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Original Article

Epithelial-mesenchymal transition markers in lymph node metastases and primary breast tumors - relation to dissemination and proliferation

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Abstract: Epithelial-mesenchymal transition (EMT) was shown to enhance metastatic abilities of cancer cells, but it remains elusive in clinical samples. Moreover, EMT is rarely studied in lymph node metastases (LNM), thus limiting our understanding of its role outside of the primary tumors (PT). We collected a set of samples including triplets - PT, circulating tumor cells (CTCs)-enriched blood samples and LNM from 108 early breast cancer patients. With immunohistochemistry we analyzed levels of EMT effectors – E-cadherin, vimentin and N-cadherin in LNM, central areas and margins of PT. Additionally, expression of EMT core regulators *TWIST1, SNAI1, SNAI2* was measured with RT-qPCR. Patients with E-cadherin loss had CTCs in 45% of the cases in comparison to 23% with normal E-cadherin level (P = 0.05). Mesenchymal phenotype of CTCs-enriched blood fractions was five-times more frequent in patients with E-cadherin loss in PT compared to PT with normal E-cadherin levels (P = 0.01). Epithelial/mesenchymal status of matched samples at different stages of dissemination was frequently discordant, especially for pairs involving CTCs, indicating high plasticity of tumor cells. LNM showed increased expression of *TWIST1, SNAI1, SNAI2* accompanied by decreased Ki67 labeling index, with median Ki67 of 15% in PT and 10% in LNM (P = 0.0002). Our findings demonstrate that E-cadherin loss, not only in PT margin, might lead to seeding of especially malignant CTCs with mesenchymal phenotype. In comparison to PT, cells in LNM re-express E-cadherin, upregulate EMT transcription factors and reduce cell division rate, which could be viewed as their long-term survival strategy.

Keywords: Epithelial-mesenchymal transition, epithelial-mesenchymal plasticity, lymph node metastases, circulating tumor cells, Ki67

Introduction

Development of distant metastases is a preliminary cause of cancer mortality. Even though extensive efforts are made to understand the mechanisms of metastatic spread, fundamental questions remain unanswered. One of the most widely discussed topics in cancer dissemination deals with the involvement of epithelial-mesenchymal transition (EMT) in metastatic cascade. Easy to observe *in vitro*, EMT still remains elusive when analysis of clinical material is concerned [1]. Nevertheless, results show that changes connected with EMT are associated with metastasis in different solid tumors [2-4]. Classically, EMT is described as a loss of cell polarity as well as firm cell-cell contacts by switching expression of E-cadherin to N-cadherin; and acquisition of migratory and invasive phenotype with vimentin (VIM) which is a trait of the mesenchymal phenotype [5, 6]. Thus, loss of E-cadherin, expression of N-cadherin and VIM are frequently used for assessing how advanced in EMT are tumor cells [1]. However,

EMT can generate a whole spectrum of epithelial/mesenchymal phenotypes, each carrying its unique invasive and metastatic properties, often complementing each other for successful metastases formation [7, 8]. Complementary markers, like EMT core regulators (transcription factors) TWIST1, SNAI1 and SNAI2 are helpful for studying EMT but their expression cannot be linked with the extent of EMT engagement in a cell [1].

In primary breast tumors, loss of E-cadherin expression correlates with poor clinicopathological characteristics [9, 10] and decreased survival [10-12], also in patients without lymph node involvement [13]. Nevertheless, E-cadherin positive cancer cells were also shown to be invasive, when co-expressing N-cadherin [14], which underlines the complexity of metastatic strategies. In gastric cancers reduced E-cadherin level was also related to the presence of CTCs detected by expression of cytokeratin 18 [15]. In a mouse model of breast cancer dissemination Bonnomet *et al* presented progressively increasing levels of VIM-positive CTCs, which coincided with an increase of VIM-positive area of the primary tumor [16]. In addition, results from breast cancer patients show that CTCs indeed may have mesenchymal phenotype. Unfortunately, studying mesenchymal CTCs remains largely an academic enterprise [8, 17], due to the limited abilities of clinically approved CTCs detection assays to capture mesenchymal CTCs [18, 19]. We have recently applied an epithelial marker-independent enrichment of CTCs from early breast cancer patients and have shown that CTCs-enriched blood fractions have mesenchymal features, with increased expression of invasion and metastasis related markers – *CXCR4* and *uPAR* [20]. We also noted that presence of both epithelial and mesenchymal CTCs correlates with lymph node involvement [20], but lymphatic dissemination is decreased in PTs expressing mesenchymal markers *SNAI1* and vimentin [21]. Thus, different mechanisms of dissemination might occur in lymphatic and hematogenous dissemination. However, research linking the occurrence of EMT in matched clinical samples with lymphatic and hematogenous dissemination is still limited. EMT process is mostly studied in primary tumors, which diminishes the significance of tumor cells seeding from sites other than primary tumor. Interestingly, gene expression profile of lymph node metastases might be more informative in terms of predicting patients survival than profiling matched primary tumors [22].

Reports exist showing comparative analysis of various EMT markers in primary breast tumors and corresponding metastases, but often they are restricted to a single EMT marker [23-28], do not evaluate CTCs [23-26, 28, 29] or concern experimental metastases in animal models [16, 30] (which do not recapitulate complexity of the metastatic cascade in humans). As multiple markers of EMT were rarely analyzed on matched cancer samples encompassing different stages of tumor dissemination, therefore, in this study we aimed at analyzing multiple effectors of EMT (E-cadherin, N-cadherin, vimentin) and its core regulators (*TWIST1, SNAI1, SNAI2*) in primary tumors and lymph node metastases and correlate them with presence and phenotype of circulating tumor cells. This knowledge might help to reveal the importance of EMT activation for tumor cells dissemination, survival and growth during clinical course of the disease.

Materials and methods

Primary tumors (PT) of non-lobular histological type (N = 108), lymph node metastases (LNM, N = 55) and CTCs-enriched blood samples (N = 98) from 108 breast cancer patients (stage I-III) were investigated; 53% of the patients had lymph nodes involved. Patients were admitted to the Medical University Hospital in Gdansk between April 2011 and May 2013, where they were operated and treated according to the current standards of care. For staging and lymph node status classification American Joint Committee on Cancer staging manual version 7 was used. Tumor grade was assessed according to the modified Bloom and Richardson system [31]. Median age of the patients was 60 years (28.3-85.6 years). Median follow-up period was 2.4 years and was last updated in May 2014. Clinico-pathological characteristics of the patients included in the study are presented in the Table S1.

