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Correlation Between Expression of Twist and Podoplanin in Ductal Breast Carcinoma

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Abstract. *Background/Aim: As a result of activation of transcription factors engaged in epithelial–mesenchymal transition (EMT), such as Twist, inhibition of epithelial markers and an increased expression of mesenchymal markers are observed. One of the specific markers of cancer-associated fibroblasts is podoplanin (PDPN) – a mucin-type membrane glycoprotein. The aim of this work was to study the localisation and intensity of expression of Twist and PDPN on the mRNA and protein level in cases of invasive ductal breast carcinoma (IDC), and its association with patients' clinico-pathological data. Materials and Methods: The study included archival material in a form of 80 paraffin IDC blocks and 11 IDC fragments frozen in liquid nitrogen. Immunohistochemical expression of Twist and PDPN was evaluated using light microscope and semiquantitative scale for evaluation of nuclear expression or immunoreactive scale (IRS) for evaluation of cytoplasmic expression. Material was isolated from frozen IDC fragments using laser micro-dissection (from cancer and stromal cells, separately) and was used to perform real-time PCR. Results: Twist expression was higher in stromal cells in comparison to cancer cells. Analysis of patients' survival rate showed, that higher expression of Twist in cancer cells was associated with shorter overall survival time and shorter event-free survival time. The expression of PDPN was also higher in stromal cells in comparison with cancer cells. In addition, positive correlation was observed between expression of Twist and PDPN in stromal cells of IDC ($r=0.267$; $p<0.05$). Conclusion: The relationship between the higher expression of Twist in both cancer and stromal cells and shorter patients' survival indicates Twist as a potential useful prognostic marker in IDC. Positive correlation of Twist and PDPN expression may indicate the role of PDPN in EMT in IDC.*

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According to the estimations of the International Agency for Research on Cancer (IARC), 14.1 million new malignant cancer cases were diagnosed and 8.2 million people deceased of malignant tumors in 2012 only. This is the second most common cause of death in the world (1, 2). Breast cancer is the most common cancer among women and it is a serious clinical problem. In 2012, its incidence was 1.67 million, accounting for 25% of overall incidence of all malignant cancers in women worldwide (2).

During the process of breast carcinoma progression, a phenomenon called epithelial–mesenchymal transition (EMT) is observed. There are 3 types of EMT distinguished on account of the variability of the biological processes it is involved in (3). Type 1 plays a key role in embryogenesis - during gastrulation and neural tube formation (4). At this stage, primary epithelial cells are transformed into primary mesenchymal cells. Next, at the target location, those mesenchymal cells undergo reverse process, mesenchymal-epithelial transition (MET), and form secondary epithelial cells. They participate in organogenesis or undergo apoptosis. Type 2 results from chronic inflammation, which stimulates formation of fibroblasts from fully-differentiated epithelial or endothelial cells. As a result, organs such as kidneys, lungs or liver become fibrous (5-7). Additionally, type 2 EMT is observed during the wound healing process. Type 3, on the other hand, is associated with carcinogenesis and distant metastases formation. It allows cancer cells in the primary site to break the basilar membrane barrier and blood vessel walls, translocate and invade other tissues. Moreover, it is believed, that the activation of EMT allows cancer cells to bypass defence mechanisms of the immune system, avoid

induction of apoptosis and determine resistance to chemotherapy (8). Potential inductors of EMT are transforming growth factor (TGFβ), hepatocytes growth factor (HGF), fibroblast growth factor (FGF), hypoxia and interactions with constituents of extracellular matrix (ECM). However, the exact activation mechanism of this process is not known (9). As a result of activation of transcription factors engaged in EMT, such as Twist, Snail and Slug, inhibition of expression of genes encoding proteins characteristic for epithelial cells and an increase in the expression of genes characteristic for mesenchymal cells are observed. This leads to the loss of intercellular connections, inter alia by lowering E-cadherin expression and increasing expression of N-cadherin, vimentin and fibronectin, which are characteristic of mesenchymal cells (10). Moreover, cytoskeleton reorganisation and production of ECM-degrading metalloproteinases occur during EMT (3). As a result of the above-mentioned processes, epithelial or cancer cells that underwent this process, acquire migration and invasion capabilities.

