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PRECLINICAL STUDY

Vimentin, zeb1 and Sip1 are up-regulated in triple-negative and basal-like breast cancers: association with an aggressive tumour phenotype

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Abstract In epithelial-to-mesenchymal transition (EMT) epithelial cancer cells achieve mesenchymal features, essentially helping them to metastasize. There is some evidence that EMT could be increased in triple-negative (TNBC) or basal-like breast cancers, although more precise mechanisms considering e.g. EMT-regulating transcription factors are largely unknown. We assessed immunohistochemically vimentin (separately in in situ areas and in invasive cells) as an indicator of EMT, and also EMT-regulating transcription factors zeb1 (separately in stroma and tumour) and Sip1 (in nuclei and cytoplasm) in histological samples of 231 women with local or locally advanced invasive breast cancer. 51.1 % of patients had TNBC and 48.9 % oestrogen and progesterone receptor-positive and HER2 negative breast cancer. Basal-like breast cancers were defined as TNBC that also expressed epidermal growth factor receptor EGFR and/or cytokeratin 5/6. Vimentin expression in invasive cells was higher in TNBCs ($p = 9 \times 10^{-12}$) compared to non-TNBC tumours. Vimentin ($p = 2 \times 10^{-6}$), nuclear Sip1 ($p = 0.035$) and zeb1 in stroma ($p = 0.013$) were overexpressed in basal-like cancers compared to non-basal-like TNBCs. In non-TNBC group findings between studied markers and clinicopathological factors were rare. However, in TNBC cases, vimentin expression in invasive cells associated with poor differentiation ($p = 0.00007$), zeb1 expression in cancer cells with higher grade ($p = 0.002$), vascular invasion ($p = 0.036$) and larger T-class ($p = 0.027$), whereas stromal zeb1 associated with lymphatic vessel invasion ($p = 0.036$) and vascular invasion ($p = 0.039$). High nuclear Sip1 expression was prognostic for poor disease-free survival ($p = 0.002$) in the whole cohort. The current results emphasize the increased role of EMT in TNBC and especially in basal-like breast cancers. These observations also support the role of studied parameters in tumour progression.

Keywords Epithelial-to-mesenchymal transition · Prognosis · Transcription factor · Triple-negative breast cancer

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Introduction

Triple-negative breast cancer (TNBC) is a tumour showing no oestrogen (ER) or progesterone (PR) receptor positivity and no HER2 amplification [1, 2]. It accounts for 15–20 % of breast cancer cases and it has a higher frequency of early relapse and a poor diagnosis compared to other carcinoma types during the first few years after cancer diagnosis [1, 3]. Most TNBCs are histologically ductal, but a minority represents other histological categories such as metaplastic, adenoid cystic, medullary or secretory types [2]. About 80 % of triple-negative carcinomas show a basal-like gene expression [3]. There is some evidence that breast cancers with basal phenotype may have increased

expression of epithelial-to-mesenchymal transition (EMT) markers [4]. Triple-negative and basal-like carcinomas show a higher frequency of BRCA1 mutations, a higher frequency of EGFR amplification and p53 mutation frequency [2]. Also, the so-called claudin-low breast cancer type is usually of triple-negative type with downregulation of E-cadherin and upregulation of vimentin [2].

Epithelial-to-mesenchymal transition is process that is physiologically involved in development, tissue regeneration and wound healing. In this process epithelial cancer cells attain mesenchymal features which make it easier for them to invade to the surrounding tissues and metastasize. EMT has also been linked to tumour stemness [5]. In breast cancer it has been shown that EMT increases the population of breast cancer stem cells. Since TNBC also shows a higher number of stem cell markers, EMT activity would be expected to be increased in these tumours.

Expression of vimentin, a cytoskeleton protein taking part in the migration of epithelial cells, is considered as feature of EMT indicating acquisition of a mesenchymal phenotype of tumour cells along with upregulation of alpha-smooth muscle actin [6, 7]. Vimentin expression and its association with ER negative phenotype in breast cancer have been reported several years ago [8]. In a recent study, vimentin and smooth muscle acting expression was detected in 24.5 and 9.8 % of triple-negative breast carcinomas, respectively, while the percentages for non-triple-negative breast tumours were 4.1 and 0.4 % [6]. According to this study EMT was a specific feature of triple-negative tumours along with a high histological grade [6].

Smad interacting protein 1 (Sip1), also known as zinc finger E-box-binding protein 2 (zeb2) or ZFHX1B, is a 140 kDa protein belonging to the δEF1/ZEB family of proteins [9]. The Sip1 gene is located at chromosome 2 and consists of ten exons and nine introns [10]. Sip1 is a transcription factor and can attach with its zinc finger domains to CACCT region of DNA sequences in the promoter regions of target genes such as E-cadherin or α4-integrin thus inhibiting their transcription and in this way contributing to EMT [11, 12]. Sip1 also inhibits Smad transcription factors which, on the other hand, are induced by TGFβ [13]. On the other hand, Sip1 may induce the expression of some genes such as vimentin, N-cadherin, MMP1 and MMP2 which contribute to EMT and tumour cell invasion [14].

