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Research article

Expression of epithelial-mesenchymal-transition markers in breast cancer

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Abstract

Aberrant expressions of proteins involved in epithelial-to-mesenchymal transition (EMT) have been described in various cancers. In this study, we sought to evaluate the expression of E-cadherin, and vimentin in breast cancer and to analyze the relationship between the expression of these markers and the stage of tumor extension. E-cadherin, and vimentin expression were investigated by immunohistochemistry in a series of 89 invasive breast carcinoma cases. We observed a decreased expression of E-cadherin in 27% of cases and an increased expression of Vimentin in 20% of cases. For clinicopathological parameters, we found that the expression of Vimentin is significantly associated with a high histological grade (p = 0.008). The expression of Vimentin by tumor cells was also significantly correlated with estrogen receptor negativity (p = 0.001), progesterone receptor negativity (p = 0.001), and triple negative tumors. However, we found no significant correlation between the expression of E-cadherin and theclinicopathological parameters studied. We also found a high prevalence of these markers in triple negative tumors (51%) compared to luminal A (2%), luminal B (9%) and HER2 (10%) (p = 0.001). Immunohistochemical analysis of the expression of E-cadherin and Vimentin allows an earlier detection of tumor progression and metastatic dissemination and leads to a better patients' therapeutic care.

Key words: *Breast cancer, Epithelial-mesenchymal transition, immunohistochemistry, E-cadherin and Vimentin*

1. Introduction

Breast cancer is a heterogeneous disease characterized by deregulation of several pathways, related to cell cycle control, apoptosis, angiogenesis, and involves several processes such as metastasis formation and cell dedifferentiation, also called the epithelial-mésenchymal transition.

The epithelial-mesenchymal transition is a process of cellular trans-differentiation that leads to generate mesenchymal phenotype cells from a structured epithelium. This process seems critical in the process of tumor invasion and metastatic dissemination (Meng and Wu 2012). Indeed, the EMT is manifested by a loss of intercellular junctions, which results in the decrease of E-cadherin expression, in the depolarization of epithelial cells and in the acquisition of cellular motility by vimentin nuclear expression (Thiery et al. 2009).

E-cadherin is a transmembrane glycoprotein that plays an important role in cell adhesion in various tissues (Birchmeier et al. 1993, Gumbiner 2000). Loss of E-cadherin has been reported to be associated with tumor aggressiveness and metastatic dissemination in breast, bladder and prostate cancer (Gamallo et al. 1993, Bringuier et al. 1993, Umbas et al. 1994, Kanai et al. 1994, Siitonen et al. 1996, Tamura et al. 2000).

Vimentin is a type III intermediate filament protein normally expressed in mesenchymal cells and in epithelial cells that migrate during embryogenesis, organogenesis and wound healing (Satelli and Li 2011). Previous studies reported an overexpression of Vimentin in various cancers including breast cancer, endometrial cancer, pancreatic, colorectal and hepatic cancers (Alfonso et al. 2005, Wu et al. 2007, Sethi et al. 2010). Indeed, these studies showed that increased Vimentin expression correlates significantly with tumor invasion and metastasis (Grille et al. 2003, Kokkinos et al. 2007).

In this context, we investigated the expression of EMT markers (expression of vimentin; loss of e-cadherin) and we evaluated its relationship with clinicopathological parameters and intrinsic molecular subgroups in a large series of breast cancer

2. Materials and Methods

2.1. Patients and specimens

This study included 89 breast carcinomas patients, the most common breast cancer type, obtained from the archives of the Department of Pathology, Farhat Hached University Hospital of Sousse (Tunisia). The cases were selected based on the availability of sufficient paraffin-embedded tissue, before any treatment.

The slides were reviewed and the cases were classified into 4 categories based on the immunohistochemical status of estrogen receptors (ER), progesterone receptors (PR), HER2 and Ki67, according to Goldhirsch et al (2013): luminal A (ER+ and/or PR+, HER2−, low Ki67), luminal B (ER+ and/or PR+, HER2+ and/or high Ki67), HER2 overexpressing (ER−, PR−, HER2+) and triple negative (ER−, PR−, HER2−) (Goldhirsch et al. 2013).