The inclusion criteria were operable breast cancer of non-lobular histological type confirmed by histological examination and a signed consent form. The study was accepted by the Independent Ethics Committee of the Medical University of Gdansk.

Peripheral blood samples (5-10 ml) for CTCs isolation were collected prior to surgery and only from neoadjuvant chemotherapy/hormone therapy naïve patients. Detailed methodology of CTCs enrichment and analysis was described previously [20]. In summary, blood samples (5 ml) were diluted with phosphate buffered saline and subjected to density gradient centrifugation followed by negative selection step for depletion of CD45-positive cells with magnetic particles (CD45 Dynabeads, Invitrogen, Oslo, Norway). After depletion, CTCs-enriched blood fractions were used for RNA isolation and gene expression analysis. CTCs recovery rate, based on cell line spike-in experiment was within 54%-72% [20].

Protocol of PT and regional LNM collection was described previously [21]. Briefly, PT and LNM removed during surgery were subjected to histopathological examination; tumor involved sections were formalin-fixed and paraffin-embedded (FFPE), and evaluated for the presence of cancerous component with hematoxylin-eosin staining under light microscope. Representative fragments indicated by a pathologist were used for immunohistochemistry and gene expression analysis.

Immunohistochemistry

Expression of E-cadherin (mouse monoclonal antibody, clone NCH 38, Dako, Copenhagen, Denmark), N-cadherin (mouse monoclonal antibody, clone 6G11, Dako) and VIM (mouse monoclonal antibody, clone V9, Dako) was analyzed on the whole FFPE tissue sections (full-face sections) to be able to capture both center and margin of the tumor (presumably invasive edge). For the purpose of this analysis the tumor margin was defined as the most peripheral 1 mm wide area of the tumor, while the central part of the tumor was the core region of the specimen separated from tumor margin by at least a 2 mm wide border zone. ER (rabbit monoclonal antibody, clone SP1, Roche, Tuscon, USA), PgR (rabbit monoclonal antibody, clone 1E2, Roche) and HER2 (rabbit monoclonal antibody, clone 4B5, Roche) were analyzed on whole slides during standard pathological assessment of the tumor. TMA, prepared as described in [32], from PT and LNM were used for Ki67 staining (mouse monoclonal antibody, clone MIB-1, Dako).

Antigen retrieval and staining was performed on the automatic devices: BenchMark GX (Ventana, Roche) for ER, PgR, HER2 staining, whereas DAKO AutostainerLink48 (DAKO, Copenhagen, Denmark) was used for E-cadherin, N-cadherin, VIM, and Ki67 staining. For negative controls, the primary antibodies were omitted. ER, PgR, HER2 was detected using UltraView DAB Benchmark XT system (Roche), Ki67, E-cadherin, N-cadherin and VIM with EnVision™ FLEX Dako Autostainer (Dako).

Previously established threshold value of 10% was used to define positive expression of E-cadherin, N-cadherin and VIM [22, 33]. Additionally, for E-cadherin, N-cadherin and VIM, H-score was calculated (0-300) by multiplying the percentage of positively stained cells (0-100%) and intensity of staining (0, 1, 2 or 3). For an H-score based cut-off value, the median H-score was taken to define positive expression. Cut-off value of 14% of positively stained nuclei for Ki67 was applied according to St Gallen recommendation [34]. ER and PgR staining was evaluated according to Allred system, with a score of at least 3 being counted as positive expression; HER2 was considered positive according to previously described criteria [35] involving immunohistochemical and fluorescent *in situ* hybridization analysis (for equivocal samples with IHC 2+ score). Only invasive carcinoma component was considered in scoring. For E-cadherin and N-cadherin moderate to intense membrane staining was observed; VIM showed moderate to intense cytoplasmic staining. Specimens were analyzed under a light microscope (Olympus BX43F, Tokyo, Japan). Two pathologists (J.Sz. and H.M.), blinded to clinical data, independently reviewed all stained slides. Discrepancies were resolved by simultaneous viewing with a multihead microscope.

Gene expression analysis

RNA was isolated using TRIzol (Invitrogen, Oslo, Norway) from freshly prepared CTCs-enriched blood fractions (described in [20]) or with RNeasy FFPE kit (Qiagen, Hilden, Germany) from freshly cut FFPE sections of PT and LNM, with tumor cell content higher than 50% (described in [21]). Reverse transcription using random hexamer primers was performed with Transcriptor cDNA First Strand Synthesis Kit (Roche, Mannheim, Germany) for all samples.

