Expression of EMT Markers SLUG and TWIST in Breast Cancer

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Abstract. *Background: The epithelial-mesenchymal transition (EMT) has been observed in progression of in situ breast cancer to the invasive form and might be initiated by snail family zinc finger 2 (SLUG) and twist family bHLH transcription factor 1 (TWIST) protein overexpression. During this phenomenon, cells lose their epithelial phenotype and acquire mesenchymal features. The aim of the study was to examine the association of EMT markers SLUG and TWIST with clinicopathological data and the possibility of using these proteins as prognostic markers of breast cancer. Materials and Methods: Immunohistochemical analysis (IHC) of SLUG and TWIST expression was performed on archival paraffin samples of 19 cases with fibrocystic breast changes (control group), 148 cases of invasive ductal breast cancer (IDC) and 26 of invasive lobular breast cancer (ILC). Laser capture microdissection for isolation of cells from 17 frozen samples of IDC was employed and subsequently SLUG and TWIST mRNA expression in cancer and stromal cells was detected separately by real-time polymerase chain reaction. Results: SLUG and TWIST expression in IDC was significant higher in stromal cells regardless of the method of quantification used ($p<0.001$ for SLUG mRNA, and $p<0.0001$ for SLUG IHC, TWIST IHC and TWIST mRNA expression). Positive correlation of SLUG and TWIST protein and mRNA expression was observed in stromal cells of IDC ($r=0.347$; $p<0.0001$ and $r=0.704$; $p<0.01$, respectively). Expression of TWIST protein in IDC was higher in cancer cells of cases with shorter event-free survival period, as well as in stromal cells of cases with shorter overall survival period ($p<0.05$ for both). Conclusion: Stromal cells could play a role in the regulation of EMT in breast cancer.*

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Breast cancer (BC) is one of the most common oncological causes of death in highly developed countries (1). In 2008, there were 421,000 newly-diagnosed cases and 129,000 cases of death from BC among the European population alone (2). Invasive forms of BC are classified into two main histopathological types: ductal (IDC, developing from the lactiferous ducts) and lobular (ILC, developing from glands forming lobules) (2). Regardless of their different origin, they are characterized by typical microarchitecture and characteristic molecular profile *e.g.* expression of membrane marker E-cadherin (present in ductal and absent from lobular BC) (4). During routine pathological examination, the histological type of cancer, grade of malignancy, presence of nodal metastases and immunohistochemical expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor type 2 (HER2) and Ki-67 antigen are determined (4-6).

The epithelial-mesenchymal transition (EMT) is a physiological phenomenon occurring in embryonic development, as well as in mature organisms (*e.g.* during wound healing). EMT also occurs during pathological processes such as fibrosis and tumorigenesis (7). During EMT, cells lose their epithelial phenotype (*i.e.* loss of intercellular adhesion, basal-apical polarity and depletion of E-cadherin expression) and acquire mesenchymal features enabling for their migration, invasion or metastasis, such as increased expression of fibronectin, vimentin and N-cadherin (7, 8). EMT can be initiated by overexpression of certain proteins, *e.g.* snail family zinc finger 1 (SNAIL), snail family zinc finger 2 (SLUG), twist family bHLH transcription factor

1 (TWIST), zinc finger E-box binding homeobox 1 (ZEB1), and zinc finger E-box binding homeobox 2 (ZEB2), in cells which have been observed in progression from *in situ* to invasive forms of BC (7-9).

SLUG is a transcriptional factor regulating expression of genes responsible for the EMT. It belongs to the SNAIL superfamily of zinc-finger transcription factors (10). Expression of SLUG in cells suppresses E-cadherin expression, which subsequently reduces intercellular adhesion and increases cell motility properties (10). The well-known role of this protein is the regulation of migration of neural crest cells during embryogenesis and commitment in wound healing or tumorigenesis in adults (10). Moreover, it was demonstrated that degradation of SLUG was promoted by binding p53 and p21, two proteins which suppress cell invasiveness (11).

The TWIST protein belongs to a family of helix-loop-helix transcription factors involved in EMT (11). TWIST is a predominant regulator of EMT, involved in the promotion of cellular differentiation, motility and proliferation, and is associated with cancer stem cell phenotype (11-13). Moreover, TWIST is also known to have an anti-apoptotic action (14). It was also shown that TWIST inhibits the p53 pathway (15). In addition, overexpression of TWIST and methylation of its promoter (which activates *TWIST* gene expression) is commonly observed in primary and metastatic cancer (16-20).