2.2. Immunohistochemical analysis of E-cadherin and vimentin

The expression of E-cadhérin (clone 36B5, dilution 1:25, Leica, Biosystems, UK) and vimentin (clone V9, dilution 1:100, Dako, Danemark), was investigated by immunohistochemistry using the EnVision Flex system (DakoCytomation, Glostrup, Denmark) according to the manufacturer's instructions.

Briefly, Paraffin-embedded breast cancer tissues were cut at 5 µm, dried overnight at 60°C and deparaffinized in Ottix Plus (Diapath, Martinengo, Italy). Subsequently, the sections were hydrated with Ottix Shapper (Diapath, Martinengo, Italy), and rehydrated in water.

For antigen retrieval, the sections were boiled in a water bath with citrate buffer (0.01 M, pH 6.0) for 40 min until the temperature reached 98°C. The sections were then allowed to cool at room temperature for 20 min. later; they were placed in EnVision Flex Wash buffer (DakoCytomation, Glostrup, Denmark)**.** The, endogenous peroxidase activity was blocked with EnVision Flex Peroxidase-Blocking Reagent for 5 min. The sections were thoroughly washed with the Wash buffer. The samples were incubated at 4°C overnight with the primary antibody. Subsequently, the sections were rinsed gently with Wash buffer.

Immunostaining was performed using the high sensitive polymer-based EnVision Flex /HRP system. The sections were then incubated in 3, 3 diaminobenzidine, a substrate–chromogen, solution for 20 min. Finally, the slides were counterstained with Mayer hematoxylin, permanently mounted, and viewed with a standard light microscope (Zeiss Axiscope A1, 400X).

2.3. Immunohistochemical Evaluation

In all the cases, immunostaining results were evaluated independently by two pathologists. E-cadherin, vimentin positive staining was evaluated in the cell membrane. For the two antibodies, a case was

considered positive if more than 10% of the cells exhibited immunostaining for this antigen, otherwise, it was negative (Zhou et al. 2016).

2.4. Statistical analysis

Statistical analysis was carried out with the SPSS software package (version 20.0; SPSS, Chicago, IL, USA). The correlation between the patients' clinicopathologic features, EMT markers expressions was investigated by the chi-square test and Fisher exact test, where appropriate. A p value ≤ 0.05 was considered to indicate statistical significance.

3. Results

3.1. Expression of E-cadherin and vimentin in breast cancer

We analyzed the E-cadherin and Vimentin expression by immunohistochemistry on a series of 89 cases of invasive breast cancer to detect the level of expression of proteins related to the epithelial-mesenchymal transition. Overall, 65/89 cases (73%) showed a positive membrane staining of E-cadherin in most tumor cells **(Figure 1a)**. However, 18/89 cases (20%) were classified as positive for Vimentin, showing a cytoplasmic expression in tumor cells with varying intensity and distribution **(Figure 1d)**. In the positive cases, the strong membranous expression of E-cadherin in the tumor cells contrasts with the absence of a detectable staining in the normal mammary epithelial cells **(Figure 1e)**. Regarding Vimentin, a focal staining was observed in some normal mesenchymal cells **(Figure 1b)**.

3.2. Correlation between the expression of E-cadherin, Vimentin and clinicopathological parameters

The distribution of breast cancer cases expressing E-cadherin and Vimentin according to clinicopathological parameters is presented in Tables I and II. We found that vimentin expression was significantly associated with a high histological grade. Indeed, 36% of the positive cases were grade III (p=0.008) while 11% and 5% of the negative cases were grade I and grade II, respectively.

The expression of Vimentin by tumor cells was significantly correlated with estrogen receptor negativity (p=0.001), progesterone receptor negativity (p=0.001) and triple negative phenotype tumors (absence of expression of estrogen, progesterone and HER2 receptors). However, we found no significant association between E-cadherin expression and clinicopathological parameters.

