

Concomitant expression of epithelial-mesenchymal transition biomarkers in breast ductal carcinoma: Association with progression

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Abstract. Epithelial to mesenchymal transition (EMT) is a process implicated in cancer progression in which the underlying cellular changes have been identified mainly using *in vitro* models. We determined the expression of some putative EMT biomarkers including E-cadherin, β -catenin, zinc finger factor Snail (Snail), transforming growth factor β 1 (TGF β 1), TGF β type II receptor (TBRII) and the HGF receptor (c-met) and their possible correlation to progression and overall survival in a series of breast ductal carcinoma *in situ* (DCIS) and invasive ductal carcinomas (IDC). Biomarkers were immunohistochemically determined in 55 IDC specimens from which 21 had lymph node metastases and in 95 DCIS specimens, 46 of these cases associated to invasive carcinoma, in a tissue microarray (TMA). Positive cytoplasmic staining of TGF β 1 (78.2%), c-met (43.6%), Snail (34.5%), TBRII (100%), membranous E-cadherin (74.5%) and membranous/cytoplasmic β -catenin (71%) were detected in the IDC samples. Metastatic lymph node samples displayed similar frequencies. A significant increase of c-met and TGF β 1 positivity along DCIS to IDC progression was noted but only TGF β 1 positivity was associated with presence of lymph node metastases and advanced stages in IDC. The evaluation of the other EMT markers in DCIS did not show differences in positivity rate as compared to invasive carcinomas. DCIS either pure or associated to IDC showed similar expression of the analyzed biomarkers. All the

carcinomas exhibited positive expression of TBRII. Associations between the markers, determined by Spearman's correlation coefficient, showed a significant association between TGF β 1 and respectively E-cadherin, β -catenin and c-met in DCIS cases, but in invasive carcinomas only cadherin and catenin were positively correlated. Kaplan-Meier survival curves revealed that none of the EMT biomarkers analyzed were correlated with survival, which was significantly determined only by clinical and hormone receptor parameters.

Introduction

Progression of breast cancer is often accompanied by changes in the pattern of gene expression of neoplastic cells, resulting in a highly tumorigenic and invasive cell phenotype. Some of these changes are reminiscent of an epithelial-mesenchymal transition (EMT), a process characterized by loss of epithelial features and gain of mesenchymal properties (1).

In vitro data suggested that mesenchymal transformation may correlate with loss of epithelial cell adhesion molecules. Reduced expression of E-cadherin is emerging as one of the most common indicators of EMT onset. The disappearance of E-cadherin results in the release of β -catenin and its cytoplasmic accumulation with further delocalization from the cytoplasm to the nucleus where it can activate LEF/TCF (lymphoid enhancer factor/T cell factor) transcription. In addition, increased production of developmental transcription factors, as Snail, Slug, Twist, FOXC2 and gooseoid, are central mediators of EMT. Snail is a transcription target of twist and it is a repressor of E-cadherin (2). Furthermore, Snail suppresses the expression of claudins and occludins (3). EMT may be triggered by an intricate interplay of extracellular signals including soluble growth factors and members of the TGF β (transforming growth factor β) family play a critical role as key effectors in cancer progression and metastasis. TGF β 1 exerts its effect by binding to the TGF β type II receptor (TBRII), enabling the formation of a complex with type I receptor, resulting in phosphorylation of SMADs which then

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regulate transcription of targets genes repressing E-cadherin and inducing expression of Snail (4). In addition to TGF β , several other tyrosine kinase receptors including c-met play a role in regulating EMT-like morphogenetic events (5). C-met is also induced by TGF β 1 (6). Dysregulation was reported of c-met receptor or over-expression of its ligand, the hepatocyte growth factor (HGF) that is produced by stromal cells, has been associated with an aggressive phenotype. Importantly HGF regulates the levels of unbound β -catenin thus overexpression of c-met may facilitate disruption of E-cadherin junctions (7).