The analysis of expression of EMT markers has predominantly been carried-out on cancer cells, even though lines of evidence suggest different roles of stromal cells and cancer cells in malignancies. The balance between stromal and cancer cells is considered to play a major role in cancer progression (21, 22). Moreover typical stromal cells *i.e.* cancer-associated fibroblasts (CAFs) are also recently suggested to be involved in chemoresistance in BC (22).

The aim of the study was to determine the localization and correlation between SLUG and TWIST proteins in IDC and ILC with regard to patients' clinicopathological data. Evaluation was conducted separately for cancer and stromal cells by immunohistochemistry (IHC) and real-time PCR method.

Materials and Methods

Patients. The study was performed on archival material of 19 cases of fibrocystic breast changes (control group), 148 paraffin-embedded samples of IDC, 26 paraffin-embedded samples of ILC, and 17 frozen tissue samples of IDC. All material was obtained from the Department of Tumor Pathology of the Maria Sklodowska-Curie Institute of Oncology in Krakow, collected in 2000-2006. All patients were treated surgically with radical mastectomy or conservative quadrantectomy followed by axillary lymph node resection. Tissue collection was performed prior to the beginning of the chemotherapy. Patients were followed up for a mean±SD of 65.91±39.02 (range=1-145) months. During this period, 28 (19.04%) patients died. Detailed clinicopathological data are presented in Table I.

Table I. *Patients and tumor characteristics of invasive ductal cancer (IDC) cases.*

Mean age (range), years	58.23±11.88 (27-84)	
Parameter	Number	%
Age		
<50 years	34	22.97
>50 years	114	77.03
Menopausal status		
Pre	43	29.05
Post	105	70.95
Grade of malignancy		
G1	9	6.08
G2	83	56.08
G3	56	37.84
pT		
T1	70	47.30
T2	74	50.00
T3	2	1.35
T4	2	1.35
pN		
pN0	54	36.49
pN1, pN2, pN3	93	62.84
pNx	1	0.67
ER		
Positive	103	69.59
Negative	45	30.41
PR		
Positive	92	62.16
Negative	56	37.84
HER2 by IHC		
0, 1	98	66.21
2, 3	50	33.78
Ki-67		
≤25%	92	62.16
>25%	56	37.84

G, Grade of malignancy; pT, pathologic size of tumor; pN, pathologic degree of regional lymph node spread; ER; estrogen receptor expression by IHC; PR; progesterone receptor expression by IHC; HER2; human epithelial growth factor receptor 2; Ki-67, marker of proliferation Ki-67

Immunohistochemistry. BC samples were fixed in 10% buffered formalin and embedded in paraffin. All sections were stained routinely with hematoxylin-eosin (HE) for diagnosis and revision of malignancy grade. IHC reactions were performed on 4-μm-thick sections using an Autostainer Link 48 instrument (DakoCytomation, Glostrup, Denmark). De-paraffinization and antigen retrieval was performed using Target Retrieval Solution (97˚C, 20 min; pH 9; DakoCytomation) 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, primary murine monoclonal antibodies directed against SLUG (sc-166476; 1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA), TWIST (ab50887; 1:50; Abcam, Cambridge, UK), were applied for 20 min

Table II. *Percentage of invasive ductal cancer (IDC), invasive lobular breast cancer (ILC) and control cases presenting immunohistochemical expression of snail family zinc finger 2 (SLUG) and twist family bHLH transcription factor (TWIST) protein in particular localizations.*

	Control	IDC		ILC		χ^2 test
Protein	Ductal cells (%)	Cancer cells (%)	Stromal cells (%)	Cancer cells (%)	Stromal cells (%)	*p*-Value
SLUG	55	31	81	29	84	<0.0001
TWIST	30	18	82	55	87	<0.0001

Table III. *Correlation (r) between snail family zinc finger 2 (SLUG) and twist family bHLH transcription factor (TWIST) mRNA expression in cancer cells (CC) and stromal cells (SC) of invasive ductal breast cancer. Significant results are shown in bold.*