Like Sip1, also zeb1 consists of two clusters of zinc finger domains in its molecular structure [15]. They both play a role in EMT and both can be inhibited by the miRNA 200 group [15]. Interestingly, p53 suppresses the EMT phenotype by inducing expression of the miRNA 200 and miRNA-192 family which downregulate both Sip1 and zeb1 [16]. Interestingly also, both can be upregulated by Snail [17]. Both Sip1 and zeb1 are detrimental for the development of vertebrates; zeb1 knockout mice develop skeletal deformities which are lethal while Sip1 knockout mice have deficiencies in neural development and show an arrest in embryonic development at E 8.5 [15]. Zeb1 induces metastasis behaviour and loss of polarity in colon carcinoma cells and it is more frequently expressed in lung metastatic disease [18, 19]. Association of zeb1 with invasion and metastatic disease has also been reported in gastric and hepatocellular carcinoma [20, 21]. In diffuse large B-cell lymphoma high expression of zeb1 protein is associated with an adverse outcome [22].

Research on Sip1 and zeb1 expression in triple-negative or basal-like breast carcinoma are very few. To gain insight in this topic we investigated the expression of Sip1 and zeb1 in a large cohort of triple-negative breast carcinomas, where cytokeratin (CK) 5/6 and epidermal growth factor receptor (EGFR) were determined to find tumours with basal-like phenotype. We compared results with a cohort of non-triple-negative cases and with expression of vimentin. The expression of these markers was also compared with the clinical and pathological data of the patients.

Methods

The material consisted of 231 women with local or locally advanced invasive breast cancer. The patients were diagnosed and treated in Oulu University Hospital, Oulu, Finland and Kuopio University Hospital, Kuopio, Finland during 2000–2010. Two hundred and eight (90.0 %) patients had ductal, 9 (3.9 %) medullar, 5 (2.2 %) tubular, 4 (1.7 %) lobular and the rest (2.0 %) invasive breast cancer of unspecified histology. The specimens had been fixed in neutral formalin, embedded in paraffin blocks and stored at the Department of Pathology in Oulu and Kuopio University Hospitals. The patients were surgically staged according to the current TNM classification system and the histological degree of tumour differentiation was classified according to the WHO Classification of Tumours [23]. The study was approved by the Local Ethics Committee of the Northern Ostrobothnia and Northern Savo Hospital District of Finland.

Immunohistochemistry and scoring

The immunostainings were performed as follows. Four-μm-thick tissue sections were cut from the paraffin-embedded blocks. After deparaffinisation and rehydration, the sections were heated in a microwave oven for 2 × 5 min in Tris–EDTA buffer (pH 9.0), incubated in a Tris–EDTA buffer for 20 min and washed twice for 5 min in phosphate buffered saline (PBS). Hydrogen peroxide (5 %, 5 min) was used to block endogenous peroxidase. Non-specific binding was

blocked with 1.5 % normal serum in PBS for 35 min at room temperature. The sections were incubated overnight at 4 °C with the mouse monoclonal anti-vimentin, anti-zeb1 and anti-Sip1 antibodies (dilutions 1:3,000, 1:500 and 1:200, respectively). The mouse monoclonal vimentin antibody was from BioGenex (clone V9, Fremont, CA, USA 94538), zeb1 antibody from GenWay (clone 416A7H10, San Diego, CA, USA) and the polyclonal rabbit anti-human antibody to Sip1 (sc-48789) was purchased from Santa Cruz (San Diego, CA, USA). The slides were then incubated with a biotinylated secondary antibody and avidin–biotin-peroxidase complex (ABC Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA). Careful rinsing was performed with PBS at each step of the immunostaining procedure. The colour was developed with diaminobenzidine tetrahydrochloride (DAP) (Sigma, St. Louis, MO, USA). The slides were counterstained with Mayer's haematoxylin, washed, dehydrated, cleared and mounted with Depex (BDH, Poole, UK). Ovarian and lung tumour tissue with known positive Sip1 and zeb1 expression was used as a positive control. In negative controls the primary antibody was omitted.

For vimentin, the cases were initially semiquantitated in four groups as follows: 0–2 % = negative; 2–10 % = +; 10–50 % = ++; over 50 % = +++. The immunoreactivity was strong and consequently no evaluation for qualitative expression was used. We also analysed vimentin expression separately in invasive cells and in situ areas, because its expression was notable in both areas.