In the available literature, the role of Twist transcription factor in EMT is well documented (11). This protein belongs to the family of transcription factors comprising a helix-turn-helix domain. It also plays an important role during embryonic development in myogenesis, limb development, as well as formation and connection of cranial sutures (12). Nuclear expression of Twist is observed in cells of cancers such as breast, lung, liver and ovarian cancer and it is associated with poorer prognosis (13-19).

Cells of cancer and its stroma closely communicate with each other, forming complex networks of signal factors engaged in the initiation and progression of cancer disease (20). Cancer microenvironment is formed by the heterogeneous group of cells consisting of fibroblasts, endothelial cells, immune cells, smooth muscle cells, adipocytes and ECM (21). A special role is given to cancer-associated fibroblasts (CAFs), which are probably actively involved in cancer progression (22). They can be found in the stroma, have spindle shape and show expression of smooth muscles actin (SMA), vimentin (VIM) and podoplanin (PDPN) (21, 23). CAFs show similarity to activated fibroblasts, which take part in wound healing and fibrosis of organs (SMA+, FN+).

In terms of histogenesis, CAFs are a heterogeneous group of cells with varied origin depending on the histological type of cancer and their localisation within the tumor (20, 24). Potential precursors of CAFs may be activated residual fibroblasts, bone marrow mesenchymal stem cells (MSCs) and cancer cells, which underwent EMT. So far, none of these theories have been fully proved (25).

Podoplanin (D2-40, PDPN) is one of the characteristic markers for CAFs. It is a membrane glycoprotein that belongs to mucin-type proteins (26). PDPN is also expressed in the endothelium of lymphatic vessels, podocytes, cells of skeletal muscles, placenta, heart and type I pneumocytes (27, 28). The role of PDPN is not fully understood. It is known, however, that it is necessary for normal development of lungs, lymphatic system and heart (26). It is believed, that PDPN may have an impact on cancer progression, however, the mechanism of such action remains unclear. CAFs (PDPN+) were observed in many cancers, such as skin, lung, breast, bile ducts, colon, cervix and oesophagus cancer (29-31). As shown previously, PDPN expression in CAFs localised within invasive ductal breast cancer (IDC) and was correlated with the microvessel count (MVC). It might suggest, that PDPN is involved in the process of cancer angiogenesis (32). It is also believed, that because PDPN has an extracellular platelet aggregating domain (PLAG) (33), which is the ligand for C-type lectin-like receptor-2 (CLEC-2) (34), it may play an important role in platelet aggregation. In the process of cancer progression, platelet aggregation on the surface of cancer cells probably enables avoidance of immune mechanisms and allows metastasis (35).

Previous studies also suggested a correlation between PDPN expression and cancer progression – both EMT-related and non-EMT-related (28, 36-37). In studies performed using oesophagus cancer cells lines it was observed, that E-cadherin expression (epithelial marker) was lower in high podoplanin-expressing cell lines (38). Similar observations were made regarding squamous cell skin carcinoma, in which increased expression of Twist, Zeb1, vimentin and beta-catenin was associated with an increased podoplanin expression. In the same cases E-cadherin delocalisation was observed, wherein the loss of its membrane localisation was associated with loss of ability to form intercellular connections (39).

The aim of this study was to investigate localisation and expression of Twist and PDPN on mRNA and protein level in cases of IDC. Additionally, the purpose was to analyse relationships between above-mentioned proteins in correlation with clinico-pathological data from IDC cases.

Materials and Methods

Patients. The studies were performed on archival material of 80 paraffin-embedded samples and 11 frozen IDC tissue samples. All materials were obtained from the Department of Tumour Pathology of the Maria Sklodowska-Curie Memorial Centre and Institute of Oncology in Cracow, sampled in 2000-2006. All patients were treated surgically (radical mastectomy or conservative quadrantectomy followed by axillary lymph node resection) and tissue specimens were collected before the beginning of the chemotherapy. The patients from IHC group were followed up for 80.68±35.06 (median=85.491; range=1-145) months. In this period, 18 patients deceaed. The clinicopathological data are presented in Table I.