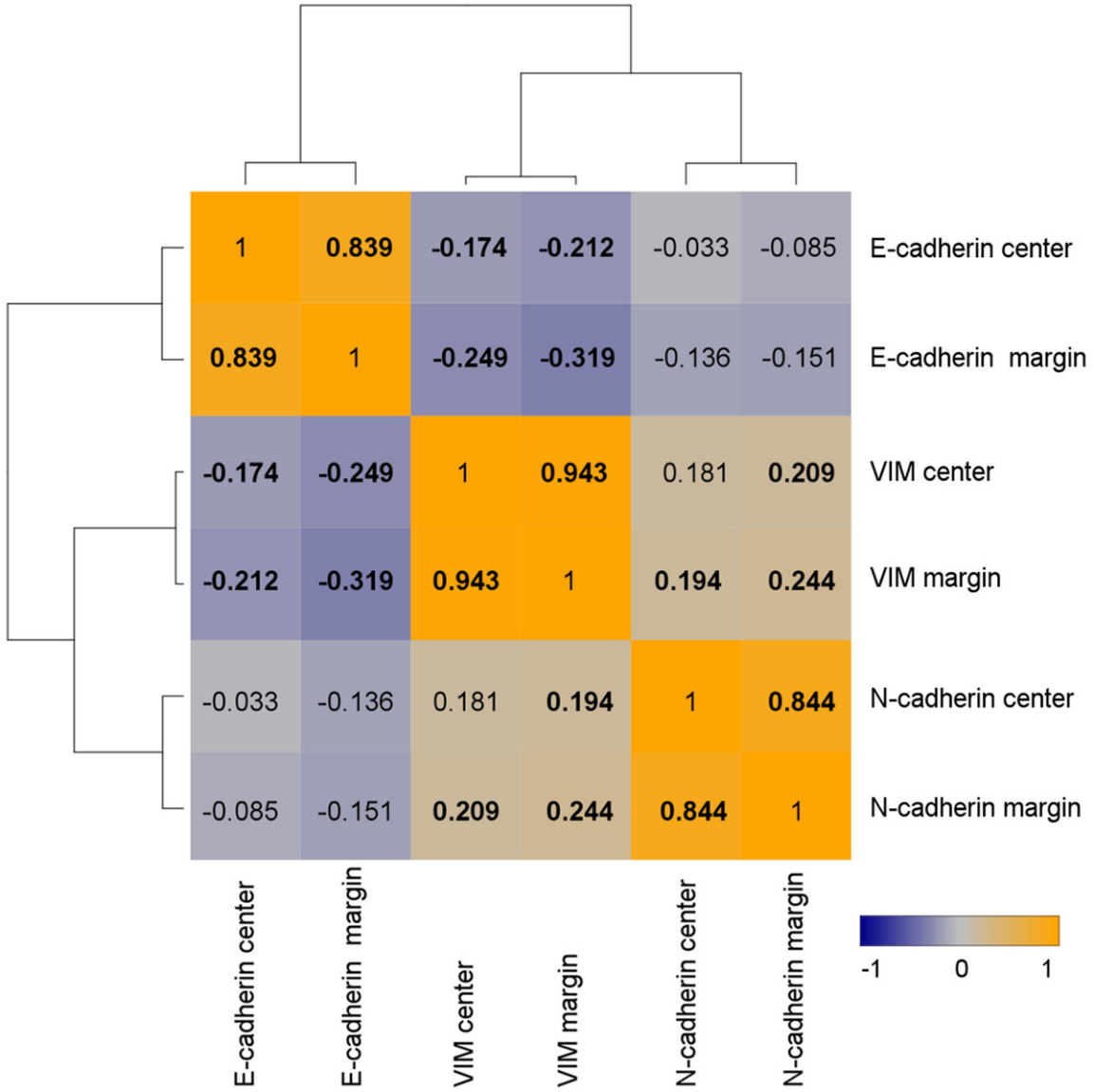

Figure 1. Heatmap of Spearman rank correlation coefficients calculated on H-scores for the analyzed EMT effectors: E-cadherin, N-cadherin, vimentin (VIM) in the primary tumor central area and margins (N = 108). Numerical values of correlations coefficients are presented in the corresponding boxes (statistically significant values in bold).

The methodology of gene expression analysis was described previously (for CTC in [20], PT and LNM in [21]). Briefly pre-designed, optimized hydrolysis probes and primers sets (TaqMan Gene Expression Assay, Applied Biosystems, Foster City, California, USA) were purchased for the analysis of expression of *TWIST1* (Hs00361186_m1), *SNAI1* (Hs00195591_m1), *SNAI2* (Hs00950344_m1) in PT and LNM. Expression of cytokeratin 19 (*CK19*; Hs01051611_gH), vimentin (*VIM*; Hs00185584_m1), mammaglobin 1 (*MGB1*; Hs00935948_m1) and *HER2* (Hs99999005_mH) was measured in CTCs-enriched blood fractions. Reference genes - *GAPDH* (Hs99999905_m1) and *YWHAZ* (Hs03044281_g1) were chosen based on their expression stability analyzed in geNorm [21]. Twenty microliter reactions were performed using TaqMan® Universal PCR Master Mix (Applied Biosystems, Branchburg, New Jersey, USA) on 96-well plates in CFX96 cycler (Bio-Rad, Hercules, California, USA). Reactions were performed in duplicates, each plate contained an inter-run calibrator, a set of no template controls and controls for detecting contaminating gDNA (only for assays which

Table 1. E-cadherin, N-cadherin and VIM and status in the central area and margins of primary tumor (PT). Conversion rates from negative to positive (- → +) and positive to negative (+ → -) status between center and margin are given as number of cases and percentages of the total samples number. Kappa coefficient of concordance is given with 95% confidence interval (CI). N- number of cases

Analyzed marker	N	Positive in PT*		Conversion center → margin			kappa coefficient (95% CI)
		center	margin	(-) → (+)	(+) → (-)	Switch total	
		N (%)	N (%)	N (%)	N (%)	N (%)	
E-cadherin	108	78 (72)	77 (71)	3 (3)	4 (4)	7 (7)	0.840 (0.726-0.954)
N-cadherin	108	11 (10)	14 (13)	4 (4)	1 (1)	5 (5)	0.774 (0.585-0.964)
VIM	108	18 (17)	19 (18)	1 (1)	0 (0)	1 (1)	0.967 (0.904-1)

*Results based on the 10% cut-off value.

could detect gDNA). Calculation of gene expression was performed in qBasePLUS (version 2.1, Biogazelle, Zwijnaarde, Belgium) using a modified ΔΔCt approach [36]. PT and LNM samples were considered positive for the analyzed marker when expression level was higher than median. For CTCs, as previously reported [20], samples being *CK19+/VIM-* and *MGB1+* or *HER2+* were classified as epithelial CTCs-positive, whereas mesenchymal CTCs-positive samples were defined as *CK19-/VIM+* and *MGB1+* or *HER2+*.

Statistical analysis

Statistical software (version 10, StatSoft) was used for the analysis of categorical data (χ^2 or Fisher's exact test, where appropriate) and continuous values (Mann-Whitney test). Kappa (κ), being a measure of the strength of agreement, was calculated using MedCalc Software (version 12.5.0.0). Interpretation of kappa coefficient was according to Altman [37], with agreement defined as poor (κ < 0.20), fair (0.21-0.40), moderate (0.41-0.60), good (0.61-0.80) and very good (0.81-1). A heatmap of Spearman rank correlation coefficients was calculated on H-scores from immunohistochemical analysis for each pairwise combination of protein markers using TMA Navigator online software [38]. The dendrogram was created by applying agglomerative hierarchical clustering with complete linkage in TMA Navigator.

Results

Comparison of EMT effectors expression in PT center and margin

As EMT is thought to preferentially occur at the tumor-stroma border [39] we have therefore analyzed full-face sections covering tumor center and margin to be able to find those aggressive cancer cells. However, expression pattern of EMT effectors was highly concordant between center and margin of the tumor (**Figure 1**). As expected, mesenchymal markers – VIM and N-cadherin clustered together in the dendrogram (**Figure 1**). In PT center E-cadherin H-score correlated negatively with VIM (rho Spearman -0.174, P = 0.03) but not with N-cadherin (rho Spearman -0.033, P = 0.66). Observed correlations were stronger in PT margin - E-cadherin vs. VIM (rho Spearman -0.319, P = 0.0001) and E-cadherin vs. N-cadherin (rho Spearman -0.151, P = 0.09; **Figure 1**). Kappa coefficient of concordance, assessing markers expression between tumor center and margin, was very good for E-cadherin (0.840) and VIM (0.967), slightly lower for N-cadherin (0.774) (**Table 1**). Exemplary photographs of E-cadherin, N-cadherin and VIM staining in PT center, PT margin and LNM are depicted in **Figure 2**.