Correlation tested	*r*	*p*-Value
TWIST in CC *vs*. *TWIST* in SC	0.618	0.006
TWIST in CC *vs*. *SLUG* in CC	0.382	0.130
TWIST in CC *vs*. *SLUG* in SC	0.494	0.037
TWIST in SC *vs*. *SLUG* in SC	0.704	0.001
TWIST in SC *vs*. *SLUG* in CC	0.110	0.670
SLUG in CC *vs*. *SLUG* in SC	0.537	0.021

at room temperature. For TWIST, the slides were additionally incubated with EnVision FLEX+ Mouse LINKER (15 min at room temperature) to enhance the signal. The visualization of the studied antigens was performed using the EnVision FLEX system according to the manufacturer's instructions. Counterstaining was performed using FLEX Hematoxylin. The preparations were mounted in SUB-X Mounting Medium. All reagents and equipment except for primary antibodies were obtained from DakoCytomation.

Histopathological examination and analysis of IHC reactions. HE- and IHC-stained sections were evaluated by two independent pathologists using a BX-41 light microscope (Olympus, Tokyo, Japan). Controversial cases were reassessed until consensus was reached. The grade of histological malignancy according to Elston and Ellis criteria (23) and presence of necrosis (regarded as positive when the area of necrosis comprised >10% of the tumor) were assessed on HE-stained sections. Nuclear SLUG and TWIST expression was evaluated semi-quantitatively based on the percentage of positively stained cells of whole section (one slide per case) and encoded as follows: 0: absence of staining; 1: 1-10% cells stained; 2: 11-25%; 3: 26-50%; and 4: 51-100%. The expression of SLUG and TWIST was evaluated in cancer and stromal cells separately.

Laser capture microdissection (LCM). The frozen tissue samples of 17 IDC cases were used for RNA extraction. Tissue sections of 10-μm thickness were prepared with use of a 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 stained with HE by using the H&E Staining Kit for LCM (MMI). LCM was performed using MMI CellCut Plus System (MMI). Dissected samples were collected onto the adhesive lid of 500 μl tubes (MMI). Total RNA was isolated from the tissue samples using RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The protocol included on-column DNase digestion to eliminate genomic DNA. First-strand cDNA was synthesized according to the QuantiTect Reverse Transcription Kit (Qiagen).

Real-time polymerase chain reaction (PCR). Expression of *SLUG* and *TWIST* mRNA was determined by quantitative real-time PCR with using a 7900HT Fast Real-Time PCR System and TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocols. As reference gene β-actin (*ACTB*) was used. The primers and TaqMan probes used were: Hs00950344_m1 for *SLUG*, Hs01675818_s1 for *TWIST*, and Hs99999903_m1 for *ACTB* (Applied Biosystems). All reactions were performed in triplicates under following conditions: activation of polymerase at 50˚C for 2 min, initial denaturation at 94˚C for 10 min followed by 40 cycles of denaturation at 94˚C for 15 s, and annealing and elongation at 60˚C for 1 min. The relative mRNA expression of studied markers was calculated with the ΔΔCt method.

Statistical analysis. Statistical analysis was performed with Prism 5.0 (GraphPad, La Jolla, CA, USA). To evaluate the expression and relationships of examined markers with clinicopathological data, X^2, Kruskal–Wallis, Mann–Whitney, Spearman rank correlation, and Fisher's exact test were utilized. The Mantel–Cox test was used to prepare Kaplan–Meier survival curves. Survival period was measured from date of diagnosis. As the event, during the event free survival period analysis, cancer recurrence was considered. Differences were considered as statistically significant at $p<0.05$.

Results

IHC. For both tested markers, nuclear localization in cancer as well in stromal cells was found (Figure 1). SLUG protein was predominantly expressed in stromal cells in both studied cancer types (81% of IDC and 84% of ILC cases), whereas in the control group, only 55% of cases presented SLUG expression ($p<0.0001$, χ^2 test; Table II). Similarly, TWIST expression was noted in nuclei of stromal cells of 82% of IDC, 87% of ILC cases and of 30% of the control group ($p<0.0001$, χ^2 test; Table II). The highest SLUG expression was noted in nuclei of IDC cases and was significantly higher as compared to ILC and the control group (Figure 2A). Moreover, in ILC cases, expression of SLUG was significantly higher when compared to that of the control group (Figure 2A). The highest TWIST expression was noted in nuclei of ILC cases and was significantly higher compared to IDC and the control group (Figure 2B). In IDC cases, higher expression of TWIST and SLUG was observed in stromal cells compared to cancer cells ($p<0.0001$ for both; Figure 2C and D, respectively). Moreover, a moderate positive correlation between both studied markers in stromal cells of IDC was noted ($r=0.347$, $p<0.0001$, Spearman correlation