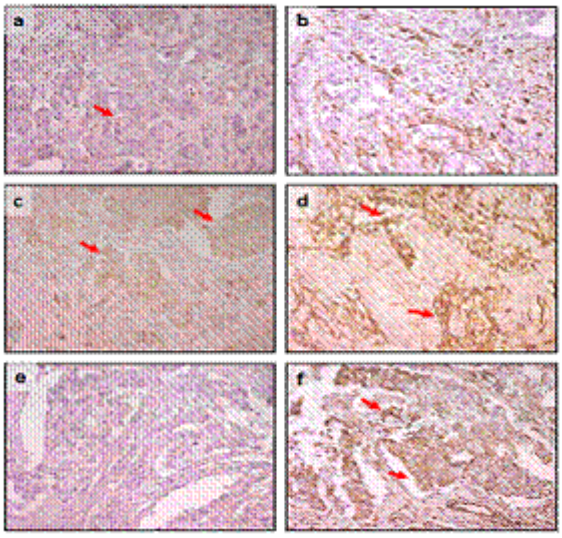

Fig. 1 Examples of immunostaining for E-cadherin (a, c x 200) and vimentin (d, f x 200) expression in breast cancer. Positivity consists of a brownish staining in tumor cells. Absence of a detectable staining in the normal mammary epithelial cells for E-cadherin (e x 200). Regarding Vimentin, a focal staining was observed in some normal mesenchymal cells (b x 200).

3.3. Association between E-cadherin and Vimentin expression and molecular subtypes

Among 89 cases studied, 39 were luminal A, 11 were luminal B, 10 were HER2 and 29 were triple negative. The study of the relationship between EMT markers and molecular subtypes in breast cancer revealed a high prevalence of these markers in triple negative tumors (51%) compared to luminal A (2%), luminal B (9%) and HER2 (10%)

Table 1: Correlation between the expression of E-cadherin, vimentin and anatomo-pathological parameters

Anatomo-pathological parameters	Total	e-cadherin expression		Vimentin expression	
		n (%)	*p*-value	n (%)	*p*-value
Age					
≤ 50	50	39(78)	0.2	8(16)	0.2
> 50	38	26(68)		10(26)	
Histological grade					
Grade I	19	14(73)	0.9	1(5)	0.008*
Grade II	34	24(70)		4(11)	
Grade III	36	27(75)		13(36)	
Tumor size (mm)					
≥2	25	18(72)	0.7	4(16)	0.7
>2-5≤	57	41(71)		13(22)	
>5	7	6(85)		1(17)	
Nodal involvement					
Positive	39	27(69)	0.5	9(23)	0.7
Negative	23	14(60)		6(26)	

p value for $\chi 2$ or Fisher exact test. *asterisks indicate significant correlations

Table 2: Correlation between EMT markers and the classic clinico-pathological parameters and intrinsic molecular subtypes.

clinico-pathological parameters	Total	e-cadherin expression		Vimentin expression	
		n (%)	*p*-value	n (%)	*p*-value
Menopausal status					
Post menopause	37	26(70)	0.6	6(16)	0.3
Pre menopause	47	35(74)		12(25)	
Estrogen receptor					
Positive	36	22(61)	0.03	1(2)	0.001*
Negative	53	43(81)		17(32)	
Progesterone receptor					
Positive	42	30(71)	0.7	16(38)	0.001*
Negative	47	35(74)		2(4)	
Her2 status					
Positive	20	13(65)	0.3	2(10)	0.1
Negative	69	52(75)		16(23)	
EGFR					
Positive	7	5(71)	0.9	3(42)	0.1
Negative	51	37(72)		9(17)	
Cytokeratin					
Positive	15	13(86)	0.1	7(46)	0.004*
Negative	43	29(67)		5(11)	
Molecular subtype					
Luminal A	39	26(66)	0.2	1(2)	0.001*
Luminal B	11	8(72)		1(9)	
HER2	10	6(60)		1(10)	
Triple Negative	29	25(86)		15(51)	
Triple Negative subtype					
Positive	20	25(86)	0.05	15(51)	0.001*
Negative	60	40(66)		3(5)	

p value for $\chi 2$ or Fisher exact test. *Asterisks indicate significant correlations. HER2: Human Epidermal Growth Factor Receptor-2
EGFR: Epidermal Growth Factor Receptor

