH 6	20 min
Ki67 CK5	K2 XM26	Leica Biosystem Newcastle Ltd, Newcastle Upon Tyne, UK	15 min	RTU	Bond Polymer Refine Detection System (Leica Biosystems, Newcastle Upon Tyne, UK)	15 min	Bond Epitope Retrieval Solution2, (Leica Biosystems, Newcastle Upon Tyne, UK)	20 min

RTU: Ready to use.

Image acquisition and data processing. Slides were evaluated on a Nikon Eclipse 80i microscope with Nikon DS-Fi1 installed camera by using Nis-elements BR 2.30 imaging software (Nikon Instruments Europe BV). A Microsoft Access 2003 database (Microsoft Office 2003 SP3) was used to store and group the data.

Statistical analysis. WINSTAT 2012.1 software (R. Fitch Software, Bad Krozingen, Germany) was used for descriptive statistics, the mean and standard error of the mean determined and for all the tests a value of $p \leq 0.05$ was considered significant. Pearson's correlation was used to determine the relationship between different variables for a value of $p \leq 0.05$. The strength of the correlation was appreciated in accordance with Evans' guide (18): 0.00-0.19: very weak; 0.20-0.39: weak; 0.40-0.59: moderate; 0.60-0.79: strong; 0.80-1.0: very strong.

Ethics. The study has been approved by the Ethics Committee of the "Nicolae Testemitanu" State University of Medicine and Pharmacy from Chisinau, Republic of Moldova, based on patients' informed consent (approval number 21/13 from 31.03.2014).

Results

We found E-cadherin to be present in all primary tumors (95.45%), out of which 82.95% of cases exhibited a strong, grade 3 expression and 12.5% a grade 2. All negative cases (4.55%) were graded as 0.

In LNM, E-cadherin was evaluated as being positive in 72.73% of cases, of which 59.09% were estimated at grade 3 and 13.64% as grade 2. The negative cases represented 27.27%, of which 19.32% were graded as 0 and 7.95% as 1 (Figure 1 and b).

After grouping our 88 cases by hormonal profile, the luminal A subtype constituted the majority-68 cases (77.27%) in the primary sites (Figure 1a) and in 63 cases (71.59%) of LNM. Luminal B and basal-like comprised nine cases each (10.23%) in the primary tumors, and seven (7.95%) and 14 cases (15.91%) of LNM. HER2-positive profile was found in two cases (2.27%) of primary tumor and in four cases (4.55%) of LNM (Figure 1 c-f).

The analysis of E-cadherin distribution by molecular subtypes shows that the highest rate (72.73%) of E-cadherin expression was found for the luminal A profile of primary tumors (Table II). In all cases of luminal B (9 cases/10.23%), basal-like (9 cases/10.23%) and $HER2^+$ (2 cases/2.27%) at the primary level, E-cadherin expression was positive. Only four (4.55%) luminal A cases did not express E-cadherin.

A different distribution of E-cadherin was determined in the LNM. From 63 (71.59%) luminal A cases, only 48 (54.55%) expressed this marker. E-Cadherin was positive in six cases (6.82%) of luminal B, eight (9.09%) of basal-like and two (2.27%) of $HER2^+$ LNM.