Comparison of EMT effectors expression in PT and LNM

When status of EMT effectors was compared between center of PT and LNM, we noticed a poor concordance rate for E-cadherin – with 63% of PT and 98% of LNM being positive for E-cadherin (κ = -0.040, 39% of discordant cases) (**Table 2**). N-cadherin status was more similar (κ = 0.556), with 11% and 17% of positive cases in PT and LNM, respectively. A switch from negative N-cadherin in PT to positive in LNM was more frequent and occurred in 9% (4/46) of the samples. VIM showed the best concordance rate between PT and LNM (κ = 0.921), with only one sample with changed status from VIM negative in PT to positive in LNM.

Similar results were observed when the status of the analyzed markers was compared between PT margin and LNM (Table S2). It was

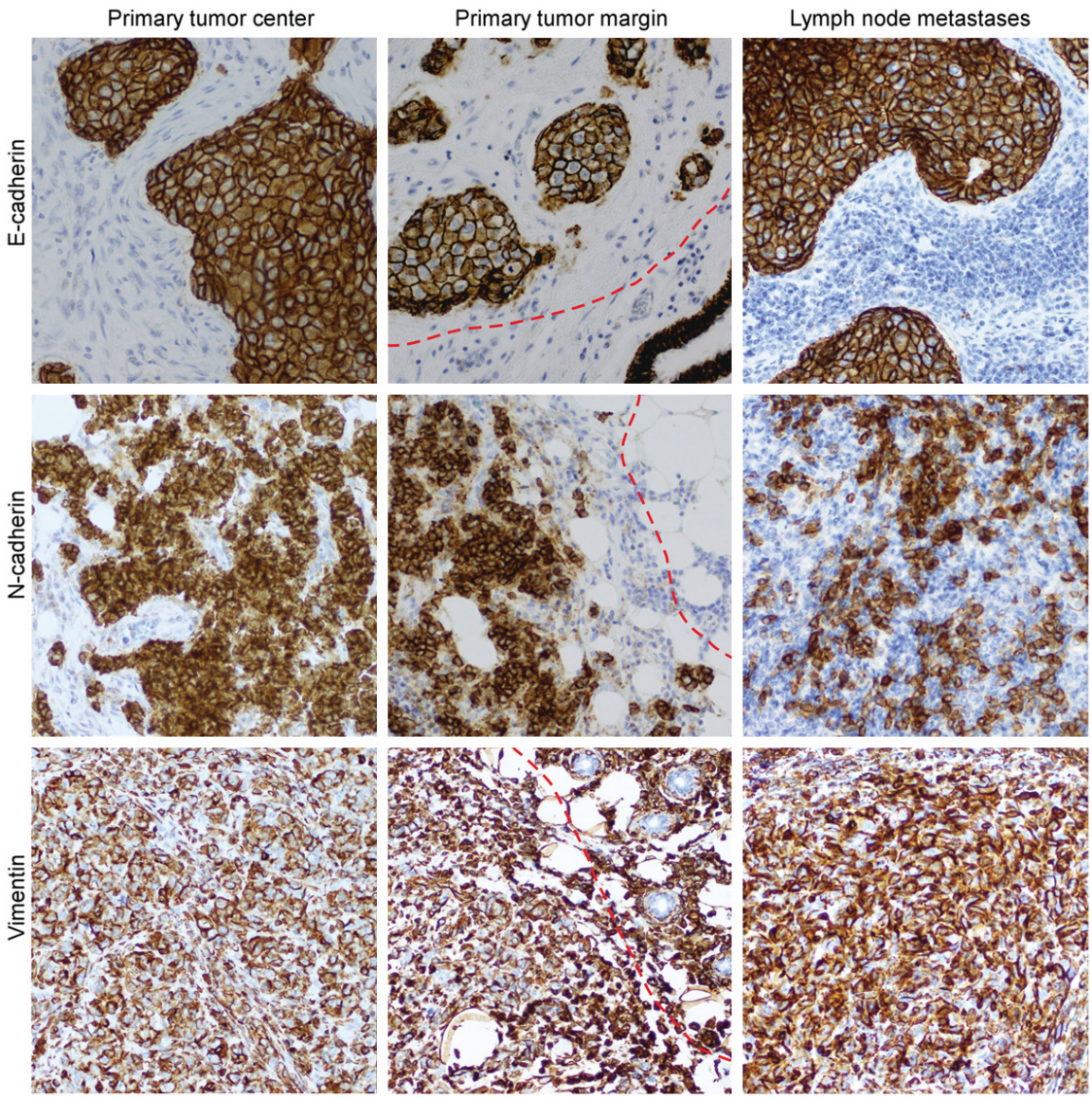

Figure 2. Exemplary photographs of E-cadherin, N-cadherin and vimentin (VIM) immunohistochemical staining in primary tumors central area, margin (showing also regions of healthy breast tissue on the right from the dotted line) and lymph node metastases.

Table 2. Comparison between E-cadherin, N-cadherin and VIM status in the central part of primary tumor (PT) and in lymph node metastases (LNM). Conversion rates from negative to positive (- → +) and positive to negative (+ → -) status between PT and LNM are given as number of cases and percentages of the total samples number. Kappa coefficient of concordance is given with 95% confidence interval (CI). N – number of cases

Marker	N	Positive in PT*	Positive in LNM*	Conversion PT → LNM			kappa coefficient (95% CI)
		N (%)	N (%)	(-) → (+) N (%)	(+) → (-) N (%)	Switch total N (%)	
E-cadherin	49	31 (63)	48 (98)	18 (37)	1 (2)	19 (39)	-0.040 (-0.117-0.036)
N-cadherin	46	5 (11)	8 (17)	4 (9)	1 (2)	5 (11)	0.556 (0.216-0.896)
Vimentin	49	7 (14)	8 (16)	1 (2)	0 (0)	1 (2)	0.921 (0.769-1)

*Results based on the 10% cut-off value.

