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Study of phospho-β-catenin subcellular distribution in invasive breast carcinomas in relation to their phenotype and the clinical outcome

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β-Catenin has a crucial role in cell–cell adhesion as well as a signaling role as a member of the Wnt pathway. The aim of this study was to examine the clinicopathological and prognostic value of phosphorylated β-catenin, as well as its relation to the tumors' phenotype, in breast cancer. Immunohistochemistry was applied on 141 paraffin-embedded breast tissue specimens for the detection of phospho-β-catenin, ER, PR, c-erbB-2, p53, Ki-67, bcl-2, uPAR and TIMP-1. For each case, a phospho-β-catenin index was determined by image analysis. Phospho-β-catenin staining was detected in the cytoplasm and the nucleus of the malignant cells. Cytoplasmic phospho-β-catenin was statistically higher in carcinomas of smaller tumor size ($P=0.030$), lower stage ($P=0.026$), decreased Ki-67 and high c-erbB-2 immunoreactivity ($P=0.052$ and $P=0.037$, respectively). Nuclear phospho-β-catenin showed a parallel correlation with ER and ERβ ($P=0.022$ and $P=0.043$, respectively), bcl-2 ($P=0.042$), uPAR in cancer cells ($P=0.041$) and TIMP-1, although the correlation was borderline ($P=0.066$). Cytoplasmic phospho-β-catenin was found to be independently correlated with prolonged disease-free and overall survival ($P=0.046$ and $P=0.002$, respectively), whereas nuclear localization was correlated with a shortened overall survival ($P=0.046$). In conclusion, phospho-β-catenin may have a different involvement in invasive breast carcinomas, according to its subcellular distribution. Nuclear localization seems to be related to an aggressive tumor phenotype, negatively affecting patients' overall survival, whereas cytoplasmic localization is associated with a favorable tumor phenotype and a longer disease-free and overall survival.
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β-Catenin is regarded as existing in three different subcellular forms: membrane-bound (as part of an adherens complex with E-cadherin), participating in homotypic cell–cell contacts,[1] cytosolic and nuclear.[2,3] Tyrosine phosphorylation of β-catenin from ErbB receptors leads to its dissociation from the adherens complex[4] and probable transfer of the protein to the cytosol where it exists in a soluble state.[2,3] Cytosolic β-catenin may subsequently be degraded or be translocated into the nucleus. The degradation of β-catenin involves binding of the protein to a complex involving the proteins APC, AXIN and glycogen synthase kinase (GSK) 3β. The latter phosphorylates β-catenin, a crucial step required to target the protein for ubiquitination and proteosomal degradation.[2,3] An important regulator of GSK-3β activity is the Wnt family. Binding of Wnt glycoproteins to their receptors leads to the inhibition of GSK-3β phosphorylating activity and the cytosolic accumulation of β-catenin, which, unphosphorylated, cannot be degraded in proteosoma.[4] In the presence of increased cytosolic levels of β-catenin, the protein is translocated into the nucleus, where, in cooperation with transcription factors, it activates transcription of target genes.[5] A considerable number of target genes of Wnt signaling have been identified to date, some of which are involved in tumorigenesis, such as c-*Myc* and *cyclin*

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D1,[6,7] or in tumor invasion, such as the receptor of the protease urokinase-type plasminogen activator (uPA), *uPAR*.[8,9]

A wide range of tumors, including breast cancer, have been examined for mutations in β-catenin as a mechanism of activation of this pathway.[10–13] In these tumors, mislocalization to the cytoplasm or the nucleus is frequent, but the clinicopathological and prognostic impact of this finding has not been elucidated yet.[14–16]

The aim of our study was to examine (a) the expression pattern of phospho-β-catenin protein in invasive breast carcinoma, (b) its possible clinicopathological and prognostic significance through its correlation with the classical clinicopathological parameters (menopausal status, histological grade, tumor size, lymph node status, stage of the disease, ER/PR) and patients' disease-free and overall survival and (c) its involvement in tumor's phenotype through its correlation with markers indicative of aggressive (c-erbB-2), proliferative (Ki-67), apoptotic (p53, bcl-2) and invasive (TIMP-1, uPAR) phenotype.