Cytoplasmic Sip1 was analysed quantitatively but since there was a variation in the strength of staining the intensity was evaluated using a three tired estimation (1 = weak, 2 = moderate, 3 = strong) and finally the quantitative and qualitative estimates were combined as follows 0 = negative; 1–2 points = 1; 3–4 points = 2; 5–6 points = 3. Nuclear Sip1 positivity was assessed only by the presence (+) or absence (−) of positivity.

For zeb1, the evaluation was performed similarly as for vimentin taking into consideration nuclear positivity. Both epithelial and stromal compartments are evaluated. The assessments were performed by experienced histopathologist (YS), who was blinded from clinical data at the time of the analysis.

Oestrogen and progesterone receptor, Ki67 and HER2

Tumours exhibiting nuclear ER/PR receptor expression in more than 9 % of invasive tumour cells were considered as steroid receptor-positive. The TNBC group did not show any ER- or PR-positivity. In other words, tumours expressing 1–9 % steroid receptors were excluded from the study. Membranous HER2 expression was also studied by means of immunohistochemistry (IHC) and if a specimen exhibited a HER2-positive result (1–3+ on a scale of 0–3+) in IHC, HER2 gene amplification status was determined by means of chromogenic in situ hybridization (CISH). Breast cancers with six or more gene copies of HER2 in cells were considered HER2-positive. Expression of Ki-67 was studied immunohistochemically as described previously [24].

Cytokeratin 5/6 was scored positive if any (weak or strong) cytoplasmic and/or membranous invasive carcinoma cell staining was observed and EGFR was scored positive if there were more than 10 % of positive cells. To detect the basal subtype among these breast cancer specimens, expression of CK 5/6 and EGFR was determined in the triple-negative tumours. The methods for these immunostainings have been published previously [25]. The triple-negative tumours that also expressed EGFR and/or CK 5/6 were classified as basal-like breast cancers (BLBCs) [26, 27]. The main patient and tumour characteristics in each of these groups are shown in Table 1.

Statistical analysis

IBM SPSS Statistics 20.0.0 was used for statistical analysis. The reported p values are from two-sided Chi square tests, except for survival analysis. Survival was analysed by using Kaplan–Meier curves with the log-rank test and only breast cancer-related death was used as an endpoint. Disease-free survival was calculated from the first operation date to the time of local relapse or detection of metastases, whichever came first. T-class was divided in statistical analyses to either T_1 or $T_{2–4}$ and nodal status to either positive or negative. Ki-67 was divided into 0–14 % or >14 % and grade was either grade I–II or grade III in analyses. Zeb1 and Sip1 were managed as either 0–1 (low expression) or 2–4 (high expression) in all statistical analyses. Since vimentin expression was significantly weaker, its expression was divided to 0–1 or more. Probability values below 0.05 were considered significant.

Results

Smad interacting protein 1 expression was assessable in 224 (97.0 %), zeb1 in 193 (83.5 %) and vimentin in 225 (97.4 %) cases. Expression of Sip1 was mainly cytoplasmic but nuclear expression was also frequently seen (Figs. 1, 2). On the contrary, zeb1 was mainly seen in nuclei in stromal cells and tumour cells stained only occasionally (Fig. 3). Vimentin expression was observed in the cytoplasm of the cells (Fig. 4). Zeb1 expression was only found in invasive tumour cells, Sip1 was seen both in

Table 1 Any positive EMT marker expression and tumour characteristics in the different subgroups used in the study