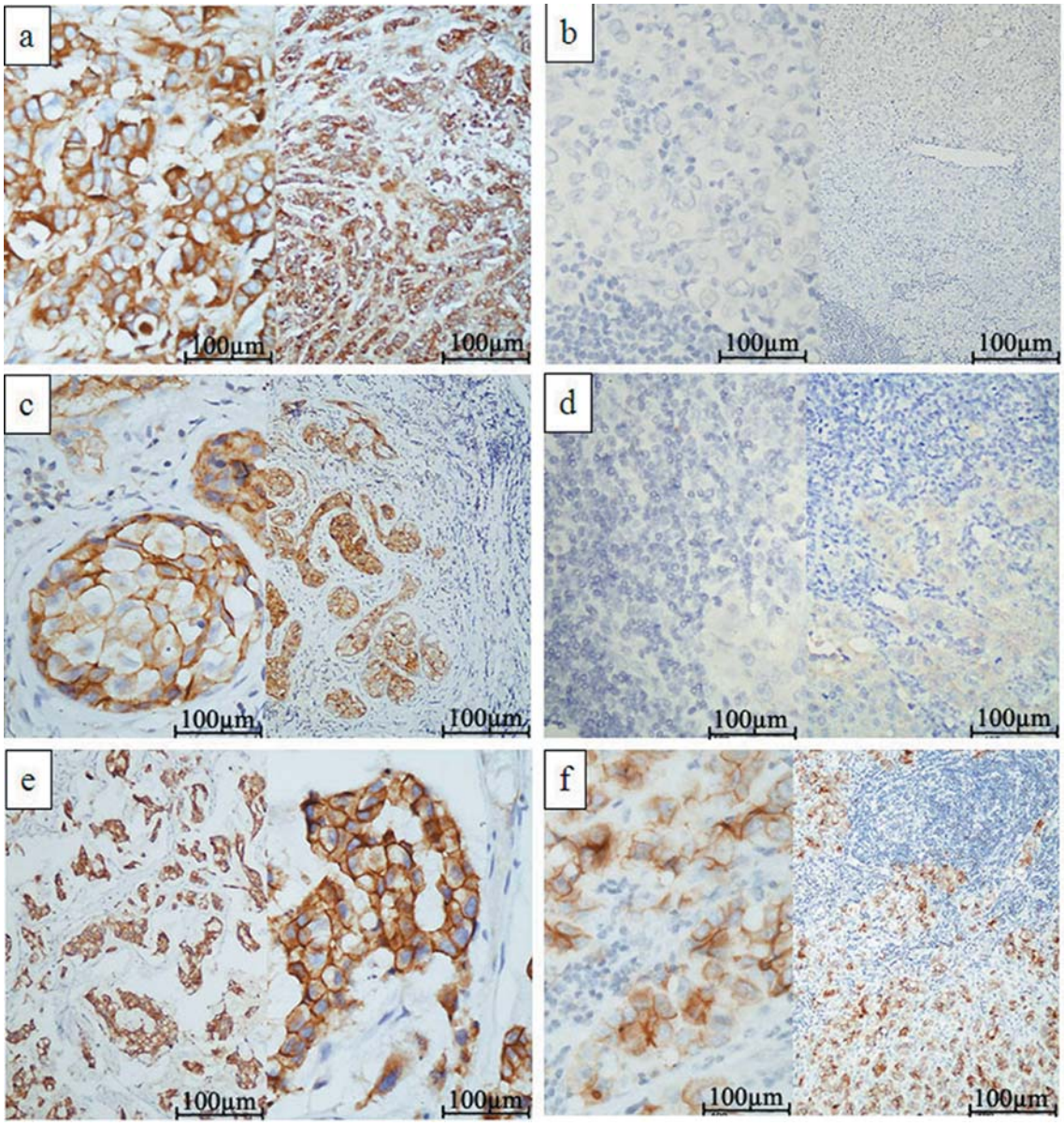

Figure 1. *The E-Cadherin expression in different molecular subtypes of breast cancer, in primary tumor and its correspondent lymph node metastasis (LNM): (a)-Invasive ductal breast carcinoma of no special type, G3, Luminal A subtype. Strong (+3) E-Cadherin expression; (b)- Lymph node metastasis of the invasive ductal breast carcinoma, Luminal A subtype, stated in (a). No (0) E-Cadherin expression; (c)-Invasive ductal breast carcinoma of no special type, G3, Her2- overexpressed subtype. Strong (+3) E-Cadherin expression; (d)-Lymph node metastasis of the invasive ductal breast carcinoma, G3 stated in (c). Her2 overexpressed subtype at the level of LNM. No (0) E-Cadherin expression;(e)-Invasive ductal breast carcinoma, no special type, G2, Luminal B subtype. Strong (+3) E-Cadherin expression; (f)-Lymph node metastasis of the invasive ductal breast carcinoma, G2, showed in (e). At the level of LNM is a Her2-overexpressed subtype determined. Strong (+3) E-Cadherin expression.*

The statistical analysis revealed no correlations of E-cadherin expression in primary tumor with ER, PR, HER2, CK5, Ki-67 markers or molecular subtype. In LNM, E-cadherin was positively correlated (0.26 at $p \leq 0.0007$) with ER. The E-cadherin level in primary sites was weakly (0.29) but statistically significantly ($p \leq 0.003$) correlatied with E-cadherin in LNM

Discussion

It is well-recognized that E-cadherin loss confirms invasive lobular carcinoma (17, 19, 20). The different pattern of E-cadherin expression in invasive ductal and lobular carcinomas suggests that this protein may play different roles in the

Table II. *Molecular subtypes and E-Cadherin expression in no special type (NST) primary breast cancer and paired lymph node metastases (LNM).*