Materials and methods

Patients and Tissue Specimens

A total of 141 paraffin-embedded breast tissue specimens were available from patients with resectable breast cancer, without distant metastasis at the time of diagnosis. We selected only women with histologically proven, clearly invasive breast carcinomas, aged 25–84 years (mean age 56.43 years). None of them had received radiation or chemotherapy preoperatively.

Routine histological examination was performed with hematoxylin–eosin staining. Conventional histological classification of the World Health Organization was applied[17] and carcinomas were recorded as invasive ductal or invasive lobular. The combined histological grade (1, 2 and 3) of invasive ductal carcinomas was obtained according to Elston.[18] Nuclear grading was based on nuclear pleomorphism and mitotic activity. Tumor staging was performed according to the TNM system of the International Union against Cancer.[19] Lymph node status was evaluated separately and, according to the number of positive nodes, three groups were formed (0, 1–4 and >4 positive axillary lymph nodes).

Follow-up was available for 136 patients. Mean survival time was 97.23 months (range 5–135 months). Patients' outcome was defined as disease-free and overall survival. All patients received conventional postoperative treatment depending on the extent of the disease, including radiation therapy and anti-estrogen therapy when indicated. Premenopausal patients with axillary involvement were treated with six courses of adjuvant chemotherapy.

Immunohistochemistry

The tumor samples were fixed in 10% buffered formalin solution for no more than 10 h. Paraffin-embedded tissue sections, 4 μm thick, were cut on poly-L-lysine-coated slides, dried, deparaffinized and incubated with 0.3% hydrogen peroxide (H_2O_2) for 30 min to block endogenous peroxidase activity. To enhance antigen retrieval, sections were microwave-treated in 0.01 M citrate buffer (pH 6.0) at 750 W for 10 min. Then, normal horse serum was applied for 40 min to block nonspecific antibody binding. Subsequently, sections were incubated overnight at 4°C with the primary antibody to phospho-β-catenin (polyclonal antibody produced by immunizing rabbits with a synthetic phospho-Ser 33/Ser 37/Thr 41 peptide corresponding to residues around Ser 37 of human β-catenin, #9561, Cell Signaling Technology Inc.) at a dilution of 1:50. In order to test manufacturing company's assurance that the used antibody does not recognize non-phosphorylated β-catenin, we performed immunohistochemistry with phospho-β-catenin antibody in cases with known positivity for β-catenin, which had been studied in the past[20] and we ascertain that the staining pattern for the two antibodies was completely different. After rinsing in TBS, sections were incubated with biotinylated anti-rabbit secondary antibody (Vector Labs, Burlingame, CA, USA) for 30 min at room temperature and then incubated with avidin–biotinylated peroxidase complex (Vectastain Elite ABC Kit, Vector Labs) for 30 min. The peroxidase reaction was developed with a 0.5 mg/ml solution of 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co, St Louis, MO, USA) supplemented with 0.01% H_2O_2. Finally, sections were counterstained with Harris hematoxylin.

Positive controls included sections from carcinomas with known immunopositivity. Negative controls had the primary antibodies omitted and replaced by (a) nonimmune, normal serum from the same species as the primary antibody and (b) TBS.

The other immunomarkers assessed in the present study in combination with phospho-β-catenin had been previously detected with the following antibodies: rabbit anti-human Ki-67 (Dako, Glostrup, Denmark) at a dilution of 1:50, monoclonal anti-c-erbB-2, clone CB11 (Biogenex, San Ramon, USA) at a dilution of 1:150, monoclonal anti-p53, clone BP53.12.1 (Oncogene, Cambridge, USA) at a dilution of 1:50, mouse monoclonal anti-bcl-2 IgG_1 antibody (Dako, Glostrup, Denmark) at a dilution of 1:100, anti-uPAR (catalog No. 3932) (American Diagnostica Inc.) at a dilution of 1:100, anti-ER (clone 1D5) and anti-PR (clone 1A6) (Dako) at dilutions of 1:450 and 1:150, respectively, Serotec anti-ERβ mouse monoclonal antibody (clone PPG5/10, Raleigh, NC, USA) at a dilution of 1:25 and monoclonal anti-TIMP-1 antibody, clone 147-6