Little is known about the concomitant expression of EMT markers in breast cancer. Since each of the mentioned EMT biomarkers have been considered to be individually important for the malignant behavior of breast cancer we reasoned that the expression of these factors changed with progression and therefore they could be prognostic markers of outcome in breast cancer patients. To this end, the expression of E-cadherin, β -catenin, Snail, TGF β 1 and c-met was immunohistochemically examined in samples of breast carcinoma *in situ* and in primary invasive tumors. In addition correlations among the expression of this EMT markers were evaluated in both DCIS and IDC the potential prognostic value of each EMT marker expression was assessed.

Materials and methods

Breast tumor samples and patient data. Formalin-fixed and paraffin-embedded tissue specimens from a total of 55 patients diagnosed with invasive ductal breast carcinoma (IDC) at the A.C. Camargo Cancer Hospital, São Paulo, Brazil, between 1993 and 2005 were included in this study after approval by its Institutional review board and was referred as the experimental set of tumors. In addition, tissue microarrays containing 95 cases of ductal carcinoma *in situ* (DCIS), 50 of these cases associated to invasive carcinoma, diagnosed at the Department of Pathology Cancer from 1980-2001, were produced. All DCIS tumor samples were also ductal carcinomas. All cases were reviewed by A.F.L., C.T.O. and F.A.S. to corroborate the diagnosis. Characteristics of these retrospective cohorts are detailed in Table I. Patients were enrolled according to the inclusion criteria consisting of suitable paraffin blocks for immunohistochemistry, adequate clinical parameters and follow-up information. None of the patients with pure DCIS had experienced recurrence or progression to an invasive cancer within the median follow-up time. Histologic typing and grade for invasive cases were defined according to the WHO and modified Scarf-Bloom-Richardson classifications. DCIS nuclear grade was estimated using the Lagios classification.

Construction of tissue microarrays (TMAs). TMAs were constructed from 1.50-mm cores of formalin-fixed, paraffin-embedded archival tissue specimens using a tissue arraying instrument (Beecher Instruments) as previously described by us (8). TMAs were then sectioned (4 μ M) and mounted on positively charged glass slides for immunohistochemistry (IHC) analysis. The presence of tumor tissue in each core was confirmed by H&E staining of TMA slides.

Table I. Data of ductal breast carcinoma patients.

Characteristics	Invasive (n=55)	<i>In situ</i> (n=95)
Median age (range)	56 (33-80)	
Histology		
Comedo		7 (7.4)
Non-comedo		88 (92.6)
N stage		
N0	20 (36.4)	
N+	35 (63.6)	
T stage		
T1 + T2	43 (78.2)	
T3 + T4	12 (21.8)	
Disease stage		
TxN0M0	16 (29.1)	
TxN1M0	15 (27.3)	
TxN2M0	8 (14.5)	
TxNxM1	16 (29.1)	
Estrogen receptors (n=42)		
Positive	27 (49.1)	58 (70.7)
Negative	15 (27.3)	24 (29.3)
Progesterone receptors (n=42)		
Positive	23 (41.8)	48 (58.5)
Negative	19 (34.5)	34 (41.5)
Differentiation		
Well	19 (34.5)	14 (14.7)
Moderate	22 (40.0)	44 (46.3)
Poor	14 (25.5)	37 (39.0)
Status		
Alive	29 (52.7)	
Deceased	26 (47.5)	

Immunohistochemistry. Monoclonal antibodies to β -catenin, E-cadherin, c-met and TGF β 1 were obtained from Novocastra (Newcastle, UK) and diluted 1:100, 1:50, 1:50 and 1:25, respectively. TBR11 (polyclonal ready for use), Snai-1 and c-met were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and diluted 1:100. Each slide was also stained with anti-ER (Neomarkers, clone 6F-11, 1:50) and anti-PR (Dako, clone PgR636, 1:500).

