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Co-expression of SNAIL and TWIST determines prognosis in estrogen receptor-positive early breast cancer patients

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Abstract

Background Epithelial mesenchymal transition (EMT) plays an important role in the development of metastases. One of the hallmarks of EMT is loss of E-cadherin and gain of N-cadherin expression, which are regulated by transcription factors, such as SNAIL, SLUG, and TWIST.

Methods We examined the prognostic value of these factors as well as E-cadherin and N-cadherin, in a well-described large cohort of breast cancer patients treated with primary surgery. Analyses were stratified by estrogen receptor (ER) status, because of its crucial role in the regulation of these transcription factors. SNAIL, SLUG, and TWIST expression were examined on a TMA containing 575 breast tumors using immunohistochemistry. Nuclear expression was quantified using a weighted histoscore and classified as high versus low expression, based on the median histoscore.

Results High expression of SNAIL, SLUG, and TWIST was seen in 54, 50, and 50% of tumors, respectively. The level of SNAIL ($p = 0.014$) and TWIST ($p = 0.006$) expression was associated with a worse patient relapse-free period, specifically in patients with ER-positive tumors (interaction Cox proportional hazards $p = 0.039$). Combining both factors resulted in an independent prognostic factor with high discriminative power (both low versus either high: HR 1.15; both low versus both high HR 1.84; $p = 0.010$). Co-expression of SNAIL–TWIST was associated with low-E-cadherin and high-N-cadherin expression, especially in ER-positive tumors ($p = 0.009$).

Conclusion This suggests that, through interactions with ER, SNAIL and TWIST may regulate E- and N-cadherin expression, thereby inducing EMT. Our results are indicative that SNAIL and TWIST play a crucial role in EMT through regulation of E- and N-cadherin expression, exclusively in ER-positive breast cancer patients.

Introduction

Breast cancer is the leading cause of death from cancer in women in the Western world.[1] Classification of breast cancer is a challenge because of considerable molecular heterogeneity. Nevertheless, this is crucial for systemic treatments decision that improves disease-free survival (DFS) and overall survival (OS) in patients with early breast cancer.[2] Current prognostic and predictive factors are not sufficient for optimal classification. Elucidation of molecular mechanisms underlying disease relapse is of importance for the development of future tailored treatment strategies for breast cancer.

Epithelial mesenchymal transition (EMT) is a process characterized by loss of epithelial cell–cell adhesions and gain of mesenchymal features.[3] This process is very important for developmental processes during embryonic development and plays an important role in wound healing. Accumulating evidence suggest that EMT also plays a critical role in the development of invasiveness and metastatic potential of cancer. E-cadherin, which maintains cell–cell contacts and the epithelial phenotype, is thought to be a suppressor of invasion in carcinoma.[4] Loss of E-cadherin and increased N-cadherin expression is a hallmark of EMT and subsequent invasion. Transcriptional regulators of this E- to N-cadherin switch include SNAIL, SLUG, and TWIST.[5-7] Studies have shown frequent expression of these transcription factors in malignant cells.[8-10] In addition, expression of SNAIL, SLUG, and TWIST has been associated with unfavorable clinicopathological parameters and worse patient outcome.[10,11,13-15,21] Various mechanisms underlie the regulation of these transcriptional factors and interplay in their effect on tumor progression and invasiveness. Pathways, such as TGFβ, Wnt, Hedgehog, and estrogen receptor (ER), have been linked to the activation of these transcription factors.[3] In ovarian cancer, the importance of the ER pathway in regulation of these factors has been shown in vitro.[15]

In this report, we investigated the expression of SNAIL, SLUG, and TWIST in a cohort of operable breast cancer patients treated at the Leiden Uni-

versity Medical Centre (LUMC). We hypothesized that high-expression levels of these transcription factors lead to increased metastasis and a worse outcome of patients, through induction of EMT via an E-cadherin to N-cadherin expression switch (cadherin switch). We, therefore, related expression of SNAIL, SLUG, and TWIST to expression of these cell adhesion molecules (CAM). We additionally stratified analyses and tested for interactions with ER status, because this has been shown to be of influence on these transcription factors in previous studies on ovarian cancer.[15] We used the REMARK-guidelines for the reporting of tumor marker studies.[22]