Table 1 The cutoff points of the various immunohistochemical markers

Ki-67 immunopositivity	≤10% of malignant cells >10% of malignant cells
c-erbB-2 expression	0 (negative): no or membrane staining in <10% of tumor cells 1+ (negative): faint/barely perceptible in >10% and stained only in part of the membrane 2+ (positive): weak or moderate, complete membrane staining 3+ (positive): a strong complete membrane staining in >10% of tumor cells
p53 expression	0–10% of malignant cells ≥10% of malignant cells
bcl-2 expression	Totally negative <10% of malignant cells 10–50% of malignant cells ≥50% of malignant cells
ERβ expression	0–10% of malignant cells 10–40% of malignant cells ≥40% of malignant cells
TIMP-1 immunoexpression	0–30% of malignant cells ≥30% of malignant cells
uPAR of malignant cells	<40% of malignant cells ≥40% of malignant cells

D11 (Daiichi Fine Chemical Co., Ltd, Medicorp, Montreal, Canada), at a dilution of 1:60.

The immunohistochemistry for the aforementioned markers was applied on serial sections from the same paraffin blocks of the patients. The immunohistochemical staining of each marker was evaluated by two independent pathologist through light microscopic observation, using a semiquantitative method. Discrepancies between the observers were found in <10% of the slides examined and consensus was reached on further review. The score resulted as the average of 10 distinct high-power fields observed under ×400 magnification. Staining intensity and the number of stained cells were taken into consideration throughout the evaluation process. Cutoff points were determined as shown in Table 1. Steroid receptor status was assessed by the H-score according to McClelland *et al*.[21]

Image Analysis Method

In each case, 500 cancer cells were evaluated and the scoring of phospho-β-catenin immunostaining was performed by image analysis. Images were acquired using a Zeiss Axiolab microscope (Carl Zeiss Jena GmbH, Jena, Germany) with a mechanical stage, fitted with a SONY-iris CCD video camera (SONY Corp., Tokyo, Japan). The video camera was connected to a Pentium II personal computer loaded with Image Scan software (Jandel Scientific, Erkrath, Germany).[22,23] Digital images were stored as JPEG files (1550×1070 pixels, 16.7 million colors). Further evaluation took place via the Color Estimator v. 2.0, a specific application developed in our laboratory in a Microsoft Visual Basic 5.0 (Microsoft Corp., Redmond, WA, USA) environment for the evaluation of histochemical and immunohistochemical color images. First, the digital images were converted into bit-map files of 256 colors; an accurate determination and a graphic representation of the total neoplastic nuclei/cells (stained and unstained) followed (with the term cells we mean cytoplasmic staining). Automatically, the immunostained nuclei were drawn in red (color code: 2630911). Finally, a ratio was calculated as the number of immunohistochemically positive neoplastic nuclei (red color in digital images) in the total number of 500 nuclei (stained and unstained) (Figure 1a and b).

Statistics

Non-parametric statistics were performed to evaluate the association between phospho-β-catenin and the various parameters of interest: (a) Mann–Whitney test to evaluate phospho-β-catenin in the dichotomous variables and (b) Kruskal–Wallis one-way analysis with ranks to assess the differences of phospho-β-catenin in ordinal variables with more than two groups (graphical representation of data is shown using box plots). Overall and disease-free survival distributions were assessed by univariate statistics (log-rank test) and by multivariate statistics (Cox's proportional hazard regression model).