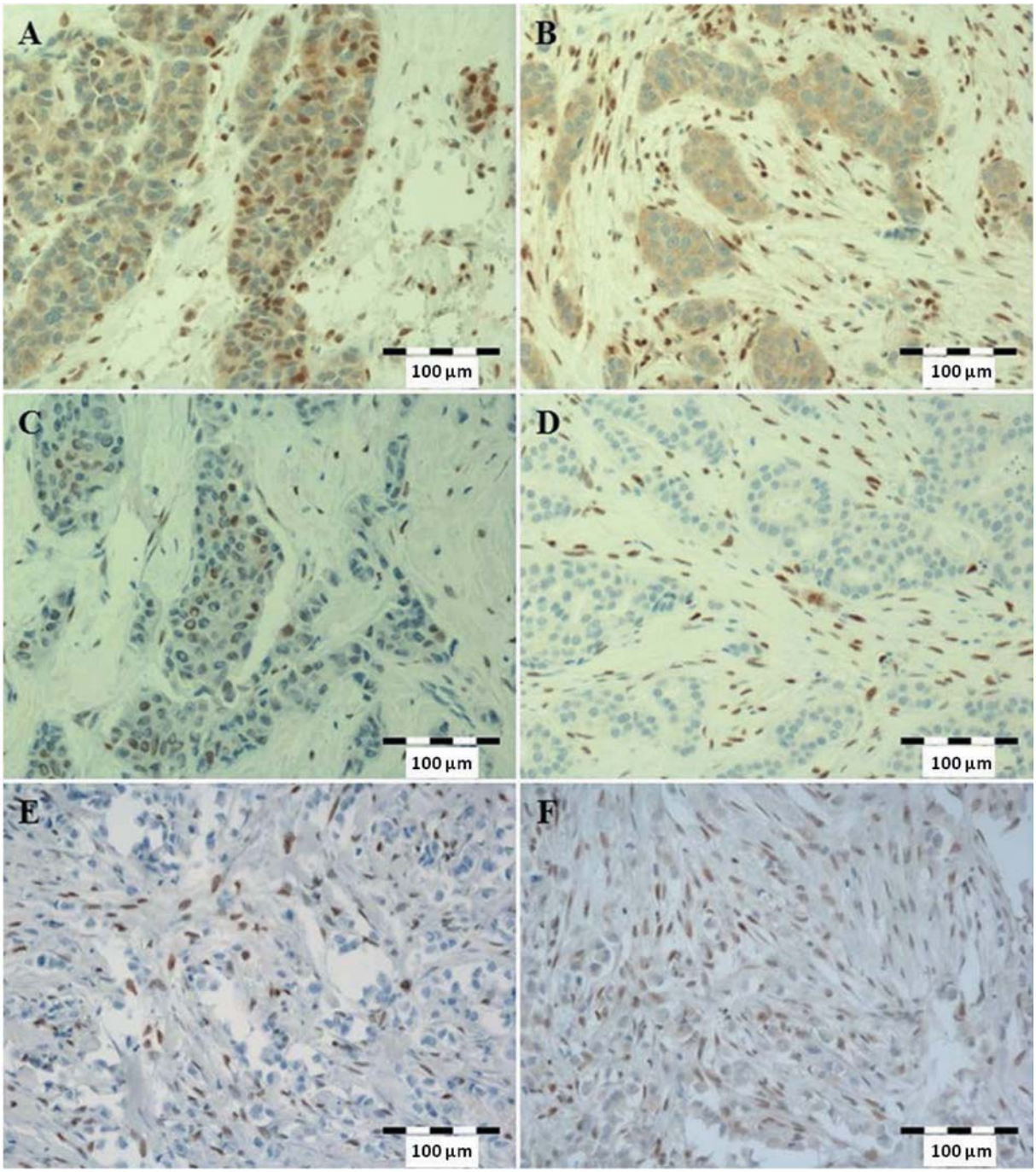

Figure 1. *Nuclear expression of snail family zinc finger 2 (SLUG) (A, B) and twist family bHLH transcription factor (TWIST) (C, D) in cancer (A, C) and stromal (B, D) cells of invasive ductal breast cancer. Nuclear expression of SLUG (E) and TWIST (F) in cancer and stromal cells of invasive lobular breast cancer (magnification, ×200).*