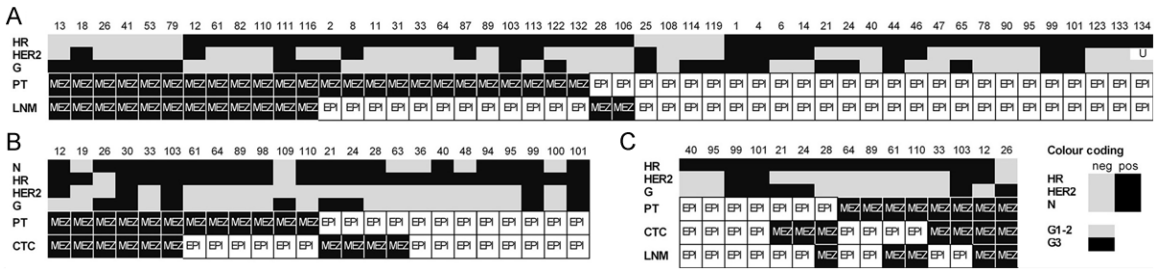

Figure 3. Epithelial/mesenchymal status of matched: (A) PT and LNM (N = 49), (B) PT and CTCs-enriched blood fractions (CTC) (N = 24), (C) PT, CTCs, LNM (N = 15). Epithelial phenotype defined as: E-cadherin present, N-cadherin and VIM negative (for PT and LNM) or as *CK19+/VIM-* and *MGB1+* or *HER2+* (for CTCs). Mesenchymal phenotype defined as a): E-cadherin loss or N-cadherin or VIM positive (for PT and LNM) or *CK19-/VIM+* and *MGB1+* or *HER2+* (for CTCs). Additionally, below patients number (top row in each subfigure) hormone receptor status (HR), HER2 status (HER2), lymph node involvement (N) is marked as negative in gray or as positive in black squares. Tumor grade (G) 1 and 2 marked in grey, grade 3 in black. U in panel A – unknown HER2 status.

Table 3. Ki67 labeling index in matched PT and LNM. N – number of cases. Statistical significance P = 0.001

	Ki67 in LNM		
Ki67 in PT	< 14% N (%)	≥ 14% N (%)	Total
< 14%	17 (94)	1 (6)	18
≥ 14%	14 (48)	15 (52)	29
Total	31	16	47

to be expected considering that no statistically significant differences were observed between EMT effectors expression between center and margin of PT.

Changes of EMT status in matched pairs and triplets of PT, CTCs and LNM

To see how the epithelial-mesenchymal status differs between PT, CTCs-enriched blood fractions and LNM, samples were divided into two groups – (1) epithelial (PT and LNM – E-cadherin present, N-cadherin and VIM negative; CTCs-enriched blood fractions – *CK19+/VIM-* and *MGB1+* or *HER2+*) and (2) mesenchymal (PT and LNM – E-cadherin loss or N-cadherin or VIM positive; CTCs-enriched blood fractions - *CK19-/VIM+* and *MGB1+* or *HER2+*). In total we collected 49 cases of matched PT-LNM, 24 cases of matched PT-CTC, 15 cases of matched LNM-CTC and 15 cases of matched PT-CTC-LNM triplets. In case of pairs PT-LNM (**Figure 3A**) mostly concordant phenotypes were observed (35/49), however switches from mesenchymal-to-epithelial (12/49) and epithelial-to-mesenchymal (2/49) phenotype were also found. Interestingly, occurrence of any phenotype switch between PT and LNM (phenotype plasticity) was detected only in PT which were hormone receptor positive (HR+ being ER+ and/or PR+ (P = 0.04)) and mostly of low histological grade (G1-2, P = 0.037). Nevertheless, LNM derived from HR- PT had mesenchymal phenotype in 60% (6/10) of the cases, whereas LNM derived from HR+ tumors were mesenchymal only in 21% (8/39) of the cases (P = 0.02). It appears though that HR+ tumors show higher propensity for phenotype plasticity, whereas HR- tumors are inherently more mesenchymal.

For pairs involving CTCs-enriched blood fractions greater phenotype plasticity was found (**Figure 3B** and **3C**), with 42% (10/24) of PT-CTC pairs and 40% (6/15) of LNM-CTC pairs having discordant epithelial/mesenchymal status. In PT-CTC-LNM triplets 27% (4/15) of the triplets consistently showed epithelial status and 13% (2/15) mesenchymal status. Another 27% (4/15) of CTCs-enriched blood fractions had their epithelial/mesenchymal-like status different from PT or LNM (**Figure 3**).

Comparison of EMT core regulators expression in PT and LNM

Knowing that EMT process may not always lead to complete loss of epithelial markers or prominent acquisition of mesenchymal markers we have quantitatively analyzed (with RT-qPCR) expression of additional markers – EMT core regulators (transcription factors) *TWIST1, SNAI1* and *SNAI2* in PT and LNM. Due to the fact that sections of tumor margin contain more stroma cell than the central part of the PT and RT-qPCR does not allow for morphological

Table 4. Median Ki67 labeling index of primary tumors (PT) and lymph node metastases (LNM) divided into positive (+) and negative (-) for the expression of particular EMT core regulators. CI – confidence interval; N-number of cases

Gene status	PT N	PT median Ki67 (95% CI)	P	LNM N	LNM median Ki67 (95% CI)	P
TWIST1-	41	20% (5-80%)	0.001	17	12% (2-25%)	0.68
TWIST1+	41	10% (2-35%)		19	8% (4-40%)	
SNAI1-	40	19% (3.5-80%)	0.45	19	10% (2-40%)	0.91
SNAI1+	42	14% (3-70%)		17	8% (4-30%)	
SNAI2-	42	20% (5-80%)	0.03	17	15% (2-40%)	0.04
SNAI2+	40	11% (2-38%)		19	8% (3-20%)	

cell differentiation, we have decided to isolate RNA for gene expression analysis only from the central part of the PT. We observed frequent status conversion of EMT core regulators between PT and LNM. A switch from negative status in PT to positive in LNM was more frequent than from positive in PT to negative in LNM – respective conversion rates were 15% and 3% for *TWIST1*, 9% and 6% for *SNAI1*, 24% and 9% for *SNAI2*. Globally, expression of all EMT core regulators was higher in LNM in comparison to PT. Median expression of *TWIST1* in PT – 0.17 (95% CI 0.003-0.89) and LNM 0.64 (95% CI 0-2.41) – P = 0.003; *SNAI1* in PT 0.14 (95% CI 0.03-0.80) in LNM 0.84 (95% CI 0-4.52) – P < 0.00001; *SNAI2* in PT 0.10 (95% CI 0-0.31) in LNM 0.67 (95% CI 0-2.14) – P < 0.00001.