Results

Phospho-β-catenin staining was generally distributed in the cytoplasm (62/141) and the nucleus (107/141) (43.97 and 75.88%, respectively) of the malignant cells (Figure 1a, c and d) and was also evident in normal epithelium adjacent to the tumor and the staining was mainly cytoplasmic with weak to moderate intensity. For the cytoplasmic localization, phospho-β-catenin indices ranged from a low of 0% to a high of 87% (mean±s.d.=12.38±20.4), and for the nuclear localization, from a low of 0% to a high of 88% (mean±s.d.=22.48±25.67). Cytoplasmic phospho-β-catenin values were statistically higher among carcinomas characterized by smaller tumor size ($P=0.030$), lower stage ($P=0.026$) (Kruskal–Wallis analysis), decreased Ki-67 staining ($P=0.052$) and high c-erbB-2 immunoreactivity ($P=0.037$) (Mann–Whitney test) (Table 2). Additionally, nuclear phospho-β-catenin values were statistically higher among carcinomas characterized by high ER and ERβ immunopositivity ($P=0.022$ with Kruskal–Wallis analysis and $P=0.043$ with Mann–Whitney test, respectively), in tumors with high bcl-2 protein expression ($P=0.042$ with

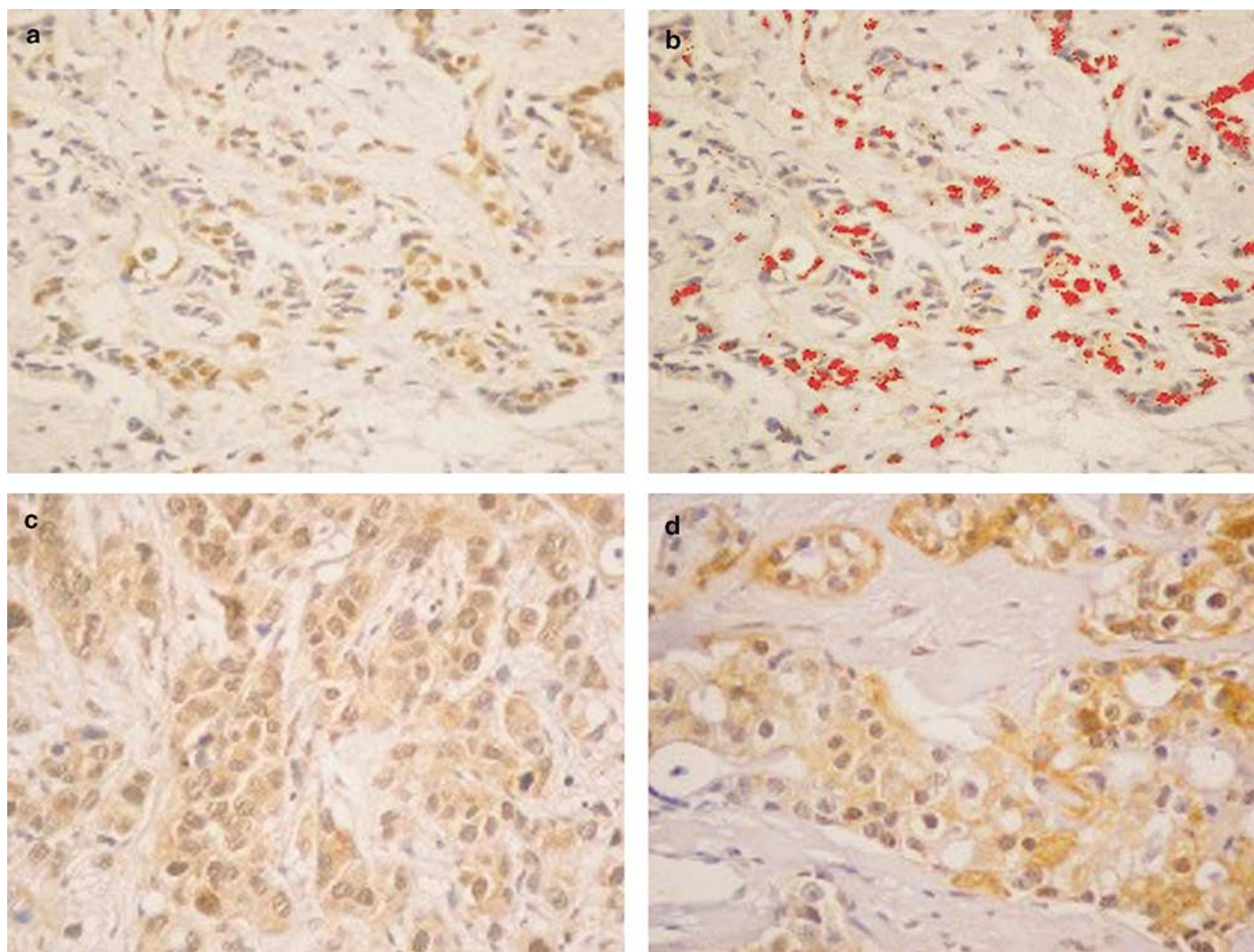

Figure 1 Phospho-β-catenin protein immunoreactivity (**a**) in the nuclei of cancer cells (ABC/HRP × 400), (**b**) the same as (**a**) elaborated with the appropriate image analysis software, (**c**) in the nuclei and the cytoplasm and (**d**) in the cytoplasm of invasive breast carcinoma cells (ABC/HRP × 400).

Kruskal–Wallis analysis) and finally among tumors with high uPAR ($P = 0.041$ with Mann–Whitney test) and high TIMP-1 proteins expression, although the latter correlation was of borderline significance ($P = 0.066$ with Mann–Whitney test) (Table 2).

On the other hand, no significant difference was detected between phospho-β-catenin values and menopausal status, histologic type and grade, lymph node status and p53 (Table 2).