test). In ILC cases, expression of SLUG and TWIST was also significantly stronger in stromal cells ($p<0.0001$ for both; Mann–Whitney test) and, similarly to IDC, there was moderate positive correlation between these markers in stromal cells ($r=0.363$, $p<0.05$, Spearman correlation test).

Additionally, by taking into account analysis of ER, PR and HER2 (receptor status) of the studied IDC cases, it was noted that significantly higher SLUG expression was observed in ER-negative, and PR-negative cases ($p<0.001$ for both). In contrast, in HER2-negative cases, SLUG

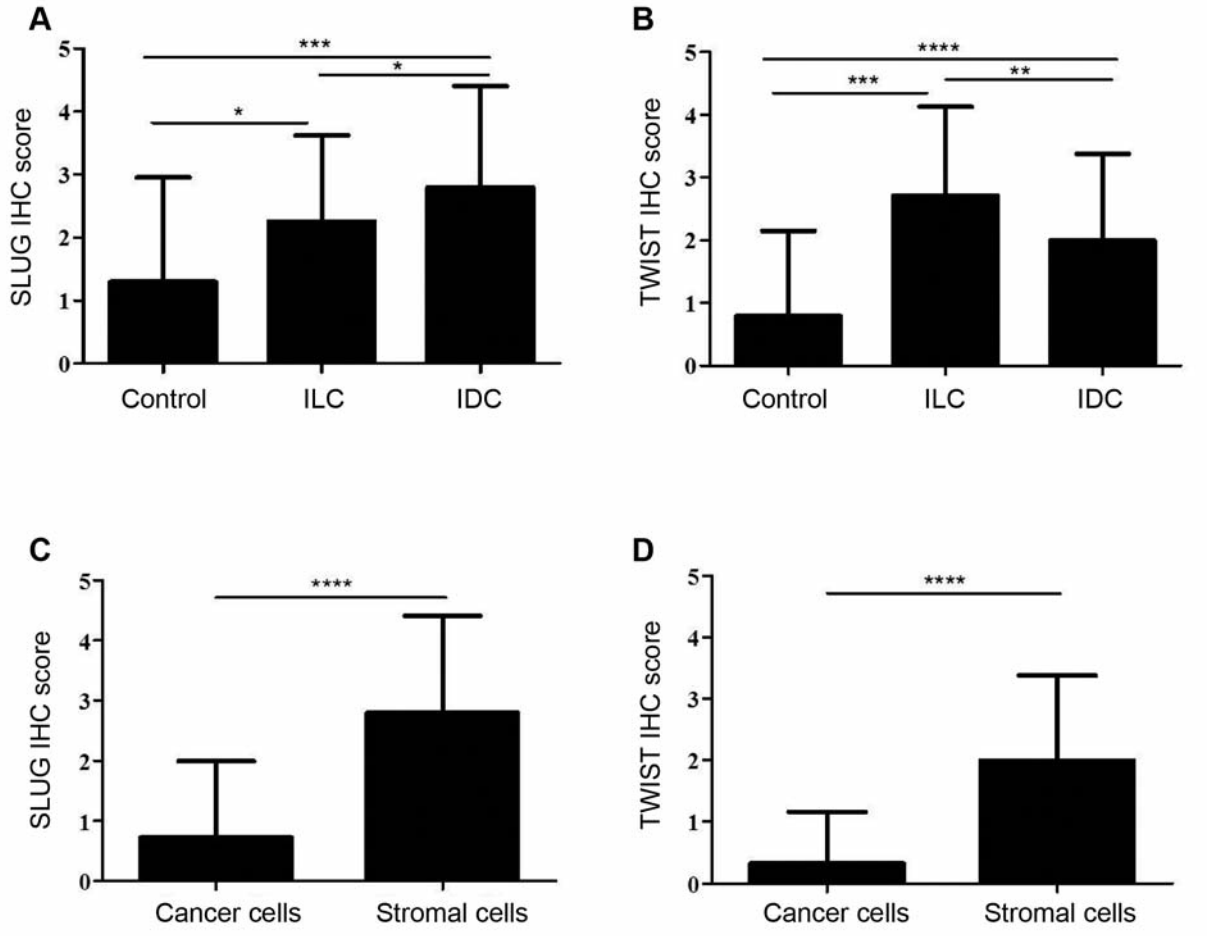

Figure 2. *Differences between snail family zinc finger 2 (SLUG) (A) and twist family bHLH transcription factor (TWIST) (B) expression in stromal cells in invasive ductal breast cancer (IDC) and invasive lobular breast cancer (ILC) compared to the control group. Differences in expression of SLUG (C) and TWIST (D) in cancer vs. stromal cells of IDC ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$, Mann–Whitney test).*

expression was non significantly higher as compared to HER2-positive cases ($p<0.051$).

Survival analysis of patients with IDC showed that higher nuclear TWIST expression in cancer cells was associated with shorter event-free survival, whereas stronger stromal nuclear expression was found in cases with shorter overall survival ($p<0.05$ for both, Mantel Cox survival analysis test). Kaplan–Meier curves are presented in Figure 3. There were no significant associations between other clinicopathological data and expression of SLUG and TWIST proteins in IDC and ILC.

Real-time PCR. By usage of LCM, the mRNA expression of both tested genes, SLUG and TWIST, was revealed in cancer, as well as in stromal cells in all examined cases. Comparison of gene expression showed significantly higher mRNA expression of SLUG as well as TWIST in stromal than in cancer cells ($p<0.01$ and $p<0.0001$, respectively; Mann–Whitney test). Furthermore, a strong positive correlation between mRNA expression of TWIST and SLUG in stromal cells was shown ($r=0.704$, $p<0.0001$; Spearman correlation test). Correlation of these two genes in both analyzed localizations can be found in Table III.

Discussion

EMT is known to be an important step of tumorigenesis. This phenomenon is responsible for the development ofdistant metastases, characteristic of advanced clinical stages of BC. The potent role of SLUG and TWIST proteins in EMT was reported in numerous articles. It was strongly suggested that increased expression of SLUG in IDC down-regulated E-cadherin expression in BC (24, 25). A similar association between TWIST and E-cadherin expression in IDC was found by Vesuna *et al*. (12). Loss of E-cadherin