Patients and methods

Patients

The patient population has been described previously and was a consecutive series of all women with non-metastatic breast cancer who received a primary surgical resection (with or without radiotherapy) in the LUMC between 1985 and 1994.[23] Patients with a prior history of cancer (other than basal cell carcinoma or in situ carcinoma) or bilateral tumors were excluded. Age at diagnosis, TNM stage, local and systemic therapy, relapses, and deaths were recorded. All tumors were histologically classified and graded by one pathologist (V.T.H.B.M. Smit) and expression of ER, progesterone receptor (PgR), human epidermal growth factor (HER2), E-cadherin, and N-cadherin was previously determined using a tissue micro array (TMA) of formalin-fixed paraffin-embedded tumor material.[23] Procedures were followed in accordance with the ethical standards of the LUMC Medical Ethics Committee.

Immunohistochemistry

TMA sections were dewaxed in xylene and rehydrated in a graded alcohol series. Antigen retrieval was carried out for 40 min in a hot water bath (at 95°C) in Trizma-EDTA buffer (pH 9.0). Slides were immersed in 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity and in a blocking solution (DAKO protein-free

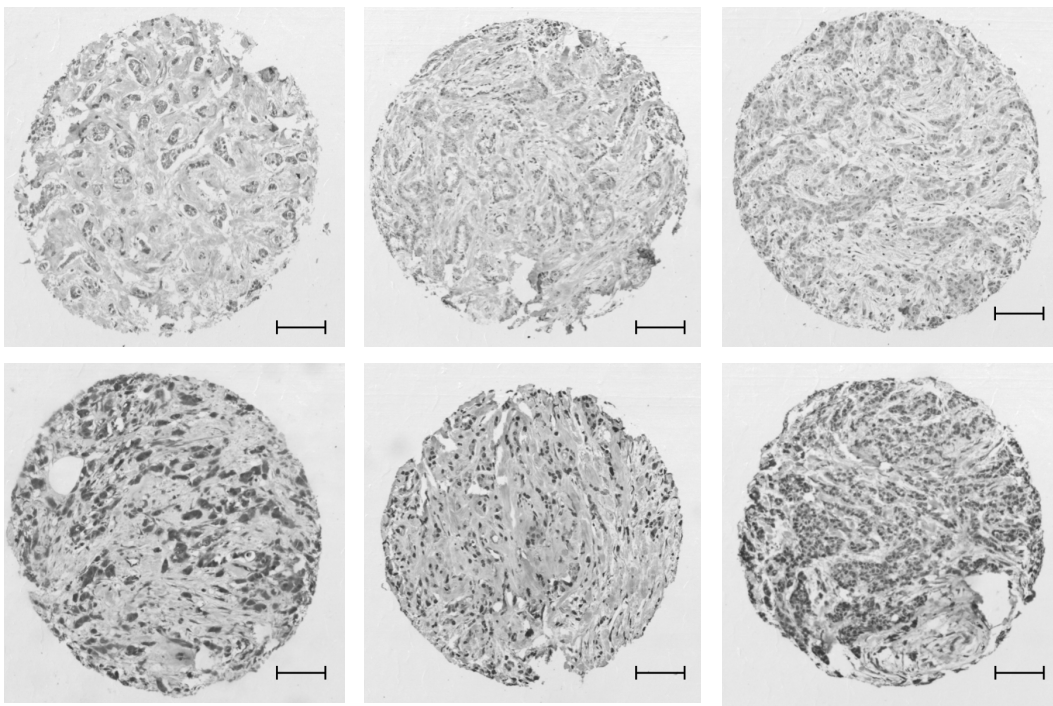

Figure 1 Representative photographs of tissue microarray punches of human breast cancer specimens immunohistochemically stained for SNAIL (a, b), SLUG (c, d), and TWIST (e, f) (x10 objective) with representative examples of lower than median expression (a, c, e, and g) and higher than median expression (b, d, f, and h). Bar represents 100 µm.