Immunohistochemistry. Tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Haematoxylin and eosin (H&E) were used to stain all sections for diagnosis and revision of

Table I. *Clinical and pathological characteristics of studied patients.*

Parameters	Patients			
	IHC N=80	%	PCR N=11	%
Age				
≤50	21	26.25	4	36.36
>50	59	73.75	7	63.64
Menopausal status				
Pre	24	30	No data	-
Post	55	68.75	No data	-
No data	1	1.25	-	-
Tumor grade				
G1	4	5	3	27.27
G2	47	58.75	5	45.46
G3	29	36.25	3	27.27
Tumor size				
pT1	38	47.5	2	18.18
pT2	40	50	6	54.55
pT3	2	2.5	0	0.00
pT4	0	0	3	27.27
Lymph nodes				
pN0	20	25	4	36.36
pN1 - pN3	60	75	6	54.55
pNx	0	0	1	9.09
Stage				
I	9	11.25	1	9.09
II	48	60	6	54.55
III	23	28.75	4	36.36
IV	0	0	0	0.00
ER				
Negative	28	35	6	54.55
Positive	52	65	4	36.36
No data	0	0	1	9.09
PR				
Negative	31	38.75	6	54.55
Positive	49	61.25	4	36.36
No data	0	0	1	9.09
HER2				
Negative	65	81.25	2	18.18
Positive	14	17.5	8	72.73
No data	1	1.25	1	9.09
Molecular tumor types				
Triple negative	16	20	4	36.36
Other types	63	78.75	6	54.55
No data	1	1.25	1	9.09
Ki-67				
Low proliferation ≤25	54	67.5	No data	-
High proliferation >25	26	32.5	No data	-

malignancy grade. Immunohistochemical reactions were performed on 4-μm-thick sections in an Autostainer Link 48 instrument (DakoCytomation, Glostrup, Denmark). In order to deparaffinise and retrieve antigen, the sections were boiled in Target Retrieval Solution (97˚C, 20 min; pH 9; DakoCytomation, Glostrup, Denmark) in Pre-Treatment Link Rinse Station. After rinsing the sections in FLEX Wash Buffer (Tris-buffered saline solution containing 0.05% Tween 20), endogenous peroxidase was blocked using EnVision FLEX Peroxidase-Blocking Reagent (5 min incubation at room temperature). Afterwards, the sections were incubated for 20 min at room temperature with primary murine monoclonal antibodies directed against Twist (ab50887; 1:50; Abcam, Cambridge, UK) and PDPN (D2-40 ready-to-use, RTU; DakoCytomation, Glostrup, Denmark). To enhance the signal for Twist, the slides were also incubated for 15 min at room temperature with EnVision FLEX+ Mouse LINKER (DakoCytomation, Glostrup, Denmark). EnVision FLEX (DakoCytomation, Glostrup, Denmark) was used to visualize the antigens. All slides were counterstained with FLEX Hematoxylin (DakoCytomation, Glostrup, Denmark). After that, the preparations were mounted in SUBX Mounting Medium (DakoCytomation, Glostrup, Denmark).

Histopathological examination and analysis of IHC reactions. All sections were evaluated by two independent pathologists using BX-41 light microscope (Olympus, Tokyo, Japan). Controversial cases were reassessed. Using HE-stained sections, the grade of histological malignancy according to Elston and Ellis criteria (40), as well as the presence of necrosis (regarded as positive when the area of necrosis comprised >10% of the tumour) was assessed. Nuclear Twist expression was evaluated semi-quantitatively based on the percentage of positively stained cancer cells of whole section, using the following scale: 0: absence of staining; 1: 1-10% cells stained; 2: 11-25%; 3: 26-50%; and 4: 51-100%. A semiquantitative immunoreactive score (IRS) method of Remmele and Stegner was utilized for the assessment of PDPN expression (41). In this study, the percentage of PDPN positive cells was defined as clearly PDPN positive area to the overall area noted in the whole IDC tissue section. The final score represents the product of the two values (the percentage of positive cells showing reaction in the whole section and the intensity of the colour reaction), ranging from 0 to 12.