After deparaffinization and rehydration of sections or tissue microarrays from formalin-fixed paraffin-embedded samples, antigen retrieval was performed in a pressure cooker. Endogenous peroxidase activity was blocked with 3% H₂O₂. Incubation with primary antibody was performed overnight at 4°C. Slides were incubated with biotinylated goat anti-mouse/rabbit Ig, or biotinylated rabbit anti-goat Ig (for SNAI 1) followed by streptABC/HRP (Dako, Denmark). Slides were developed by diaminobenzidine (Sigma, USA) and 6% H₂O₂.



Protein expression	<i>In situ</i> (%)		Invasive (%)	P-value
	Pure	With invasive component		
TGFβ1				
Negative	23 (46.9)	26 (56.5)	12 (21.8)	0.001
Positive	26 (53.1)	20 (43.5)	43 (78.2)	
E-cadherin				
Negative	9 (18.4)	10 (21.7)	14 (25.5)	0.68
Positive	40 (81.6)	36 (78.3)	41 (74.5)	
β-catenin				
Negative	14 (28.6)	18 (39.1)	16 (29.1)	0.46
Positive	35 (71.4)	28 (60.9)	39 (70.9)	
Snail				
Negative	34 (70.8)	36 (78.3)	36 (65.5)	0.37
Positive	14 (29.2)	10 (21.7)	19 (34.5)	
c-Met				
Negative	45 (91.8)	40 (87.0)	31 (56.4)	<0.001
Positive	4 (8.2)	6 (13.0)	24 (43.6)	

Expression: negative, no expression to +; positive, ++ to +++; excepting for β-catenin were negative, no expression and positive, + to +++.
P, statistical significance by χ^2 test.

and counterstained with Harris hematoxylin. Negative controls were performed by omission of the respective primary antibody. Sections from tissues previously recognized as positive for the selected antibodies were used positive controls. Evaluation of immunostaining scoring was performed by light microscopy. Expression of the biomarkers was semi-quantitatively analyzed by two of the pathologists (A.F.L. and F.A.S.). Specimens that exhibited a complete absence of staining or $\leq 10\%$ of positive cells were considered as negative. Slides were scored according to the percentage of cells showing positivity: 10–25%, +; 25–50%, ++; and $>50\%$, +++. For practical and statistical purposes we grouped the cases presenting 10–25% of positive cells (weakly positive) with the negative cases. Cases graded as moderately (++) and strongly positive (+++) were considered as positive tumors.

Statistical methods. The Spearman's rho pairwise bivariate correlations test was used to estimate the relationship between staining patterns of the different antibodies used. Correlation between antigen expression and other clinicopathological parameters was studied by the χ^2 test. Survival probabilities were estimated by the univariate Kaplan-Meier method, survival curves were compared by the log-rank (Mantel-Haenszel method). P-values <0.05 were considered to be statistically significant. Analysis was performed with SPSS for Windows (release 10.0; SPSS Inc., Chicago, IL, USA).

Results

Table II summarizes protein expression results in *in situ* or in invasive breast ductal carcinoma. Immunohistochemical

analysis revealed TGFβ1 in 78.2% of the invasive cases and the staining was localized in the cytoplasm of tumor cells. β-catenin displaying membranous and or cytoplasmic staining was observed in 70.9% of the cases and was not found in the nucleus. Membranous E-cadherin staining was positive in 74.5% of cases but complete loss was recorded in only 5 cases, c-met and Snail were expressed in respectively 43.6 and 34.5% of invasive breast cancer samples irrespective of nodal involvement and the staining pattern with both antibodies was cytoplasmic (Fig. 1). Most of the positive cases demonstrated a moderate staining for Snail. The evaluation of E-cadherin and Snail expression did not show significant differences in percentage of positivity in DCIS cases as compared to invasive carcinomas. On the other hand, we noted a highly significant increase in positivity of TGFβ1 and in c-met expression along DCIS to IDC progression (53.1 and 8.2% in DCIS versus 78.2 and 43.6%, respectively). β-catenin staining was predominantly membranous in DCIS whereas the staining pattern of the other markers was the same as that seen in the invasive carcinomas. All *in situ* and invasive carcinomas exhibited positive expression of TBR1 (Fig. 2). DCIS either pure or associated to invasive carcinoma did not show differences in frequency of expression of the analyzed biomarkers.