EMT core regulators expression and cell proliferation in PT and LNM

Interestingly, LNM showed overall lower cell division rates – median value of Ki67 labeling index was 15% (95% CI 3-70%) in PT and 10% (95% CI 3-25%) in LNM (P = 0.0002). Applying 14% Ki67 labeling index as a threshold for positivity [34] we noticed that almost all (17/18) low cycling PTs had low-cycling LNMs, but 48% (14/29) of high cycling PTs had low-cycling LNMs (P = 0.001) (**Table 3**). Increased expression of *TWIST1* and *SNAI2* in PT correlated with decreased division rate of the PT measured by Ki67 labeling index (**Table 4**). Median Ki67 labeling index in *TWIST1*-positive PT was 10%, and 20% for *TWIST1*-negative PT (P = 0.001). Similar rates - 11% and 20% of median Ki67 labeling index were found for *SNAI2*-positive and negative PT (P = 0.03), respectively. In LNM only *SNAI2* expression correlated with cell division rate, with Ki67 labeling index of 8% in *SNAI2*-positive and 15% in *SNAI2*-negative LNM (P = 0.038) (**Table 4**).

The association of EMT markers with hematogenous and lymphatic spread

To evaluate if expression of EMT markers (both EMT effectors and core regulators) in PT and LNM influences hematogenous seeding efficiency, we correlated status of the analyzed markers with the presence of CTCs markers (and the phenotype of CTCs-enriched blood fractions). Only E-cadherin loss in PT correlated with CTCs presence. Depending on the cut-off level for defining E-cadherin loss (no staining in at least 10% of cells or H-score < 300) we observed correlation between CTCs presence, phenotype of CTCs-enriched blood fractions and E-cadherin loss in the center of the tumor (with both cut-offs used) and in tumor margin (only with the H-core < 300 cut-off) (**Table 5**). Using the 10% cut-off, 45% (9/20) of patients with E-cadherin loss in PT center had CTCs markers detected, in patients with normal E-cadherin levels 23% (15/66) of the patients had CTCs markers (P = 0.05). Interestingly, proportions of CTCs-enriched blood fractions phenotypes changed between tumors without and with E-cadherin loss (P = 0.01). In PT with at least 10% E-cadherin loss in the central part, mesenchymal and epithelial phenotypes were detected in 30% (6/20) and 15% (3/20) of the cases, respectively (P = 0.01). In tumors with normal E-cadherin levels in the center, mesenchymal and epithelial phenotypes were found in 6% (4/66) and 17% (11/66) of the cases, respectively. E-cadherin loss was then related to the increase in mesenchymal phenotype of CTCs-enriched blood fractions (from 6% to 30%), and a minimal change in epithelial phenotype (from 17% to 15%) (**Table 5**).

Table 5. Correlations between analyzed markers status in PT and presence of hematogenous (CTCs) and lymphatic metastases. Results for both percentage-based cut-off and median H-score cut-off are shown for the analyzed proteins. For *TWIST1, SNAI1* and *SNAI2* median gene expression level was a cut-off value. SC – immunohistochemical H-score

Marker status in PT	CTC			CTC phenotype				Lymph node status		
	Absent	Present	P	No CTC	Mesenchymal	Epithelial	P	Negative	Positive	P
E-cadherin center										
Negative (≤ 10%)	11	9	**0.05**	11	6	3	0.01	8	22	0.008
Positive (> 10%)	51	15		51	4	11		43	35	
E-cadherin center										
Negative (SC < 300)	13	10	**0.05**	13	7	3	0.005	10	23	0.02
Positive (SC = 300)	49	14		49	3	11		41	34	
E-cadherin margin										
Negative (< 10%)	14	6	0.81	14	4	2	0.33	10	21	0.048
Positive (> 10%)	48	18		48	6	12		41	36	
E-cadherin margin										
Negative (SC < 300)	17	8	0.59	17	6	2	0.04	13	23	0.10
Positive (SC = 300)	45	16		45	4	12		38	34	
VIM center										
Negative (< 10%)	54	20	0.73	54	8	12	0.83	42	48	0.80
Positive (≥ 10%)	8	4		8	2	2		9	9	
VIM center			0.27							
Negative (SC ≤ 2)	34	10		34	4	6	0.54	27	27	0.56
Positive (SC > 2)	28	14		28	6	8		24	30	
VIM margin										
Negativee (< 10%)	54	20	0.73	54	8	12	0.83	42	47	0.99
Positive (≥ 10%)	8	4		8	2	2		9	10	
VIM margin										
Negative (SC ≤ 2)	35	12	0.59	35	5	7	0.86	28	31	0.96
Positive (SC > 2)	27	12		27	5	7		23	26	
N-cadherin center										
Negative (< 10%)	55	21	1	55	8	13	0.62	45	52	0.61
Positive (≥ 10%)	7	3		7	2	1		6	5	
N-cadherin center										
Negative (SC = 0)	43	19	0.36	43	8	11	0.66	39	40	0.46
Positive (SC ≥ 1)	19	5		19	2	3		12	17	
N-cadherin margin										
Negative (< 10%)	56	19	0.27	56	7	12	0.20	45	49	0.73
Positive (≥ 10%)	6	5		6	3	2		6	8	
N-cadherin margin										
Negative (SC = 0)	41	17	0.68	41	7	10	0.91	38	37	0.28
Positive (SC ≥ 1)	21	7		21	3	4		13	20	
TWIST1										
Negative	19	9	0.16	19	3	6	0.38	18	23	0.38
Positive	29	6		29	2	4		22	19	
SNAI1										
Negative	20	9	0.21	20	3	6	0.46	16	24	0.12
Positive	28	6		28	2	4		24	18	
SNAI2										
Negative	22	7	0.95	22	2	5	0.93	18	24	0.27
Positive	26	8		26	3	5		22	18	

Ten percent loss of E-cadherin in the center (P = 0.008) and margin of PT (P = 0.048) correlated also with lymph node involvement, although the effect was more prominent for the center of PT. Seventy three percent (22/30) of tumors with E-cadherin loss in the PT center

Table 6. Correlations between the analyzed markers status in LNM and presence of CTCs and number of lymph nodes (LN) involved; SC – immunohistochemical H-score