With regard to patients' survival, univariate analysis showed that the presence of cytoplasmic phospho-β-catenin immunostaining correlated with a prolonged disease-free and overall survival ($P = 0.0060$ and $P = 0.0012$, respectively) (Figure 2). This correlation was further verified by the multivariate analysis where cytoplasmic phospho-β-catenin emerged as an independent prognosticator of a prolonged disease-free and overall survival ($P = 0.046$ and $P = 0.002$, respectively), whereas nuclear localization was found to be an independent prognostic factor of a shortened overall survival ($P = 0.046$) (Table 3a and b).

Discussion

In the present study, we investigated the immunoexpression of phospho-β-catenin in a series of invasive breast carcinomas and we found its different localizations to be related to different tumors' phenotype and different effects on patients' survival.

Phospho-β-catenin was immunodetected in both the cytoplasm and the nuclei of tumor cells. The finding of phospho-β-catenin in the nucleus is peculiar because the literature suggests that the phosphorylated form of the protein should be rapidly degraded and the unphosphorylated form is the one that activates transcription in the nucleus.[4,5] Nevertheless, our immunodetection is in accordance with that of Kielhorn *et al*[15] and Chung *et al*,[16] who, interestingly, found phospho-β-catenin expression to be almost exclusively localized in the nuclei of both cultured cells and human melanoma and colorectal cancer tissue, respectively. The nuclear localization is possibly related to either an overexpression of the protein, which overwhelms

Table 2 Phospho-β-catenin protein expression in the cytoplasm and the nuclei of cancer cells as related to clinicopathological parameters and immunohistochemical markers