	All patients ($n = 231$)	TNBC ($n = 118$)	ER+/PR+/HER2− ($n = 113$)	Basal-like ($n = 93$)	TNBC, non-basal-like ($n = 14$)
Sip1	214 (95.5 %)	105 (95.5 %)	59 (52.2 %)	85 (94.6 %)	14 (100 %)
Nuclear Sip1	53 (23.7 %)	27 (23.7 %)	26 (23.6 %)	12 (13.3 %)	5 (35.7 %)
Vimentin in in situ areas	113 (51.2 %)	75 (65.8 %)	38 (33.6 %)	65 (72.2 %)	4 (28.6 %)
Vimentin in invasive cells	98 (43.6 %)	75 (63.6 %)	23 (20.4 %)	64 (71.1 %)	5 (35.7 %)
zeb1 in stroma	194 (100 %)	99 (100 %)	95 (100 %)	78 (100 %)	13 (100 %)
zeb1 in tumour cells	77 (33.7 %)	44 (44.4 %)	34 (30.1 %)	38 (48.3 %)	4 (30.8 %)
Tumour size					
T_1	107 (46.3 %)	47 (39.8 %)	60 (53.1 %)	32 (34.4 %)	9 (64.3 %)
$T_{2–4}$	123 (53.2 %)	71 (60.2 %)	52 (46.0 %)	61 (65.6 %)	5 (35.7 %)
Unknown	1 (0.4 %)		1 (0.9 %)		
Nodal status (N)					
N_0	126 (54.5 %)	68 (57.6 %)	58 (51.3 %)	53 (57.0 %)	7 (50.0 %)
$N_{1–3}$	105 (45.5 %)	50 (42.4 %)	55 (48.7 %)	40 (43.0 %)	7 (50.0 %)
Grade					
I-II	75 (32.5 %)	20 (16.9 %)	55 (48.7 %)	11 (11.8 %)	6 (42.9 %)
III	155 (67.1 %)	98 (83.1 %)	57 (50.4 %)	82 (88.2 %)	8 (57.1 %)
Unknown	1 (0.4 %)		1 (0.9 %)		
Lymphatic vessel invasion					
Yes	29 (12.6 %)	25 (21.2 %)	4 (3.5 %)	21 (22.6 %)	1 (7.1 %)
No	133 (57.6 %)	93 (78.8 %)	40 (35.4 %)	72 (77.4 %)	13 (92.9 %)
Unknown	69 (29.9 %)		69 (61.1 %)		
Blood vessel invasion					
Yes	21 (9.1 %)	19 (16.1 %)	2 (1.8 %)	15 (16.1 %)	1 (7.1 %)
No	141 (61.0 %)	99 (83.9 %)	42 (37.2 %)	78 (83.9 %)	13 (92.9 %)
Unknown	69 (29.9 %)		69 (61.1 %)		
ER expression					
Positive	113 (48.9 %)	0 (0 %)	113 (100 %)	0 (0 %)	0 (0 %)
Negative (<1 %)	118 (51.1 %)	118 (100 %)	0 (0 %)	93 (100 %)	14 (100 %)
PR expression					
Positive	113 (48.9 %)	0 (0 %)	113 (100 %)	0 (0 %)	0 (0 %)
Negative (<1 %)	118 (51.1 %)	118 (100 %)	0 (0 %)	93 (100 %)	14 (100 %)
Ki-67					
<15 %	64 (27.7 %)	7 (5.9 %)	57 (50.4 %)	5 (5.4 %)	1 (7.1 %)
≥15 %	111 (48.1 %)	56 (47.5 %)	55 (48.7 %)	51 (54.8 %)	3 (21.4 %)
Unknown	56 (24.2 %)	55 (46.6 %)	1 (0.9 %)	37 (39.8 %)	10 (71.4 %)
HER2					
Positive	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)
Negative	231 (100 %)	118 (100 %)	113 (100 %)	93 (100 %)	14 (100 %)
Histology					
Ductal	208 (90.0 %)	100 (84.7 %)	108 (95.6 %)	83 (89.2 %)	9 (64.3 %)
Other	23 (10.0 %)	18 (15.3 %)	5 (4.4 %)		

invasive and in situ areas and there was no significant difference in the staining. In breast carcinomas stronger Sip1 positivity was usually seen in the invasive front of the tumours (Fig. 1a, b).

One hundred eighteen (51.1 %) of the 231 studied cases were TNBC. Ninety-three (75 % of TNBC cases with EGFR-1 and/or CK 5/6 available) exhibited the BLBC phenotype, as they expressed either CK 5/6 or EGFR-1.

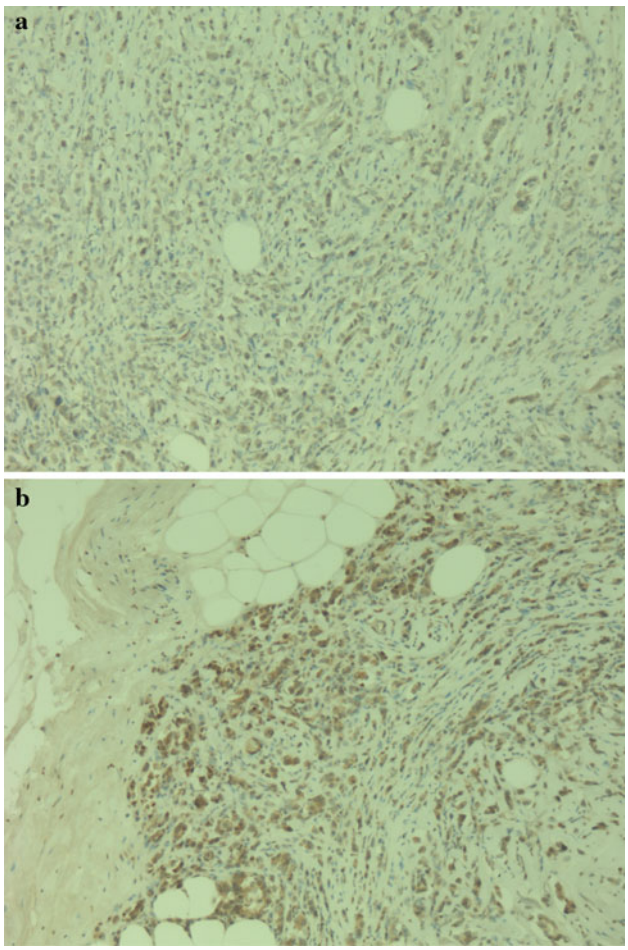

Fig. 1 Sip1 expression in breast carcinoma. The expression of Sip1 is weaker in the central area of the tumour (**a**) compared with tumour cells from the invasive front of the carcinoma (**b**). (*Size bar* 320 μm)