Markers in LNM	CTC			CTC phenotype				Number of LN involved		
	Absent	Present	P	No CTC	Mesenchymal	Epithelial	P	≤ 3	> 3	P
E-cadherin										
Negative (≤ 10%)	0	1	0.41	0	1	0	0.11	0	1	0.37
Positive (> 10%)	22	14		22	6	8		31	17	
E-cadherin										
Negative (SC < 300)	5	3	1	5	3	0	0.13	2	8	0.003
Positive (SC ≥ 300)	17	12		17	4	8		29	10	
VIM										
Negative (< 10%)	18	14	1	18	6	8	0.97	25	16	0.69
Positive (≥ 10%)	3	2		3	1	1		6	2	
VIM										
Negative (SC ≤ 0)	13	9	0.73	13	3	6	0.59	21	10	0.39
Positive (SC > 0)	8	7		8	4	3		10	8	
N-cadherin										
Negative (< 10%)	17	12	0.63	17	4	8	0.36	24	14	0.69
Positive (≥ 10%)	2	3		2	2	1		4	4	
N-cadherin										
Negative (SC ≤0)	15	12	1	15	4	8	0.58	23	13	0.48
Positive (SC >0)	4	3		4	2	1		5	5	
TWIST1										
Negative	7	5	0.88	7	3	2	0.73	15	5	0.37
Positive	10	8		10	3	5		13	8	
SNAI1										
Negative	9	6	0.71	9	2	4	0.65	17	4	0.07
Positive	8	7		8	4	3		11	9	
SNAI2										
Negative	9	5	0.43	9	2	3	0.69	15	5	0.37
Positive	8	8		8	4	4		13	8	

had involved lymph nodes, in comparison to 45% (35/78) of PT with normal E-cadherin levels (**Table 5**). To be able to quantify efficiency of lymph node colonization in relation to expression of EMT markers in LNM themselves we correlated expression of the analyzed markers in LNM with the number of lymph nodes involved. Loss of E-cadherin (H-score < 300) correlated with more than 3 LN involved in 80% (8/10) of the patients, in LNM with normal E-cadherin level only 26% (10/39) had more than 3 LN involved (P = 0.003) (**Table 6**).

Expression of *TWIST1, SNAI1* and *SNAI2* did not correlate with CTCs detection rate, phenotype of CTCs-enriched blood fractions or lymph node status/number of LN involved when measured in PT or LNM (**Tables 5** and **6**).

Correlation of clinicopathological characteristics with molecular markers in PT, LNM and CTCs

As *TWIST1, SNAI1* and *SNAI2* showed elevated expression levels in LNM in comparison to PT, such different expression pattern might underlie distinct biological features. We were interested to see if expression of these EMT core regulators is linked with different clinicopathological characteristics depending on their expression in PT and LNM. Indeed, observed correlations were not overlapping – positive *TWIST1* status in PT and *SNAI2*-positive status in LNM correlated with lower tumor grade (G1-2, P = 0.04 for both, **Table 7**). Additionally, *SNAI2*-positive PT were more frequently presenting with lower T stage (P = 0.02).

Table 7. Correlations between clinicopathological tumor characteristics and expression status (negative – Neg. or positive – Pos.) of EMT transcription factors *TWIST1, SNAI1* and *SNAI2* in primary tumors and lymph node metastases

	Gene expression in primary tumor									Gene expression in lymph node metastases								
	TWIST1			*SNAI1*			*SNAI2*			*TWIST1*			*SNAI1*			*SNAI2*		
Clinical variable	Neg.	Pos.	P	Neg.	Peg.	P	Neg.	Pos.	P	Neg.	Pos.	P	Neg.	Pos.	P	Neg.	Pos.	P
T stage																		
T1	14	20	0.21	15	19	0.54	12	22	0.02	2	7	0.13	4	5	0.72	3	6	0.45
T2-4	26	21		24	23		29	18		18	14		17	15		17	15	
Grade																		
G1-2	21	31	0.04	24	28	0.53	23	29	0.10	8	13	0.22	8	13	0.08	7	14	0.04
G3	20	10		16	14		19	11		12	8		13	7		13	7	
HR status																		
Negative	11	5	0.16	8	8	0.91	9	7	0.65	5	3	0.39	4	4	1	5	3	0.45
Positive	30	36		32	34		33	33		15	18		17	16		15	18	
HER2 status																		
Negative	29	27	0.63	26	30	0.77	28	28	0.73	13	12	0.74	11	14	0.33	12	13	0.74
Positive	11	13		12	12		13	11		7	8		9	6		8	7	

Due to relatively short follow up period (median – 2.4 years) extended survival analysis could not be performed. Nevertheless out of 89 patients with available survival data four patients died. In all four patients CTCs were detected – three with mesenchymal and one with epithelial CTCs-enriched blood fraction (P = 0.003, log-rank test for two groups CTC-positive vs. no CTCs).

Discussion

In this work we analyzed expression of EMT effectors – E-cadherin, N-cadherin, VIM and EMT-related core regulators (transcription factors) *TWIST1, SNAI1, SNAI2* in non-lobular PT and LNM of early breast cancer patients. Due to the fact that lobular tumors are often characterized by E-cadherin loss [40] we decided to include only non-lobular tumor subtypes to be able to analyze true biological value of E-cadherin changes related to EMT. Analyzed factors were correlated with presence of CTCs markers, phenotype of CTCs-enriched blood fractions and lymph node involvement. To reduce the possibility of false-negative results in detecting EMT effectors by IHC, staining was performed on full-face sections of PT and LNM, rather than TMA, on which presence of heterogeneously expressed markers might be underestimated [41]. In our study, reduced expression of E-cadherin in both primary breast tumors center and margin was related to presence of lymph node metastases. Our results also showed that expression of EMT effectors did not differ significantly between center and margin of PT. Similarly, Alkatout *et al* recently showed no differences in expression of TWIST1, SNAI1, SNAI2 and ZEB1 proteins in center and margins of primary breast tumors [42]. Explanation of this observation might come from the theory of cancer self-seeding, which experimentally was presented by Kim et al [43]. In this model primary tumor is repopulated (from outside or 'margins') by CTCs released into the circulation by other cancer cells-seeding centers, what in turn makes the primary tumor mass a conglomerate of multiple masses rather than a single mass growing and invading from inside to outside. In this model cancer should be viewed as having multiple peripheral surfaces, not necessary adjacent to tumor stroma [44, 45].