	N	*Phospho-β-catenin*					
		Cytoplasm			*Nuclei*		
		Median	*Interquartile range*	*P-value*	*Median*	*Interquartile range*	*P-value*
Menopausal status							
Before menopause	47	4	0–21	NS	7	0–40	NS
After menopause	94	0	0–20		12	0–42	
Stage							
1	26	11	0–32	0.026	37	2–54	NS
2	90	0	0–20		8	0–40	
3	22	0	0–13		6	0–34	
Histologic type							
Ductal	113	0	0–21	NS	8	0–42	NS
Lobular	26	0	0–6		19	0–41	
Nuclear grade							
1	51	7	0–26	NS	18	0–46	NS
2	49	0	0–7		10	0–37	
3	39	0	0–20		6	0–29	
Histologic grade							
1	18	11	0–28	NS	23	0–42	NS
2	63	0	0–21		8	0–42	
3	32	0	0–14		5	0–28	
Tumor size							
<2 cm	37	10	0–26	0.030	20	0–51	NS
2–5 cm	83	0	0–20		8	0–40	
>5 cm	17	0	0–0		6	1–32	
Lymph nodes							
Non-infiltrated	56	2	0–21	NS	13	1–42	NS
Infiltrated	82	0	0–20		8	0–41	
ER							
0	58	0	0–19.3	NS	5.5	0–32	0.022
1+2	41	0	0–30		5	0–39	
3	41	0	0–24		27	7–49	
ERβ							
Negative	31	0	0–11	NS	5	0–26	0.043
Positive	48	6	0–30		18	1–58	
PR							
0	67	0	1–15	NS	12	0–41	NS
1+2	60	0	0–26		5.5	0–38.8	
3	13	0	0–22.5		40	3.5–69	
p53							
Negative	94	0	0–22	NS	8	0–41	NS
Positive	43	0	0–18		11	2–42	
Ki-67							
Negative	61	7	0–25	0.052	10	0–42	NS
Positive	43	0	0–15		6	0–40	
c-erbB-2							
Negative	56	0	0–10	0.037	6	0–39	NS
Positive	82	3	0–22		12	0–43	
bcl-2							
0	40	0	0–12	NS	6	0–23.5	0.042
1	24	0	0–13.3		5.5	0–34	
2–3	40	9	0–36.5		25.5	0.3–59.8	
uPAR of cancer cells							
0–40%	51	0	0–10	NS	7	0–33	0.041
≥40%	83	1	0–24		14	2–45	
TIMP-1 protein stromal cells							
Negative	42	0	0–14	NS	6	0–31	0.066
Positive	55	0	0–26		20	0–42	