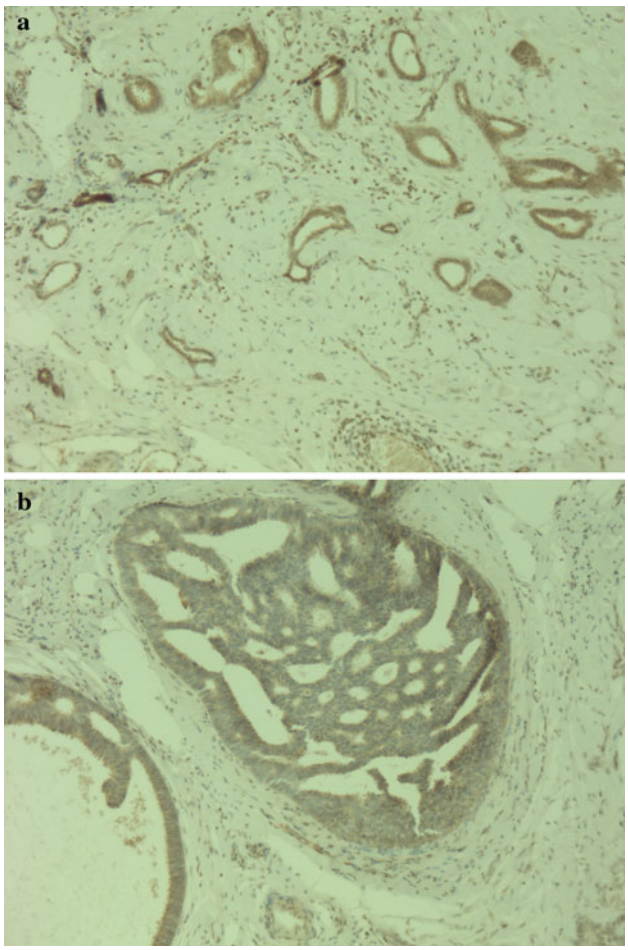

Fig. 2 Sip1 expression in ductal carcinoma of the breast (**a**). The in situ component of the xame tumour appears to stain somewhat weaker (**b**). (*Size bar* 320 μm)

This proportion is in line with previous literature [3]. The non-triple-negative control cases ($n = 113$) had both ER and PR expression over 9 % and were all HER2-negative. TNBC tumours were larger ($p = 0.037$), had a higher grade ($p = 2.0 \times 10^{-6}$) and increased Ki-67 expression ($p = 1.6 \times 10^{-7}$) compared to non-TNBC group. In TNBCs first site of distant metastasis was more frequently in visceral sites (liver, lung, brain, distant lymph nodes) than elsewhere (in bone or multiple synchronous metastases) compared to non-TNBCs ($p = 0.0073$). Nodal status was similar in both groups. BLBC tumours were larger ($p = 0.032$) and had poorer differentiation ($p = 0.0031$) compared to non-BLBC group. Patients with TNBC phenotype had worse breast cancer-specific survival than the receptor-positive control group ($p = 0.0012$). There was an association between larger tumour size and nodal status in steroid receptor-positive tumours ($p = 0.038$), which was not present in TNBC group ($p = 0.27$). The mean follow-up time was 64.1 months.

Associations between vimentin, Sip1 and zeb1 and clinicopathological prognostic factors

Of the studied EMT markers, vimentin was the most significantly associated with traditional clinicopathological prognostic factors (Table 2). Vimentin expression in invasive cancer cells was observed especially in highly proliferating ($p = 0.00051$) and poorly differentiated ($p = 0.000038$) cancers. Vimentin expression in in situ areas associated less significantly to high proliferation rate ($p = 0.046$) and high grade ($p = 0.018$). Sip1 expression associated with smaller tumour size ($p = 0.029$). Zeb1 associated with higher Ki-67 expression ($p = 0.051$). Interestingly, all cases except one with high zeb1 stromal expression had both vascular and lymphatic vessel invasion, whereas there were no tumours with low zeb1 stromal expression and vessel invasions (for lymphatic vessel invasion $p = 0.057$; for vascular invasion $p = 0.030$). Zeb1 positivity in tumour cells was associated to higher grade ($p = 0.00048$). None of the studied markers were