When it comes to hematogenous spread we noted that E-cadherin loss in PT center (independently of the definition of a loss i) no expression in 10% of the cells or ii) H-score below 300) was related to more efficient dissemination of CTCs. It was especially evident in increased occurrence of mesenchymal phenotypes of CTCs-enriched blood fractions, which have more aggressive characteristics and are more efficient in establishing lymph node metastases, as we showed before [20]. In the case of LNM, reduced E-cadherin H-score correlated with more than three lymph nodes involved, which might suggest more efficient tumor dissemination within regional lymphatics. Additionally, we noticed frequently discrep-

ant E-cadherin status between PT and LNM - 37% of matched LNM converted from negative to positive E-cadherin status, leaving almost all (98%) LNM with normal E-cadherin level. Our results corroborate correlations between E-cadherin loss and lymph node involvement [46, 47] as well as re-expression of E-cadherin in metastases [24, 47, 48]. A vast number of research convey that disrupted cell-cell adhesion facilitates cell motility and invasion, and is believed to be crucial for actively establishing new metastases [49, 50], whereas reverting to epithelial state at a distant site might be necessary for tumor cells survival [25]. Another strategy might also be employed by tumor cells in order to survive at a distant site. Evdokimova *et al* described that low proliferation of cancer cells with EMT-like changes might play an important role in cancer dissemination and survival [51]. Despite their aggressiveness, tumor cells with mesenchymal features might have decreased cell division rate. Vega *et al* showed that SNAI1 can cause G1/S cell cycle arrest and protect from cell death [52], also Liu *et al* recently presented that expression of mesenchymal markers in breast cancers is linked with decreased Ki67 levels [53]. We showed that LNM, compared to PT, have globally increased expression of *TWIST1, SNAI1, SNAI2* and are dividing slower (decreased Ki67 labeling index). At the same time however, LNM had normal E-cadherin level. Interestingly, EMT-like phenotype does not always present with complete loss of E-cadherin, even if accompanied by expression of EMT core regulators TWIST1, SNAI1 or SNAI2 [16, 54, 55]. Therefore, our results suggest that tumor cells in LNM might employ both survival strategies – on one hand they reduce cell division rate, possibly via induction of pathways involving TWIST1, SNAI1 and SNAI2, on the other hand they express E-cadherin, which activates prosurvival signals at the ectopic site necessary for establishing metastases [12]. Possible inhibitory effect of EMT transcription factor on global tumor cells division rate in LNM can be considered, however 37% of the tumors in our study were inherently low-cycling, independently of the compartment. Even though our results showing increased Ki67 labeling index in PT in comparison to LNM are supported by existing reports [56], studies describing reverse correlations exist [57, 58]. It cannot be excluded that during continuous growth and evolution of LNM a switch from low proliferating (survival stage) to high proliferating clones will occur. Another result from our work, which would persuade the role of studied EMT core regulators in inhibiting cell division rate relates to their correlation with lower grade and lower T stage. Low grade tumors divide slower [59], and as a result can be smaller.

We have also presented the connection between epithelial and mesenchymal states of matched pairs (PT-LNM, PT-CTC) and triplets (PT-CTC-LNM), which highlighted the phenotypical plasticity of samples in various compartments. Detection of mesenchymal phenotype in CTCs in the absence of mesenchymal markers in PT and LNM might suggest a dynamic induction of EMT in CTCs by e.g. platelets secreting TGFβ and NFκB [60]. Moreover, differing epithelial/mesenchymal status of pairs and triplets might suggest plasticity of tumor cells phenotypes at different stages of dissemination, which might be more informative than just the knowledge of the activation of EMT process, as plasticity (ability to switch between epithelial and mesenchymal states) is being more often regarded as crucial property in metastases establishment [61-64].

Limitations of our study linked to technically challenging detection of EMT need to be mentioned, although we tried to compensate it by analyzing, large, full-faced sections of tissues. Nevertheless, the possibility exists that not all EMT events were captured. In case of CTCs, which are rare especially in early breast cancers, stochastic events and cell losses might have impacted their detection efficiency. Additionally, sample size (especially those of triplets) is relatively small, thus reproduction study with increased number of matched samples would add additional confidence.

In summary, our results show that in PT E-cadherin loss, but not induction of mesenchymal effectors N-cadherin and vimentin, correlates with lymph node involvement and CTCs dissemination, especially those expressing mesenchymal markers. Additionally, changes in epithelial/mesenchymal status frequently occur in tumor cells at different stages of dissemination, underlining plasticity of their phenotypes. In comparison to PT, LNM re-express E-cadherin, upregulate transcription factors *TWIST1, SNAI1, SNAI2* and reduce cell division rate, what could be viewed as their long-term survival strategy.

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Disclosure of conflict of interest

None to declare.

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Table S1. Clinico-pathological characteristics of patients (N = 108)

Variable	Number of cases (%)	
Age – median (range)	60	(28-86)
T stage		
T1	47	(43)
T2	55	(51)
T3	3	(3)
T4	2	(2)
Missing data	1	(1)
N stage		
N-	51	(47)
N+	57	(53)
Grade		
G1	14	(13)
G2	54	(50)
G3	40	(37)
HER2 status		
Negative	77	(71)
Positive	29	(27)
Missing data	2	(2)
HR status		
Negative	21	(19)
Positive	87	(81)
Histological type		
Ductal	97	(90)
Mucinous	8	(7)
Papillary	1	(1)
Cribriform	1	(1)
Neuroendocrine	1	(1)

Table S2. Comparison between E-cadherin, N-cadherin and VIM status in the marginal part of primary tumor (PT) and in lymph node metastases (LNM). Conversion rates from negative to positive (- → +) and positive to negative (+ → -) status between PT and LNM are given as number of cases and percentages of the total samples number. Kappa coefficient with 95% confidence interval (CI); N – number of cases.

Marker	N	Positive in PT*	Positive in LNM*	Conversion PT → LNM			kappa coefficient (95% CI)
		N (%)	N (%)	(-) → (+) N (%)	(+) → (-) N (%)	Switch total N (%)	
E-cadherin	49	32 (65)	48 (98)	17 (35)	1 (2)	19 (39)	-0.040 (-0.116-0.036)
N-cadherin	46	7 (15)	8 (17)	3 (7)	2 (4)	5 (11)	0.602 (0.287-0.917)
Vimentin	49	8 (16)	8 (16)	1 (2)	1 (2)	2 (4)	0.851 (0.649–1)

*Results based on the 10% cut-off value.