Subtype	E-Cadherin score	Primary tumor			LNM		
		No. of cases	%		No. of cases	%	
Luminal A		64	72.73		48	54.55	
Luminal B	+2/+3	9	10.23	95.45	6	6.82	72.73
Basal-like		9	10.23		8	9.09	
HER2+		2	2.27		2	2.27	
Luminal A		4	4.55		15	17.05	
Luminal B	0/+1			4.55	1	1.14	27.27
HER2+					2	2.27	
Basal-like					6	6.82	

development of each type of tumor. The absence of membranous E-cadherin expression in invasive lobular carcinomas may determine the morphological features such as the characteristic cellular arrangement of lobular carcinoma cells, as well as the distinct pattern of stromal invasion of invasive lobular carcinomas, typically as single cells or rows of cells (9). By comparing two sites, we determined that E-cadherin is expressed at normal levels at the distant metastatic site regardless of the level of expression at the site of primary invasive ductal carcinoma. Invasive lobular carcinomas have a different pattern of E-cadherin expression at primary and metastatic sites, which suggests a different role of E-cadherin in this form of cancer. Positive staining may not completely exclude the presence of lobular carcinoma because E-cadherin expression may be retained in a minority of cases with characteristic morphological features of lobular carcinoma. We consider that E-cadherin positivity favors ductal differentiation in ambiguous cases. In contrast, partial loss of E-cadherin expression in some poorly differentiated ductal carcinomas is not of diagnostic significance.

The absence of E-cadherin expression is frequently described in correlation with tumor size and stage, lymph node status, tumoral recurrence, and grade of differentiation. Moreover, Goldstein considered that its lack of expression explains the distinctive growth patterns in metastases (21). Kowalski *et al*. consider that a reduction in E-cadherin expression is frequent in invasive ductal carcinomas that will progress and develop distant metastases (9).

By studying E-cadherin expression in primary breast tumors and their corresponding metastases to liver, lung and brain, Chao *et al*. showed an increased expression (62%) in the metastases compared to the primaries (22). In addition, it has been observed that metastatic foci commonly appear to be more differentiated than the corresponding primary tumor, suggesting that cancer cells may further undergo a mesenchymal to epithelial reverting transition in the secondary organ environment.

However, our results showed E-cadherin expression to be less frequent in LNM (72.73%) than in primary sites (95.45%). E-Cadherin loss in the lymph node environment supports the possibility of epithelial-mesenchymal transition in breast carcinomas metastasis. Although it was shown that abnormality of E-cadherin expression and methylation of its gene is associated with an aggressive clinical course, Hollestelle *et al*. concluded that loss of E-cadherin is not a necessity for epithelial-mesenchymal transition in human breast cancer (23).

The loss of E-cadherin expression has an unfavorable prognostic significance. Its absence is frequently associated with metastatic lymph node status, tumor recurrence, low grade of differentiation, and advanced stage of tumor (24). It has been demonstrated that low colony-forming activity of human breast cancer cells of the luminal subtype is accompanied by increased adhesive properties of these cells due to a high level of E-cadherin expression (11). High tumorigenicity of cells of the basal subtype is related to weakening of adhesive contacts that is caused by abnormalities of E-cadherin expression.

Recently several studies have described the possibility of a phenotype switch during the metastatic process. It is hypothesized that the molecular profile of breast canceris not stable throughout tumor progression. Moreover, Falck *et al*. found that when a shift in molecular subtype between primary tumor and metastatic lymph node occurs, the prognosis (for 10 years) follows the subtype from metastasis (25).

Nowadays, E-cadherin is considered to be an independent marker of triple-negative breast cancer, a molecular subtype characterized with poor prognosis and short survival (12). In our investigation the molecular subtypes of the primary sites did not correspond 100% with those of their LNM. In particular, we observed a shift from luminal to triple-negative. This transition is supported by a decrease in the E-cadherin-positive rate from 95.45% in primary tumors to 72.73% in LNM (Table II) and higher incidence of the triple-negative subtype in LNM (15.91% *versus* 10.23% in primary sites). However, statistically confirmed correlation between E-cadherin and the molecular subtype of primary carcinoma and LNM was not found. Our results are contradictory to certain recent data (13), which revealed statistically significant differences in E-cadherin expression by molecular subtype. Bertolo *et al*. found a positive correlation of E-cadherin and HER2 receptor, but in our study, E-cadherin in primary sites correlated positively only with E-cadherin in LNM (26). This contradiction may be due to the selected study material. Authors selected cases without distant metastasis at the time of primary diagnosis. In addition, we processed a higher number of cases (88 *versus* 60).

E-Cadherin in LNM correlated only with ER at the same site. It should be noted that despite the obtained results being statistically significant ($p \leq 0.05$), correlations were graded as weak. E-Cadherin correlation with the molecular profile in breast carcinomas needs further investigation by other groups.

Conclusion

The E-Cadherin expression changes during metastatic process. A different expression between primary tumor and metastases could have a clinical impact with important role in recurrences and tumors' molecular profile changing. This marker could also have a predictive role. Loosing E-Cadherin expression at the metastases level suggests that primary tumor is not homogenous by its cellular composition; breast carcinoma has tumor cells with different metastatic potential. This marker is not a target in personalized therapy yet, but results highlighted in the present study suggest its role in metastasis development. Low E-Cadherin expression and evidence of switch from luminal A to triple-negativ subtype in metastases, purpose idea that this marker could be involved in changing of breast carcinoma molecular subtype.

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