Laser capture microdissection (LCM). For RNA extraction, the frozen tissue samples of 11 IDC cases were used (separately from tumour and stroma). Tissue sections (10-μm-thick) were prepared with the use of Leica CM1950 cryostat (Leica Microsystems, Wetzlar, Germany) and placed on a polyethylene terephthalate membrane slide (MMI, Glattbrugg, Switzerland). The slides were fixed in 70% isopropyl alcohol and then stained with HE using the H&E Staining Kit for LCM (MMI). Laser capture microdissection was performed using MMI CellCut Plus System (MMI). Dissected samples were taken onto the adhesive lid of 500 μl tubes (MMI). Total RNA was isolated from the tissue samples using RNeasy Micro Kit (Qiagen, Hilden, Germany). The protocol included on-column DNase digestion to eliminate genomic DNA. First-strand cDNA was synthesized according to the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany).

Real-time polymerase chain reaction (RT-PCR). Expression of *Twist* and *PDPN* mRNA was determined by quantitative real-time PCR with the use of 7900HT Fast Real-Time PCR System and TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA). β-actin (*ACTB*) was used as a reference gene. The primers and TaqMan probes used were: Hs01675818_s1 for *Twist*, Hs00366766_m1 for *PDPN* and Hs99999903_m1 for *ACTB* (Applied Biosystems, Foster City, CA, USA). All reactions were performed in triplicates under the following conditions: activation of the polymerase (50˚C for 2 min), initial denaturation (94˚C for

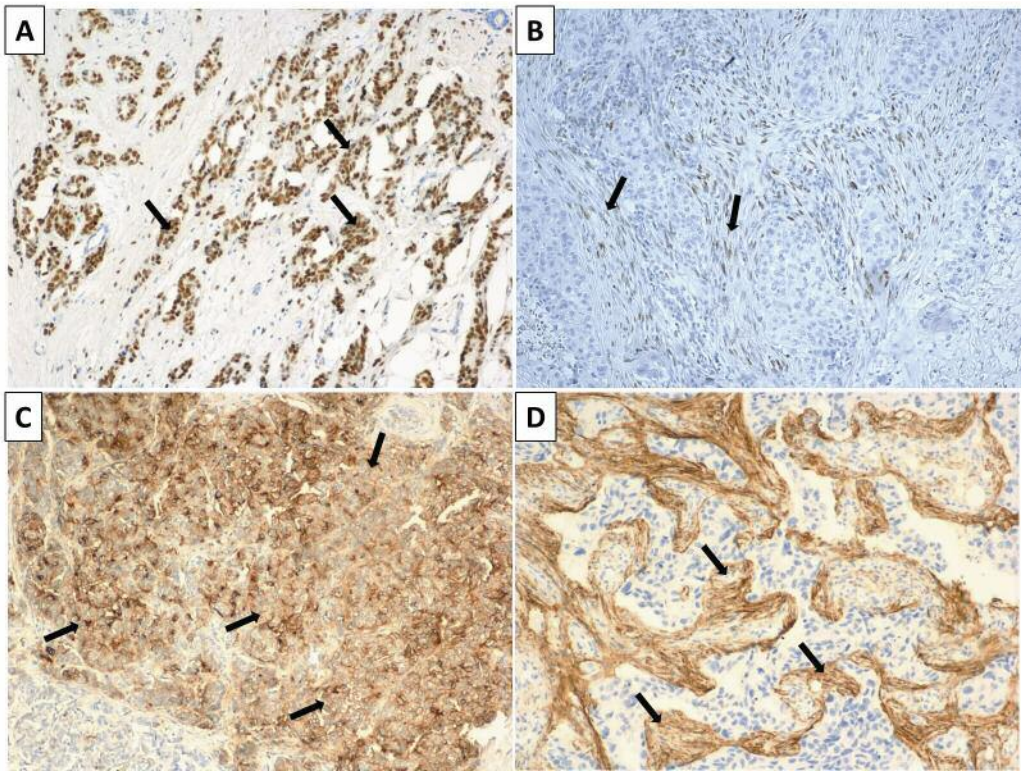

Figure 1. *Nuclear expression of Twist in cancer cells (A) and stromal cells of IDC (B). Cytoplasmic-membrane immunohistochemical expression of podoplanin (PDPN) in cancer (C) and stromal cells of IDC (D). Arrows indicate positive immunohistochemical reaction (magnification ×100).*

10 min) followed by 40 cycles of denaturation (94˚C for 15 s), and then annealing and elongation (60˚C for 1 min). The ΔΔCt method was used to calculate the relative mRNA expression of studied markers.