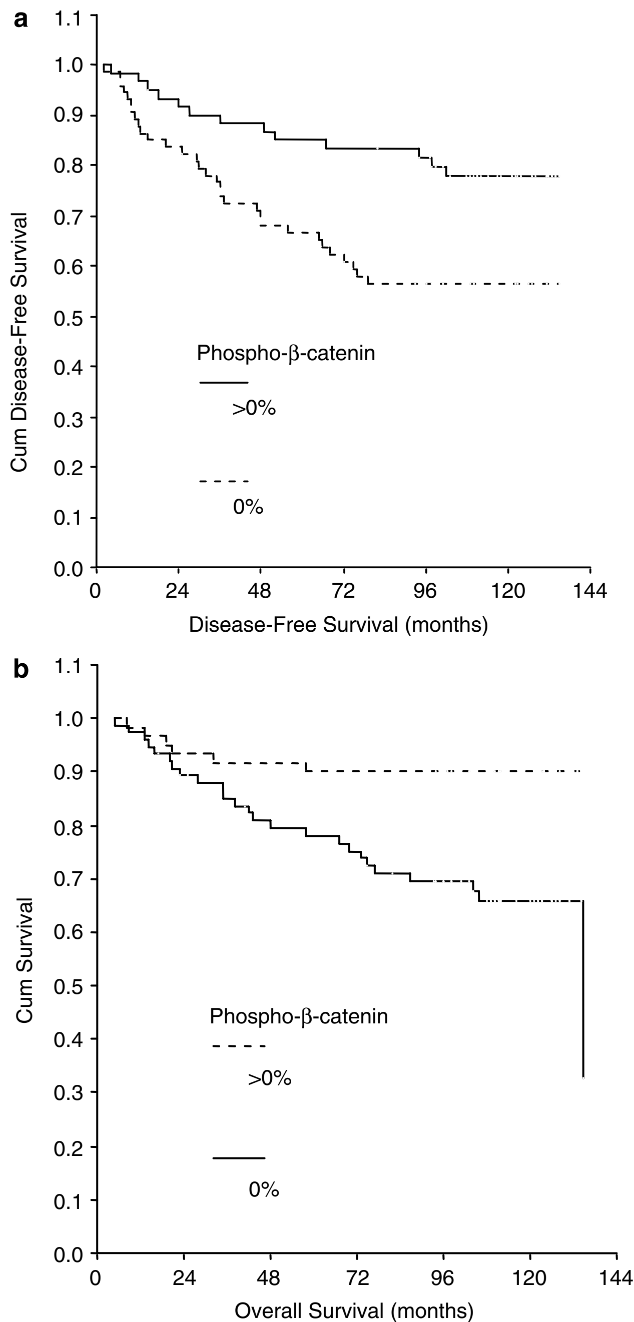

Figure 2 The effect of cytoplasmic phospho-β-catenin expression on both disease-free (**a**) and overall survival (**b**) of the patients (Kaplan–Meier curves).

the normal degradative mechanisms and results in nuclear translocation, independently of its phosphorylation status, or to alterations at other stages of the signaling pathway and the degradation machinery.[15,16] Furthermore, we immunodetected phospho-β-catenin in the cytoplasm of the tumor cells as well, in agreement with the literature suggesting that the phosphorylated form of β-catenin should be present in the cytoplasm in order to be degraded. However, we are in disagreement with the aforementioned authors,[15,16] who mentioned phospho-β-catenin expression to be exclusively localized in the nuclei of melanoma and colorectal cancer tissue, a discrepancy that may be attributed to the different tissues and methods used.

In the present study, cytoplasmic phospho-β-catenin was inversely correlated with tumor size ($P = 0.030$) and stage ($P = 0.026$), two well-established prognosticators in cancer, and Ki-67 ($P = 0.052$), a biological marker of active cell proliferation. In other words, this study is the first to mention the relation of the cytoplasmic phospho-β-catenin immunoexpression to a less aggressive tumor phenotype through its inverse correlation with tumor size, the stage of the disease and Ki-67. Interestingly, cytoplasmic phospho-β-catenin was found to have a parallel correlation with c-erbB-2, a well-established marker of aggressive tumor phenotype, which is contradictory to the aforementioned suggestion of cytoplasmic phospho-β-catenin's participation in the emergence of a less aggressive tumor phenotype. However, this parallel association could be related to the fact that ErbB receptors can induce tyrosine phosphorylation of β-catenin,[6,7] which prevents the binding of β-catenin to E-cadherin, potentially transferring β-catenin to the cytosol, from where it may subsequently be degraded or be translocated into the nucleus.[2,3] In other words, the parallel correlation between c-erbB-2 and cytoplasmic phospho-β-catenin could depict the contribution of c-erbB-2 to the increase in the cytoplasmic pool of β-catenin.

Moreover, the nuclear immunolocalization of phospho-β-catenin showed a parallel correlation with ER and ERβ, bcl-2, uPAR as well as with TIMP-1 proteins expression. The parallel association between nuclear phospho-β-catenin and ER is in conformity with studies,[24–26] that have shown the physical, transcriptional and genetic interaction *in vivo* between ER and β-catenin on different tissues. However, this is the first study to find a positive correlation between β-catenin and ERβ, implying a possible interaction between ERβ and β-catenin, similar to the aforementioned interaction between ER and β-catenin. Furthermore, bcl-2 is a well-established antiapoptotic factor that is able to enhance cell survival through apoptosis inhibition.[27] In other words, in the present study, we found a parallel correlation between nuclear localization of phospho-β-catenin and an antiapoptotic marker, a finding that is in accordance with the general notion that nuclear β-catenin signaling has an antiapoptotic effect[28] in that mechanisms that reduce such signaling, such as overexpression of APC or AXIN protein,[29] also induce apoptosis.