Our next step was to analyze the possible correlation between expression of the different EMT markers in either DCIS or in IDC cases (Table III). In invasive carcinomas no significant correlation between all studied markers was observed except for a strong association between E-cadherin and β-catenin ($P=0.007$). On the other hand in DCIS cases, significant positive association between TGFβ1 and

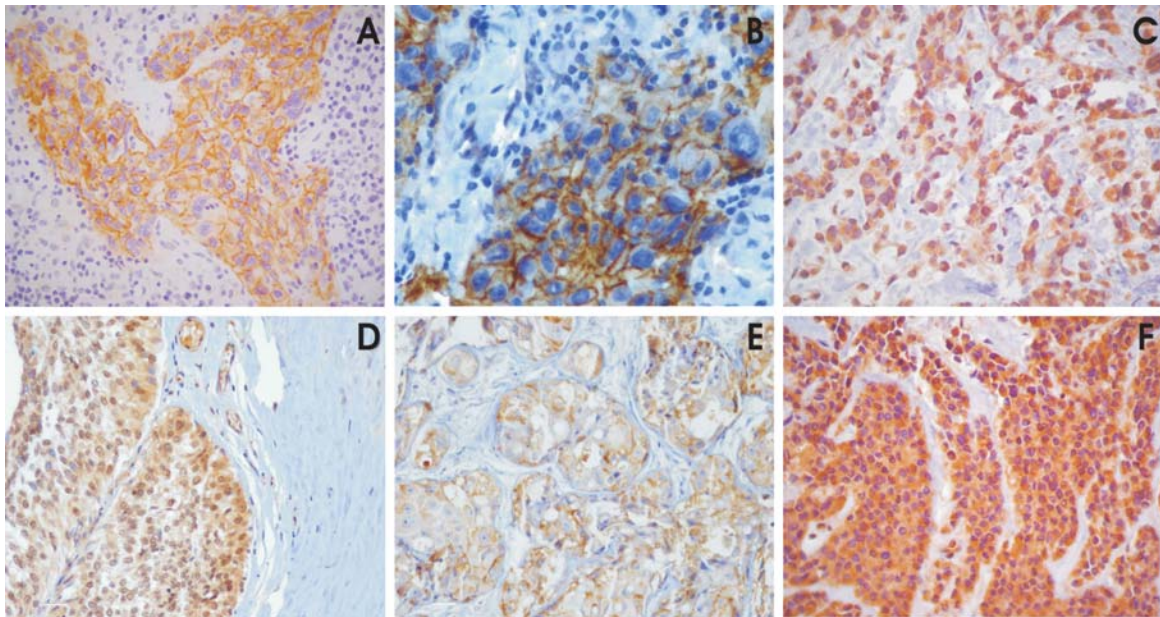


Figure 1. Immunohistochemical detection of EMT proteins in primary breast ductal invasive carcinoma (IDC). (A) Preserved membrane immunoreactivity for E-cadherin (x40) and (B) β -catenin (x100); note a redistribution of β -catenin to the cytoplasm at the interface of the invasion front. (C) Diffuse cytoplasmic staining of TGF β 1 (x40) and (D) TBR1 (x40); (E) granular and cytoplasmic pattern of Snail (x200) and (F) c-met.