4. Discussion

In breast cancer, the triple negative subtype, which is a very aggressive type associated with poor clinical outcomes (Perou et al. 2000, Sørlie et al. 2001), is related to the epithelial-mesenchymal transition. Indeed, previous studies have reported that increased expression of mesenchymal markers, including vimentin, smooth muscle actin (SMA), osteonectin, and N-cadherin, as well as reduced expression of epithelial markers such as Ecadherin, β-catenin, and cytokeratin, was more common in triple negative breast cancer than other subtypes (Charafe-Jauffret et al. 2006, Blick et al. 2008, Sarrió et al. 2008, Jeong et al. 2012). In this context, we analyzed the relationship between the expression of EMT markers (expression of vimentin, loss of E-cadherin) and tumor extension to verify its role in metastatic dissemination.

This data showed an expression of Vimentin in 20% of cases and a loss of E-cadherin in 27% of cases. Previous studies have reported variable expression levels of the EMT markers ranging from 30 to 50% for E-cadherin and from 12 to 60% for Vimentin in breast cancer (Li et al., 2014, Wang et al. 2015)

This discrepancy in terms of prevalence was explained by the heterogeneity of the series tested, by the different experimental protocols used and the threshold values adopted. In our study, we used a cutoff value of 10% as proposed by many other studies (Siitonen et al. 1996, Kavgaci et al. 2010, Zhou et al. 2016) while others used 5% and 4% (Charpin et al. 1998, Pedersen et al. 2002).

Regarding clinicopathological parameters, we found that Vimentine expression was significantly associated with a high histological grade (p= 0.008). This finding agrees with the results described by several authors where Vimentine expression is associated with high grade, tumor progression and lymph node involvement (Bloemendal and Pieper 1989, Domagala et al. 1990, Lee et al. 2015).

We also observed that the expression of Vimentin by tumor cells was significantly correlated with estrogen receptor negativity (p=0.001), progesterone receptor negativity (p=0.001), and triple negative tumors. These observations are in agreement with findings reported by Cattoretti et al (Cattoretti et al. 1988).

Several studies have shown a significant association between E-cadherin expression and clinical aggression parameters such as high tumor size, high histological grade, and lymph node involvement (Kavgaci et al. 2010, Eljuga et al. 2012). However, our results are not able to confirm theses association.

Furthermore, we investigated whether an association exists between the expression of epithelial-mesenchymal transition markers and the intrinsic molecular subtypes in breast cancers. We found a high prevalence of these markers in triple negative tumors (51%) compared to luminal A (2%), luminal B (9%) and HER2 (10%) (p= 0.001). This result is consistent with earlier studies ((Sato et al. 1990, Yamashita et al. 2013, Tsang et al. 2013). It is well documented that triple negative tumors was associated with poor prognosis compared to other molecular subtypes (Li et al. 2014,Yamashita et al. 2013, Tsang et al. 2013) .

Expression of mesenchymal markers, and in particular vimentin, is accompanied by an invasive cellular phenotype, however, its deletion has a protective role (Xue et al. 2003). The regulation of the physiological or pathological mechanisms of the epithelio-mesenchymal transition is the result of signaling pathways deregulation, particularly, protein kinase (Protein kinase B) family. Indeed, this protein leads the stabilization of Snail (Bachelder et al. 2005), to inhibit E-cadherin expression (Villagrasa et al. 2012), and the nuclear translocation of NF-κB factors which induces the expression of Slug (Conacci-Sorrell et al. 2003), and consequently the expression of vimentin and β-catenin (Min et al. 2008) . Thus cells acquire a mesenchymal character that require alteration of the intercellular junctions, modification of the extracellular matrix (ECM) and reorganization of the actin cytoskeleton and intermediate filaments (Katsuno et al. 2013).

In our study, we found that mammary tumors expressing EMT markers (vimentin expression and loss of e-cadherin) were significantly associated with clinical aggressiveness parameters. We also observed that the expression of these markers was found in triple negative subtype (p=0.001). These results suggest that immunohistochemical analysis of the expression of E-cadherin and Vimentin allows an earlier detection of tumor progression and metastatic dissemination and leads to a better patients' therapeutic care.

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