The parallel correlation between nuclear phospho-β-catenin and the expression of uPAR in malignant cells is in accordance with the identification of uPAR as a target gene of β-catenin signaling pathway and the *in vivo* finding of good correlation between β-catenin mRNA and uPAR protein among colorectal adenocarcinomas.[9] However, this is the

Table 3 Contribution of parameters of statistical significance to patients' (a) disease-free and (b) overall survival through stepwise forward Cox's proportional hazard regression model adjusted for the following parameters: menopausal status, tumor size, histological type, grade, stage, lymph node status, ER/PR, c-erbB-2, Ki-67, bcl-2, uPAR, TIMP-1, received therapy, cytoplasmic and nuclear phospho-β-catenin

	B	*s.e.*	*Sig.*	*Exp*(B)	*95% CI for Exp*(B)	
					Lower	*Upper*
(a) *Disease-free survival*						
Cytoplasmic phospho-β-catenin	−0.849	0.425	0.046	0.428	0.186	0.984
Stage	0.832	0.362	0.022	2.297	1.130	4.671
Lymph node status	0.511	0.230	0.026	1.667	0.062	2.619
(b) *Overall survival*						
Cytoplasmic phospho-β-catenin	−2.626	0.863	0.002	0.072	0.013	0.392
Nuclear phospho-β-catenin	0.024	0.012	0.046	1.025	1.000	1.049
bcl-2	−0.687	0.288	0.017	0.503	0.286	0.884

first study to show a correlation between nuclear phospho-β-catenin and uPAR protein expression in invasive breast carcinomas, suggesting a possible involvement of nuclear phospho-β-catenin in breast carcinomas' invasive phenotype.

Furthermore, nuclear phospho-β-catenin was found to be positively related to TIMP-1. TIMP-1 belongs to TIMPs, the endogenous inhibitors of metalloproteinases (MMPs), which, through non-covalent binding of the active forms of MMPs, play a pivotal role in the turnover of the extracellular matrix.[30] However, the TIMP family may be bifunctional, exerting both proteinase inhibitory and cell-stimulatory functions.[30] TIMP-1 in particular may promote cell growth activity on several cell types[31,32] and inhibit apoptosis in breast epithelial cell lines.[33] In other words, the parallel correlation between nuclear phospho-β-catenin and the multifunctional TIMP-1 may indicate the possible participation of nuclear phospho-β-catenin in tumor's invasive, antiapoptotic as well as growth-promoting phenotype.

With regard to the survival, this is the first study to show a different impact of the different subcellular localizations of phospho-β-catenin on patients' survival. Thus, whereas cytoplasmic phospho-β-catenin was found to be an independent prognosticator of a prolonged disease-free and overall survival ($P = 0.046$ and $P = 0.002$, respectively), the nuclear one emerged as an independent prognostic factor of a shortened overall survival ($P = 0.046$). In the literature, the prognostic value of β-catenin has not been elucidated yet, as the results of the various studies are contradictory. There are studies that have correlated the nuclear phospho-β-catenin expression with the decrease in the survival of patients with melanoma or breast cancer[14,15] and other, that have associated it with a good prognosis in colorectal cancer.[16] No other study has correlated cytoplasmic phospho-β-catenin with the prognosis of the disease. In our opinion, the different effects of phospho-β-catenin on patient's survival according to its subcellular distribution is in line with the knowledge that cytoplasmic phospho-β-catenin is destined for ubiquitin-mediated proteosomal degradation, decreasing the β-catenin pool disposable for transcription activation in the nucleus, where it regulates cell proliferation, apoptosis and invasion.[28]

In conclusion, the present study indicates, for the first time, the fact that phospho-β-catenin seems to have a different involvement in tumors' phenotype and different prognostic value, according to its subcellular distribution. Thus, nuclear phospho-β-catenin may be implicated in the emergence of an aggressive and invasive tumor phenotype through its positive correlation with the antiapoptotic marker bcl-2, ER, ERβ[34] and uPAR, negatively affecting the patients' overall survival. On the contrary, cytoplasmic phospho-β-catenin seems to be associated with a favorable tumor phenotype through its inverse correlation with tumor size, stage and Ki-67, prolonging disease-free and overall survival. Finally, we suggest that more investigation is required in order to elucidate the mechanism by which phospho-β-catenin is localized in the nucleus of cancer cells and whether, even if phosphorylated, it is able to serve as a transcriptional activator, affecting tumor's biological behavior.

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