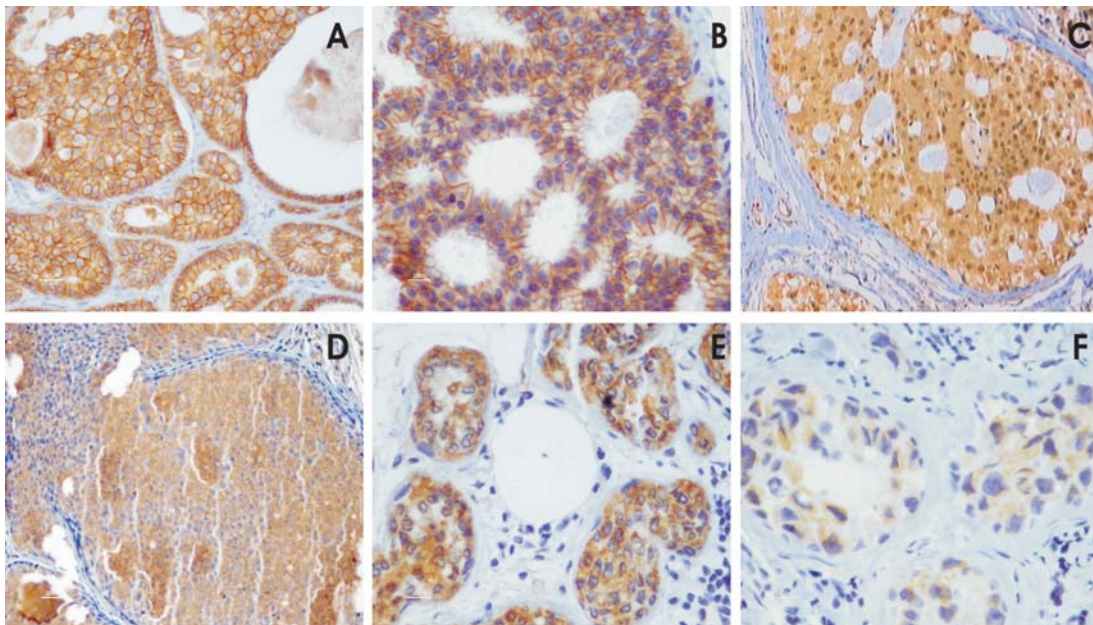


Figure 2. Immunohistochemical detection of EMT proteins in breast ductal carcinoma *in situ* (DCIS). (A) Preserved membrane staining for E-cadherin (x40); (B) β -catenin (x100); (C) Diffuse cytoplasmic staining of TGF β 1 (x40) and (D) TBR1 (x200); (E) granular and subtle cytoplasmic pattern of Snail (x200) and (F) c-met (x200).

respectively E-cadherin ($P=0.001$), β -catenin ($P=0.004$) and c-met ($P=0.035$) were noted.

The prognostic value of all proteins was evaluated by comparing the expression of each EMT marker to classical prognostic variables (presence of metastasis, histological differentiation and immunohistochemical estrogen and progesterone receptor status). TGF β 1 positive cases were associated with advanced stage and presence of compromised lymph nodes (Table IV). No correlation was found between

any EMT protein expression and clinicopathological features of the cases.

In DCIS, no correlation was found between EMT protein expression and tumor histology, presence of ER or PR or differentiation status. In 21 invasive carcinoma cases, samples of the metastatic lymph nodes were obtained. The expression of EMT markers in lymph nodes and in the matched primary tumors was compared and a similar frequency of all biomarkers was observed (Table V).



Variable	E-Cadherin	β -catenin	Snail	c-Met
<i>In situ</i>				
TGF β 1				
Spearman correlation	0.326	0.289	0.123	0.217
P-value	0.001	0.004	0.239	0.035
E-cadherin				
Spearman correlation		0.089	0.173	0.171
P-value		0.391	0.095	0.097
β -catenin				
Spearman correlation			0.158	0.169
P-value			0.126	0.100
Snail				
Spearman correlation				0.037
P-value				0.719
<i>Invasive</i>				
TGF β 1				
Spearman correlation	0.096	-0.048	-0.079	-0.68
P-value	0.488	0.730	0.566	0.623
E-cadherin				
Spearman correlation		0.361	-0.014	0.178
P-value		0.007	0.917	0.195
β -catenin				
Spearman correlation			0.044	0.160
P-value			0.748	0.243
Snail				
Spearman correlation				-0.022
P-value				0.871

Spearman's rho test. A two-sided P-value <0.05 was considered statistically significant.