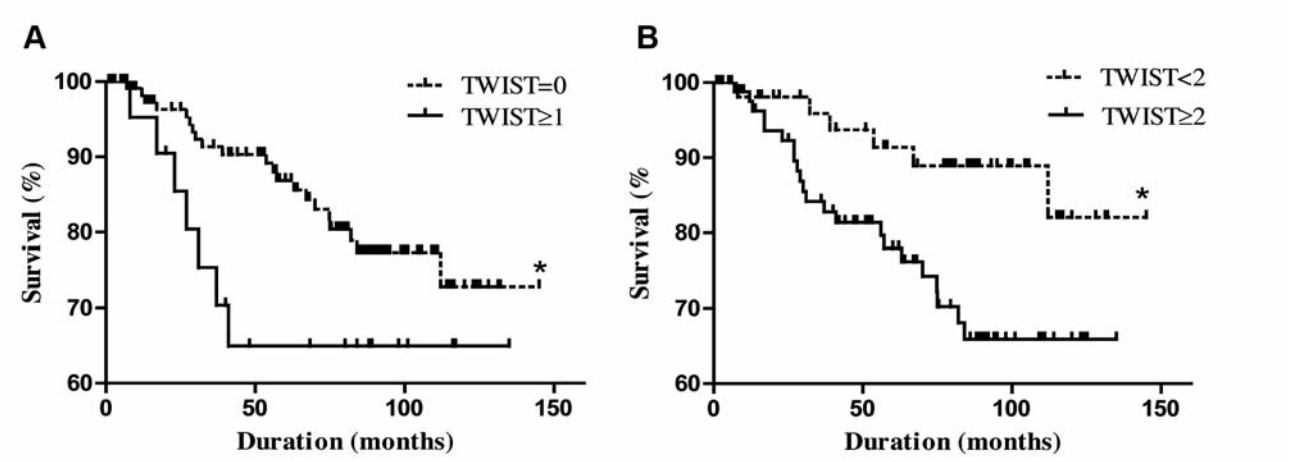

Figure 3. *Survival analysis of patients with twist family bHLH transcription factor (TWIST) expression in cancer and stromal cells. Patients with lack of TWIST expression in cancer cells lived significantly longer without recurrence of invasive ductal breast cancer than did patients with expression of TWIST (A). Patients with lower TWIST expression score in stromal cells lived significantly longer than patients with higher TWIST expression score (B) (*$p<0.05$, Kruskal–Wallis test).*

expression resulted in reduction of epithelial cell integrity by reducing cell to cell adhesion. Finally, the metastatic potential of tumor cells increased and hence cancer could enter the metastatic phase (26).

The role of stromal cells of cancer in EMT has also been suggested. It was shown by Hu *et al*. that grafting of MCF10A cell line, a model of human ductal BC *in situ* with fibroblasts to female mice resulted in BC progression to invasive carcinoma, promoted by the presence of fibroblasts (9). Liu *et al*. also indicated interaction between BC stem cells and bone marrow-derived mesenchymal stem cells in tumor growth and metastasis, suggesting the main role of interleukin 6, produced by mesenchymal stem cells (27). The development of invasive cancer seems to be a complex process with a high impact of interactions between cancer and stromal cells, mainly by complex intercellular signals (28, 29). The regulation of intestinal homeostasis between the epithelium, the microenvironment and stem cells has also been demonstrated in colorectal cancer (30). Moreover, inappropriate regulation of the balance between epithelial cells and stromal cells can result in development of cancer, as well as tumor progression and metastasis (30-32).

In our research, we observed expression of both tested EMT markers, SLUG and TWIST, in almost all cases of BC analyzed at the protein and mRNA level. For the first time in BC to our knowledge, stronger SLUG and TWIST protein as well mRNA expression in stromal compared to cancer cells was demonstrated. Moreover, increased mRNA expression of both markers in stromal cells was associated with their increased expression in cancer cells. In line with the features of EMT stated above, analysis of the studied markers in regard to their localization may provide additional information about the process of metastasis.

The potential of stromal cells to induce EMT in cancer cells was also shown in an *in vitro* model (33). The authors showed that nuclear SLUG expression appeared in fibroblasts culture during their co-cultivation with cancer cells or bone marrow cells (33). Moreover it has been suggested that SLUG overexpression in BC stem cells was regulated by tumor growth factor β1 and tumor necrosis factor α produced by stromal cells of BC under hypoxia and promoted self-renewal of BC stem cells (34). SLUG overexpression in cancer stem cells promotes their dissemination and seeding to BC stroma (34). These findings could partially explain our observation of SLUG expression in stromal cells actively involved in the EMT.

Zhao *et al*. showed nuclear and cytoplasmic TWIST protein expression in BC cells (35). Similarly, our results confirmed a nuclear expression of TWIST in BC cells but in addition, we also demonstrated nuclear expression of this protein in stromal cells. Furthermore, survival analysis revealed that patients with strong TWIST expression in cancer and stromal cells lived a significantly shorter period of time. Our results are in line with results obtained by Zhao *et al*. (35) and Martin *et al*. (36) who indicated that increased TWIST expression was associated with shorter metastasis-free survival of patients with BC. *TWIST* mRNA expression was shown to be an independent poor prognostic marker in lymph node-negative BC cases with ER-positive status (37). Román-Pérez *et al*. (31) and van Nes *et al*. (38) demonstrated that a higher percentage of TWIST-positive cells in cancer and stroma of patients with BC with ER-positive status was associated with shorter survival. In our

work, we observed that patients with ER-positive IDC and presence of nuclear TWIST expression lived for a shorter period than did patients without TWIST expression what partially confirmed their observation (data not shown).

In conclusion, the present work described as far as we are aware for the first time, the detailed localization and differences of SLUG and TWIST expression in BC. The results suggest that not only could stromal cells be crucial in IDC development but also that stromal TWIST expression could be a potential negative prognostic marker in IDC. Further studies on EMT markers in BC are necessary.

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