Statistical analysis. Statistical analysis was performed with Prism 5.0 (GraphPad, La Jolla, CA, USA). Kruskal–Wallis, Mann–Whitney, Wilcoxon, X2, Spearman rank correlation and Fisher's exact test were used to evaluate the expression and relationships of markers with clinicopathological data. The Mantel–Cox test was used to compare Kaplan–Meier survival curves. The date of diagnosis was used to measure survival period. During the analysis of event-free survival period, cancer recurrence was considered as the event. Differences were considered as statistically significant at $p<0.05$.

Results

IHC. Nuclear expression of Twist was observed in both IDC and stromal cells (in 16.25% and 72.5% of cases, respectively, Figure 1A and B). It was significantly higher in stromal cells in comparison with IDC cells ($p<0.0001$, Figure 2A). Despite a noticeable upward trend for the expression of Twist in stromal cells in stages with higher histological malignancy grade, no statistically significant differences were found (Figure 3A). Moreover, no statistically significant differences were found in cases with higher clinical stages of the disease or in triple-negative cases (ER^-, PR^-, Her-2^-). However, a weak positive correlation was found between expression of Twist in stromal cells and expression of Ki-67 in IDC cells (r=0.229, $p<0.05$, Figure 4A. Also shown was a weak negative correlation between expression of Twist and patients' age (r=-0.291, $p<0.01$; Figure 4B).

Analysis of patients' survival rate showed, that higher expression of Twist in IDC cells was associated with shorter overall survival (OS) and shorter event-free survival (EFS) (p=0.055 and $p<0.05$, Figure 5A and C, respectively). Similarly, expression of Twist in stromal cells was correlated with shorter OS and EFS in comparison to cases, in which no Twist expression was found ($p<0.05$ and $p<0.01$; Figures 5B and D, respectively).

Varied, positive expression of PDPN was observed in both IDC and stromal cells (2.5% and 75% of cases, respectively, Figure 1C and D). Moreover, the expression of PDPN was higher in stromal cells in comparison with IDC cells

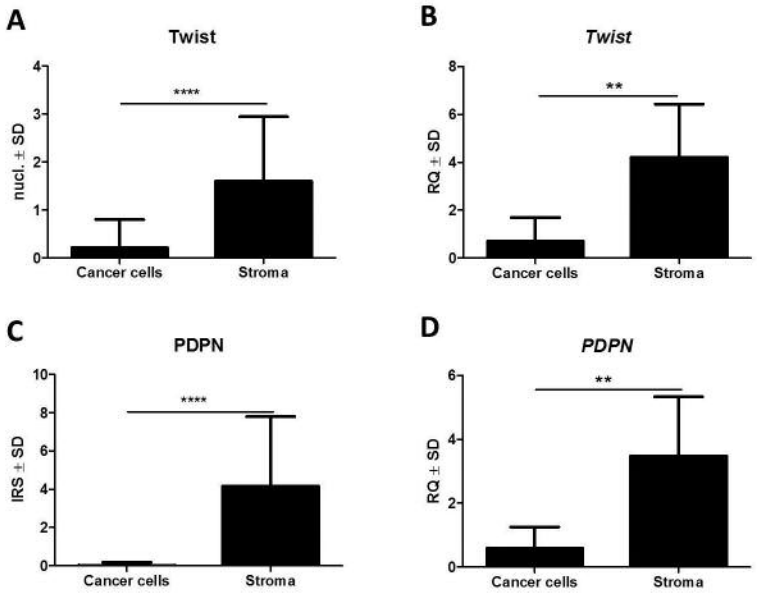

Figure 2. *Higher Twist expression in stromal cells of IDC in comparison to cancer cells at the protein (A) and mRNA (B) level. Higher expression of podoplanin (PDPN) in stromal cells than in cancer cells on protein (C) and mRNA (D) level, ****p<0.0001, **p<0.01.*