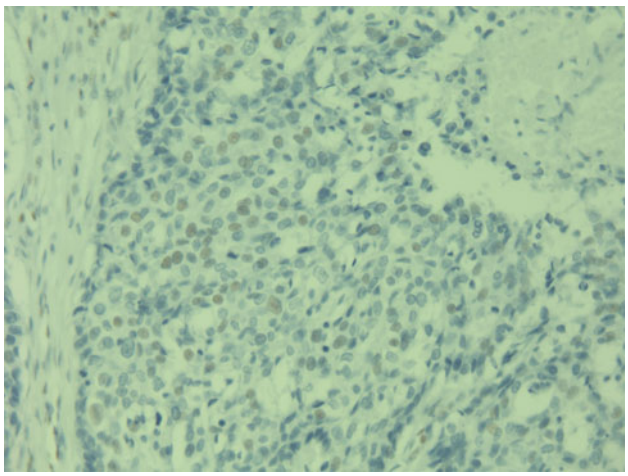

Fig. 3 Zeb1 expression in breast carcinoma. In the tumour cell compartment positively stained nuclei can be seen. (*Size bar* 100 μm)

associated with nodal status. Table 2 shows these associations separately in TNBC and non-TNBC patients. There was no association between vimentin expression and either zeb1 or Sip1 in the whole study group, although vimentin (in invasive cells) and zeb1 (in tumour) had a significant co-expression in non-TNBC tumours ($p = 0.017$).

None of the studied markers had significant prognostic significance in terms of breast cancer-specific survival, although trends to poorer survival with EMT marker expression were observed (vimentin in invasive cells $p = 0.056$; Sip1 nuclear expression $p = 0.060$). Nuclear Sip1 expression, however, predicted poor disease-free survival ($p = 0.0024$) (Fig. 5). This was observed in both TNBC ($p = 0.046$) and ER+/PR+/HER2− ($p = 0.003$) groups.

Associations between TNBC and non-TNBC groups

All studied EMT markers were significantly overexpressed in TNBC group versus ER+/PR+/HER2−, especially vimentin in invasive breast cancer cells ($p = 9.3 \times 10^{-12}$) (Table 3). Table 1 shows the expression of these markers in TNBC and non-TNBC groups in more detail.

Discussion

A characteristic feature for EMT is a gene switch resulting in downregulation of E-cadherin and upregulation of vimentin, smooth muscle actin and N-cadherin. By detecting such markers in tumours, a population of tumour cells undergoing EMT activity in a tumour cell population can be roughly estimated.

We report here a significantly higher number of vimentin expressing breast tumours in TNBC than in non-TNBC cases. This is in line with the concept that ER receptor loss leads to upregulation of EMT related markers and appearance of cytoplasmic vimentin as suggested in previous studies [28]. Both vimentin expression in in situ areas and in invasive cells was also strongly associated with tumour grade, but this was observed only in TNBC group. Vimentin also had trend towards poorer survival even though this association was present in the whole material and not separately in TNBC or non-TNBC. Recently Jeong et al. [6] reported an overexpression of EMT markers including vimentin in TNBC cohort. Also in line with the current study performed on whole histological sections, their tissue microarray material demonstrated a connection between EMT phenotype and higher tumour grade. Smaller studies have also previously reported vimentin expression in tumours with poor differentiation [29, 30], although an association with vimentin and high proliferation has been varied from study to study [29, 31]. In our material, vimentin was also significantly associated

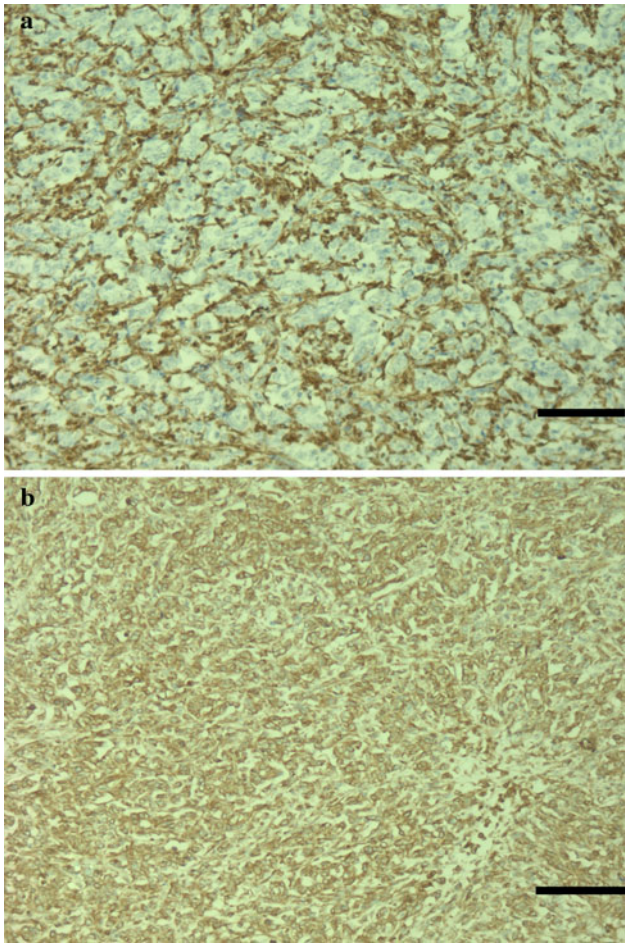

Fig. 4 A case of a ductal carcinoma showing no expression of vimentin in tumour cells (**a**). A case of a ductal carcinoma showing strong cytoplasmic positivity for vimentin (**b**). (*Size bar* 180 μm for both)