To determine whether the expression of each of the proposed EMT markers may affect the survival of the patients we prepared Kaplan-Meier survival curves and analyzed them by the log-rank test. Because several clinical parameters are known to affect survival we also analyzed clinical parameters. Survival was found to be reduced in patients presenting compromised lymph nodes ($P=0.018$) with moderate/poor differentiated ($P=0.001$) or with negative receptors ($P<0.001$). However, none of the analyzed EMT markers showed a significant correlation with survival.

Discussion

In this study the expression of several markers associated with the EMT phenotype was concomitantly analyzed in a well defined cohort of breast tumors diagnosed as ductal carcinomas. We sought to document whether the expression

of these factors changed with progression of breast carcinoma and to analyze their utility as biomarkers for overall survival of invasive ductal breast carcinoma patients.

Among the EMT markers evaluated, we observed an increased proportion of c-met and TGF β 1 positivity along the DCIS to invasive ductal breast cancer suggesting their association with tumor progression.

Positivity rate of c-met in invasive and *in situ* breast carcinoma was in the range of two previous studies using immunohistochemistry (9,10). We did not find c-met expression useful in predicting overall survival, although a correlation between c-met and adverse outcome has been reported (10). In this aforementioned study the authors focused on total c-met expression (cytoplasmic and membrane staining) and in our cases we observed only cytoplasmic staining. As we did not find differences in TGF β 1 positivity between pure *in situ* cases and those associated

Table IV. Correlation of TGFβ1 expression in invasive ductal breast carcinoma with other prognostic factors.

Characteristics	TGFβ1 expression		P-value
	Negative (%)	Positive (%)	
N stage			
N0	8 (66.7)	12 (27.9)	0.018
N+	4 (33.34)	31 (72.1)	
T stage			
T1 + T2	9 (81.8)	34 (79.0)	1.00
T3 + T4	2 (18.2)	9 (21.0)	
M stage			
M0	12 (100.0)	27 (62.8)	0.012
M1	0 (0)	16 (37.2)	
Differentiation			
Well	3 (25.0)	11 (25.6)	0.10
Moderate	2 (16.7)	20 (46.5)	
Poor	7 (58.3)	12 (26.9)	
Estrogen receptors			
Positive	4 (57.1)	23 (65.7)	0.68
Negative	3 (42.9)	12 (34.3)	
Progesterone receptors			
Positive	5 (71.4)	18 (51.4)	0.43
Negative	2 (28.6)	17 (48.6)	

P, statistical significance by χ^2 test. A two-sided P-value <0.05 was considered statistically significant. M0, absence of metastasis and M1, presence of metastasis.

to invasive tumors it appears that TGFβ1 status is not a predictor of the clinical course of DCIS in our series. Although our results suggested an association between TGFβ1 and aggressive tumor phenotype (compromised lymph node and advanced stages), implying that an increased TGFβ1 production by tumor cells may augment certain aspects of their malignant phenotype, no relationship with overall survival was found. An elevated expression of TGFβ1 in invasive breast cancer was reported to be associated with disease progression in some studies whereas, others did not describe this predictive values (reviewed in ref. 11). Different methodologies of TGFβ1 evaluation may in part explain different results. Alternatively TGFβ1 may affect survival indirectly, by inducing the expression of other cytokines that confers metastatic ability as recently suggested (12) explaining perhaps the conflicting results of a number of studies engaged to evaluate the potential prognostic value of TGFβ1. Oncogenic properties of TGFβ1 seem to involve loss of TGFβ1 receptor proteins (13) but again TBR11 expression was retained in our cases. However, we can not discard the possibility of alterations in the SMAD signal transducers or activation of non-SMAD signaling pathways by TGFβ1 (4).

Table V. Comparison of protein expression markers between primary invasive ductal breast carcinoma and lymph node metastases.