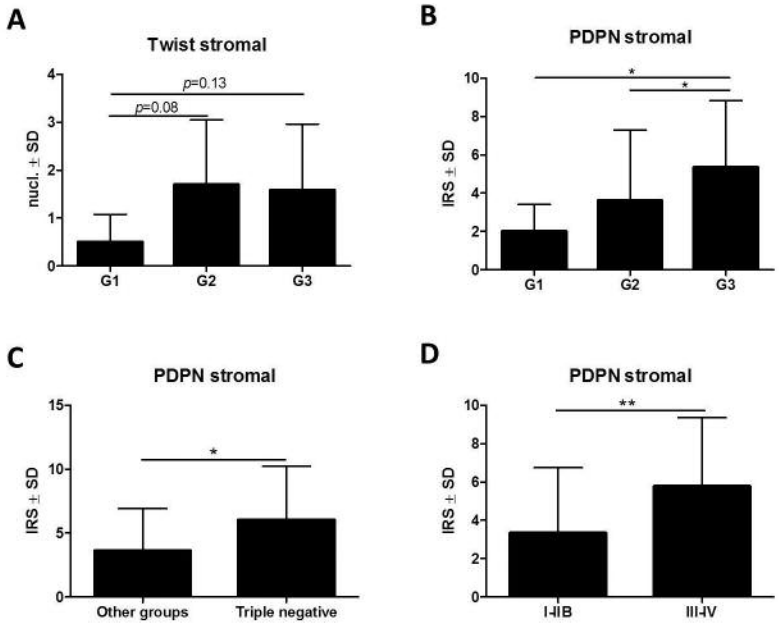

Figure 3. *Upward trend for Twist expression in G2 and G3 cases in comparison to G1 (A). Higher podoplanin (PDPN) expression in groups G2 and G3 in comparison to group G1 (B). Expression of PDPN is significantly higher in triple-negative group than in the other molecular subtypes (C). Similarly, podoplanin expression in the stroma of IDC is higher in clinical stages III-IV in comparison to stages I-II (D), *p<0.05, **p<0.01.*

(p<0.0001, Figure 2C). Higher expression of PDPN was found in stromal cells of IDC with higher grade of histological malignancy G (p<0.0001, Figure 3B), in triple-negative cases (p<0.05, Figure 3C) and with higher clinical stages of the disease (p<0.01, Figure 3D). Additionally, correlation analysis showed positive correlation of PDPN expression in stromal cells of IDC with the expression of Ki-67 antigen (r=0.362, p<0.001, Figure 4C).