Table 2 Sip1, vimentin, zeb1 and the presence of EMT area in tumour are compared to tumour size, nodal status, grade, Ki-67 expression, lymphatic vessel invasion and vascular invasion

All cases	*T*	*N*	Grade	Ki-67	LVI	Vascular invasion
Sip1	0.029 ↓					
Nuclear Sip1						
Vimentin in in situ areas			0.018 ↑	0.046 ↑		
Vimentin in invasive cells			0.000038 ↑	0.00051 ↑		
Zeb1 in stroma					0.057 ↑	0.030 ↑
Zeb1 in tumour cells			0.00048 ↑	0.051 ↑		
TNBC cases	***T***	***N***	**Grade**	**Ki-67**	**LVI**	**Vascular invasion**
Sip1	0.0064 ↓					
Nuclear Sip1			0.024 ↓			
Vimentin in in situ areas			0.000071 ↑			
Vimentin in invasive cells			0.000071 ↑			
Zeb1 in stroma					0.036 ↑	0.039 ↑
Zeb1 in tumour cells	0.027 ↑		0.0020 ↑		0.066 ↑	0.036 ↑
ER+/PR+/HER2− cases	***T***	***N***	**Grade**	**Ki-67**	**LVI**	**Vascular invasion**
Sip1						
Nuclear Sip1			0.021 ↑			
Vimentin in in situ areas						0.025 ↑
Vimentin in invasive cells						
Zeb1 in stroma				0.053 ↑		
Zeb1 in tumour cells				0.045 ↑	0.0060 ↑	0.057

Inverse correlations are marked with ↓ and positive correlations with ↑. *p* values are from 2-sided Chi square test and only (near-) significant *p* values are reported

LVI lymphatic vessel invasion

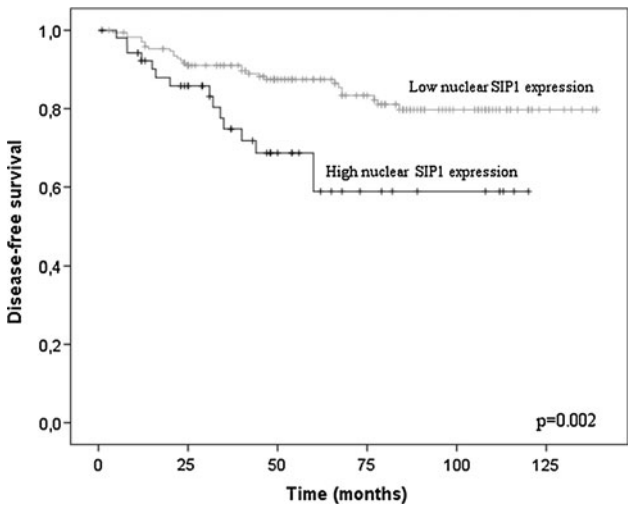

Fig. 5 Kaplan–Meier curve showing disease-free survival according to nuclear Sip1 expression. Both TNBC and ER+/PR+/HER2− patients are included to the analysis. *Crosses* indicate censored cases

Table 3 Differences in Sip1, vimentin and zeb1 expression between TNBC versus non-TNBC (ER+/PR+/HER2−) tumours and basal-like compared to non-basal-like TNBC tumours

	TNBC versus ER+/PR+/HER2−	Basal-like versus non-basal-like
Sip1		
Nuclear Sip1		0.035
Vimentin in in situ areas	2.2×10^{-6}	0.0013
Vimentin in invasive cells	9.3×10^{-12}	0.0091
Zeb1 in stroma		0.013
Zeb1 in tumour		

p values are from 2-sided Chi square test and only significant *p* values are reported. In all associations with significant *p* value higher expression is in either TNBC or basal-like tumours compared to control group

with BLBCs versus non-basal-like TNBCs but not with the TNM status of the tumours. Previous studies have reported contradictory results whether vimentin could be used to delineate basal-like breast carcinoma from the other TNBCs [29, 32]. Our results, however, give support for the hypothesis that EMT (roughly measured as vimentin expression) could be in induced in BLBCs more than CK 5/6-negative and EGFR-negative TNBCs. Feasibility of

vimentin expression to differentiate basal-like from non-BLBC should be studied with larger materials, since these two subtypes have significantly different clinical course and probably also different treatment in the future [33].