Protein expression	Invasive (%)	Lymph node (%)	P-value
TGFβ1			
Negative	2 (14.3)	2 (14.3)	1.00
Positive	12 (87.7)	12 (87.7)	
E-cadherin			
Negative	7 (33.3)	3 (14.3)	0.28
Positive	14 (66.7)	18 (85.7)	
β-catenin			
Negative	6 (28.6)	11 (52.7)	0.20
Positive	15 (69.7)	10 (47.6)	
Snail			
Negative	17 (85.0)	18 (90.0)	1.00
Positive	3 (15.0)	2 (10.0)	
c-Met			
Negative	10 (76.9)	8 (61.5)	1.00
Positive	3 (23.1)	5 (38.5)	

Expression: negative, no expression to +; positive,++ to +++; Fisher's exact paired test. A two-sided P-value <0.05 was considered statistically significant.

In accordance to several previous reports we found that E-cadherin expression (membrane linked) was preserved in a considerable proportion of invasive breast cancer, and a similar frequency was displayed by *in situ* tumors (14-20). We did not show that E-cadherin deficiency is a prognostic determinant for survival in agreement with some studies but in contrast to others (21). In spite of being considered as a transcriptional repressor of E-cadherin we failed to verify an indirect relationship between Snail and E-cadherin in ductal breast carcinomas as demonstrated by recent data analyzing Snail messenger RNAs (22). Because of the low repression capacity of Snail on this promotor, it was suggested that other cell specific corepressors are need for complete shutdown of the E-cadherin transcription (23). It was suggested that a function of Snail is to provide cells with survival properties (24) but, we did not find any association between Snail and poor prognostic factors conflicting with few previous studies that reported an association between Snail and presence of metastasis (24,25).

In the present study, β-catenin staining pattern was predominantly membranous in DCIS whereas in the central parts of invasive breast carcinoma it was also localized in the membrane and cytoplasm and absent in the nucleus consistent with other studies (15,16,26-30). However, at the invasive front, in advanced tumors, we have observed a loss of plasma membrane β-catenin accompanied by cytoplasmic accumulation, suggesting that specific signals from the tumor environment may regulate locally the intracellular β-catenin distribution. We chose to evaluate the total level of β-catenin expression as the sum of membrane and cytoplasmic



as described previously (27), but we did not find a correlation between reduction in immunoreactivity of β -catenin and poor prognostic markers, a result aligned with several authors, but in disagreement with others. Discrepant results in the literature, reviewed by Dolled-Filhart *et al* (28), might be due to the lack of detailed information considering different localizations of β -catenin and emphasizing the necessity of analysis of total tumors sections.

In summary, when analyzing the individual prognostic value of the proposed EMT markers in the present study, in comparison with results of other groups, we observed a large variability arising from heterogeneity and size of the cohorts, nature of the antibodies selected, distinct scoring systems and detection methods.

The expected association among EMT was not found in IDC suggesting that *in vivo*, the expression level of each variable in a given tumor assessed as part of the EMT phenotype is a result of specific complex regulatory pathways. Only an association between E-cadherin and β -catenin was observed indicating that perhaps the sustained E-cadherin/ β -catenin axis might be important for disease progression in this particular type of breast carcinoma (16). Statistical co-expression of some different EMT markers that were found in DCIS (such as c-met versus TGF β 1), but disappears in IDC, suggested that such markers may have different functions during tumor progression.

Axillary lymph node metastasis is considered an important event in the metastatic process of breast cancer. We verified that the EMT signature analyzed in the present study could not distinguish the metastatic samples from primary tumors. For instance we found that lymph node metastases continue to express E-cadherin, β -catenin and c-met in accordance with previous studies (31-33), suggesting that the gene expression profiles displayed by primary breast carcinomas and by matching lymph nodes are highly alike (34).

In conclusion, none of the EMT biomarkers analyzed by immunohistochemistry in the present study showed significant prognostic value whereas the prognosis was significantly determined by clinical and hormone parameters.

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