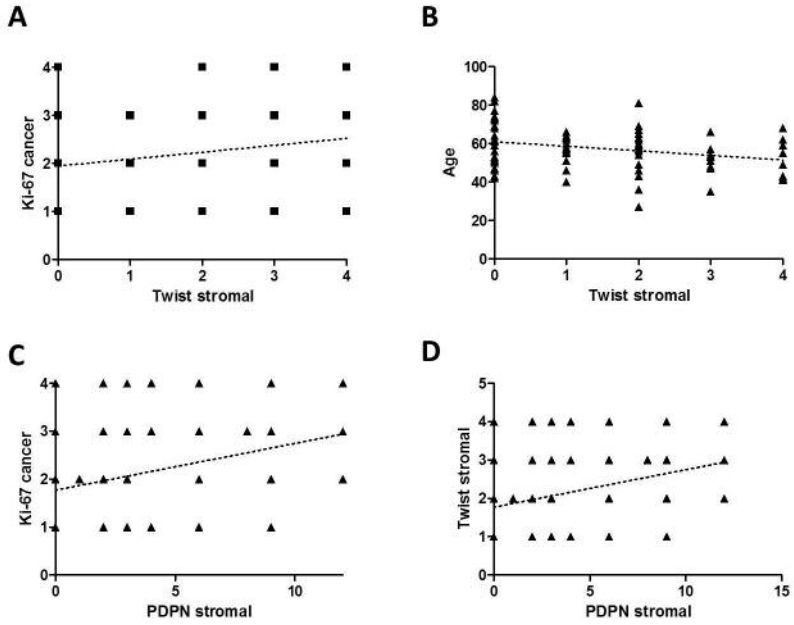

Figure 4. *Correlation analysis showed a weak positive correlation between expression of Twist in stromal cells and expression of Ki-67 in invasive ductal breast cancer (IDC) cells (r=0.229, p<0.05). Moreover, a negative correlation was found between expression of Twist in stromal cells and the age of IDC patients (r=–0.291, p<0.01). Expression of podoplanin (PDPN) in stromal cells is positively correlated with the expression of Ki-67 in cancer cells (r=0.362, p=0.001) (C). Positive correlation between PDPN and Twist expression in the stroma of IDC (r=0.267, p<0.05) (D).*

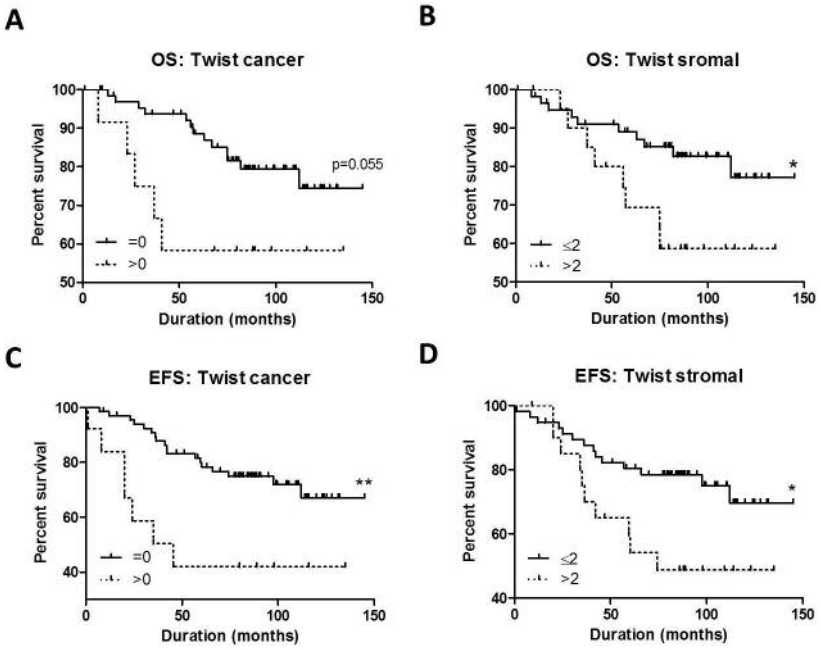

Figure 5. *Significantly higher mean overall survival (OS) of patients with lower immunohistochemical expression of Twist in cancer cells (A) and stromal cells (B), as well as event-free survivals (EFS) in both cancer and stromal cells (C, D, respectively). *p<0.05, **p<0.01.*

Also, a positive correlation was observed between expression of Twist and PDPN in stromal cells of IDC (r=0.267; $p<0.05$, Figure 4D).

Real-time PCR. Statistical analysis of the results showed significantly higher expression of mRNA for both Twist and PDPN in stromal cells in comparison with IDC cells

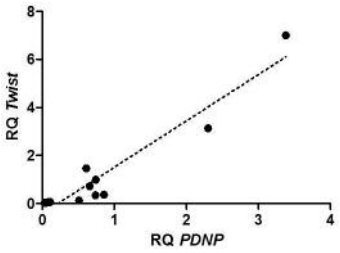

Figure 6. *Correlation of expression of mRNA for podoplanin (PDPN) and Twist in IDC cancer cells (r=0.89, p<0.0001).*

(p<0.01, Figure 2B and D). Additionally, a high positive correlation was observed between expression of mRNA for Twist and PDPN in IDC cells (r=0.89, p<0.0001, Figure 6).