Epithelial-to-mesenchymal transition is regulated by transcription factors such as snail, slug, twist, zeb1 and Sip1 which are able to downregulate genes associated with cellular adhesion and upregulate genes related to mesenchymal traits such as vimentin. According to our knowledge, Sip1 has not been previously evaluated in TNBCs or BLBCs. Nevertheless, importance of Sip1 in EMT regulation has been recognized several years ago [10, 11]. In a previous study, Sip1 was induced especially in vimentin expressing breast cancer cells and Sip1 expression appeared to increase vimentin expression [34]. In the current material no association between vimentin and either zeb1 or Sip1 expression was observed. The previous study also indicated a role for Sip1 and vimentin in cell migration. We found that Sip1 was associated with a smaller size of the breast carcinomas, but only among TNBC cases. Nuclear Sip1 positivity was, however, associated with a worse survival of the patients. This apparent inconsistency may be explained by one characteristic feature of TNBCs, namely poor correlation between tumour size and clinical course [3]. On the other hand, it was specifically cytosolic Sip1 that associated with small tumour size; in nuclear Sip1 this association was not observed. There are not very many studies on the influence of Sip1 on patient prognosis in cancer material. In non-small cell lung carcinoma Sip1 predicted a poor survival and associated with advanced stage of the tumours, however [35].

Zeb1 expression in tumour cells was associated with vimentin in non-TNBC but not in TNBC. In line with this association, vimentin has been shown to be a zeb1 responsive gene in lung carcinoma [36]. It has previously been shown that expression of zeb1 is low in breast carcinoma [37] and its expression in TNBC was 16 % while that of Snail was 80 % [38]. We detected zeb1 positivity in about 35 % of TNBC but there was no significant difference between the expression of zeb1 in TNBC and non-TNBC. Instead, zeb1 was significantly overexpressed in BLBC compared to non-basal-like TNBCs. The difference in the frequencies between our and previous studies depends on that we accepted a low number of positive tumour cells as representing positive cases and on the fact that we did not use array based samples. As previously shown the expression of zeb1 in tumour associated fibroblasts was many times stronger and this stromal expression was nearly significantly associated with vessel invasion in tumours in TNBC while Ki-67 expression was positively associated with it in non-TNBC. Tumour cell zeb1 expression also associated with higher grade in both TNBC and non-TNBC groups. All cases with high zeb1 expression in stroma had both vascular and lymphatic vessel invasion, but no tumours with low zeb1 stromal expression showed any vessel invasions. This association is especially interesting since a part of zeb1 positive stromal cells may represent mesenchymal-like stem cells [37]. This finding is emphasized by the fact that TNBCs do not show a higher frequency of vessel invasion than non-TNBC [39]. Interestingly also, in colon carcinoma, zeb1 induced EMT-like features and vascular mimicry in tumour cells which was abolished by zeb1 knockdown [40]. Knockdown of zeb1 also reduced invasion and locomotion of the tumour cells and provoked EMT in cell culture studies [40]. However, in our material zeb1 stromal positivity did not associate with survival in the whole material ($p = 0.21$) or separately in TNBC ($p = 0.26$) even though there was a tendency for a worse survival in both groups.

Triple-negative breast cancer is known to have aggressive clinical course [1]. This was also shown in our material where survival of TNBC was significantly worse compared to the non-TNBC cohort. As previously reported in most of the studies, triple negativity was also in our material associated with tumour size, high proliferation and high grade but not with the presence of axillary metastases [reviewed in 3]. The association of TNBC with such aggressive features may partly be explained by a heightened EMT-like activity in these tumours. First site of distant metastasis was in visceral sites in 86.7 % of TNBC women, but in non-TNBC patients only in 33.3 %. This is also in line with previous literature [3]. One of the problems considering clinical decision making of TNBC risk assessment is especially poor association between tumour size and nodal status [26]. Again in our study, there was no association between these two important prognostic factors in TNBC cohort, although it was present in women with ER/PR-positive disease.

One of the strengths in this study was careful examination of vimentin, zeb1 and Sip1 in different tumour compartments. In the light of the current results, Sip1 seems to have the most important role in nuclei of the cancer cells, which is logical with its function as a transcription factor. However, stromal zeb1 seems to have influence on lymphovascular invasion and especially stromal zeb1 expression is induced in BLBCs. Vimentin expression in non-invasive areas may also be contributed to vascular invasion, at least in non-TNBCs. Therefore, we suggest that in future studies also EMT marker assessment should be not done only for cancer cells. On the other hand, one of the most prominent weaknesses of the pure immunohistochemical studies includes poor definition of causalities. Taken together, our results suggest a strong association between vimentin (as an EMT marker) and EMT-regulating transcription factors and poor prognostic factors, especially in TNBCs, although not having clear effect on breast cancer-specific survival.

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