Discussion

In the present study, we showed higher expression of Twist in stromal cells in comparison to IDC cells. This was also reported in our previous studies (42), as well as by other authors (6, 7). Similar correlations have also been observed in other carcinomas, *e.g.* oesophagus or colon cancer (8, 43). Twist transcription factor is considered as a potential repressor of E-cadherin gene and a marker of EMT process, which suggests, that it plays an important role in cancer progression (11, 44, 45). Our studies suggest, that high Twist expression in breast cancer stromal cells is observed in higher malignancy grades and also in cases with significantly shorter patients' survival time and in the period before recurrence. It may suggest involvement of stroma in the progression of this type of cancer. Similar suggestions were made before in gastric cancer cases (46). Other authors also have reported mutual interaction between cancer cells and cancer-associated fibroblasts (CAFs). For example, a decrease in e-cadherin expression, accompanied by an increase in expression of vimentin and fibronectin with simultaneous increase of expression of Snail, Slug, Twist and Zeb1 transcription factors (EMT regulators) was observed in breast cancer cells cultured together with CAFs (24, 47-49).

Moreover, CAFs can produce numerous factors potentially affecting cancer progression: fibroblast-specific protein 1 (FSP-1), fibroblast-activating protein (FAP), vimentin, smooth muscle alpha-actin (alpha-SMA), interleukin 6 (IL-6), transforming growth factor beta (TGF beta), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), epithelial growth factor (EGF), fibroblast growth factor (FGF) or platelet-derived growth factor receptors (PDGFRs) (50). Expression of Twist in CAFs was also reported, thus confirming our results (51).

Similarly to the results of previous studies, we also confirmed, that PDPN expression is significantly higher in stromal cells in comparison to breast cancer cells (26, 27, 28). The results obtained in studies by Pula *et al*. suggest, that PDPN may be involved in breast cancer progression and may be a marker for poor prognosis. Additionally, high PDPN expression correlates with higher tumor size, higher malignancy grade, lymph node metastases and higher expression of Ki-67 (29). In our studies, higher PDPN expression in CAFs of breast cancer was associated with higher grade and triple negative cases, and was also increased in advanced clinical stages of the disease. Moreover, as in the work of Pula *et al*. (29), PDPN was correlated with Ki-67 expression in IDC cells.

The observed correlation between expression of Twist and PDPN in both breast cancer cells (mRNA) and stromal cells (IHC) suggests a functional relationship between those proteins. So far it has been reported, that PDPN may affect cancer progression by inducing EMT, but no attention has been drown to the possible relationship between PDPN and Twist in breast cancer (28, 36, 52). The *in vitro* studies using pancreatic cancer cells showed, that PDPN expression was present in cells with increased migration ability (53). Moreover, in these same cells alpha-SMA (marker characteristic for mesenchymal cells) expression was observed, possibly resulting from EMT. Indirect evidence of correlation between EMT and podoplanin may be another *in vitro* experiment. In this experiment, relationship between increased PDPN expression in type II MDCK cell line (epithelial cell line isolated from dog kidney), caused by plasmid transfection, and induction of migration and invasiveness of those cells by, inter alia, inducing phosphorylation of proteins from ezrin-radixin-moesin family (54) was observed. In squamous cell skin carcinoma, higher PDPN expression was positively correlated with the presence of metastases and higher expression of EMT markers, such as vimentin, Zeb1 and Twist (39). However, breast cancer was not included in the publications. In our work, on the other hand, we showed similar correlations in IDC and stromal cells. Maybe, in contrary to other cancers, stroma in IDC plays a more important role, which in turn may be a proof of EMT regulation specificity.

Mutual influence between CAFs and tumor cells may occur in cancer progression (47, 57). Tumor cells induce and enhance fibroblasts activation, therefore newly-formed CAFs may produce growth factors and cytokines increasing cancer progression by inducing cell proliferation, angiogenesis processes and EMT (47, 57). Correlation of expression of PDPN and Twist, both in tumor and stromal cells, may suggest similar mechanism of action engaged in the regulation of EMT in IDC.

Acknowledgements

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