serum block) for 60 min to block non-specific binding sites. Sections were incubated overnight at room temperature with primary antibodies (SNAIL: kindly provided by Professor Ismo Virtanen; SLUG: AbCAm: ab27568; TWIST: AbCam TWIST2C1a, ab50887) diluted in antibody diluent (DAKO) (SNAIL 1:50; SLUG 1:16,000; TWIST 1:100). One tumor TMA slide was stained with and another slide without antibody as a positive and negative control, respectively. Sections were treated with envision (DAKO) and visualization was performed using DAB (DAKO). Finally, tissues were counterstained with hematoxylin and dehydrated through graded alcohol and xylene.

Evaluation of immunostaining

Immunohistochemical staining was quantified independently and in a blinded manner by two observers using a weighted histoscore. The proportion of cells with nuclear staining was multiplied by the intensity of staining to provide a score of 0–300. Score = (0 x percentage not stained) + (1 x percentage weakly stained) + (2 x percentage moderately stained) + (3 x percentage strongly stained).[24] The interobserver variability analyzed using the Cohen's kappa coefficient was for expression of SNAIL1 0.906, SLUG 0.966, and TWIST 0.972. The mean score of all cores scored by the first investigator was used for analysis.

Study design

The design comprises a retrospective cohort study of all women diagnosed with non-metastatic breast cancer who received a primary surgical resection (with or without radiotherapy) in the LUMC between 1985 and 1994. No matching or stratification was used. The end of follow-up period was 18 January 2011, death, or date of loss of follow-up. The median follow-up of patients alive was 20 years (range 0–26).

Objectives of these retrospective analyses are assessment of the association of the transcription factors with patient's survival and their relationship with ER. In our analyses, the following known clinicopathological parameters were included: age, tumor and nodal status, tumor grade, morphology, ER, PgR, HER2 overexpression, surgery, and radiotherapy.

Statistical analysis

All data were analyzed using the statistical package SPSS for Windows 17.0 (SPSS Inc., Chicago, IL, USA). Descriptive data are given as median (range). For statistical analysis, we categorized expression of SNAIL, SLUG, and TWIST into two equal groups (high and low expression) based on the median histoscore. Associations between expression of these transcription factors with each other and with E-cadherin and N-cadherin were assessed with a chi-square test. Relapse-free period (RFP) was calculated from the date of surgery up to the first date of locoregional or distant recurrence, and is reported as cumulative incidence functions, after accounting for death as competing risk.[25] To examine if the transcription markers were associated with RFP, univariate Cox proportional hazards analyses were performed. Multivariable analyses were performed using the Cox proportional hazards model entering SNAIL, SLUG, and TWIST with other known clinicopathological parameters of influence on outcome according to univariate analysis (defined as those with $P < 0.1$). All testing was two-tailed with 0.05 as level of significance.[26] Cases with missing data were not used for analyses.

Results

Patients

A total of 667 patients with non-metastatic breast cancer were treated with a primary surgical resection in the LUMC during the study period. Formalin-fixed paraffin-embedded tumor material was available and included in the TMA of 575/667 (86%) patients. Of these patients, 370 (64%) did not receive any systemic treatment and were only treated with local therapy. The clinicopathological and local treatment characteristics of the population were published previously (Table 1).[23]

SNAIL, SLUG and TWIST in tumor tissue

Nuclear staining was successful for SNAIL in 90%, for SLUG in 91%, and for TWIST in 89% of tumors (cores were missing, folded, or contained no invasive tumor in 10, 9, and 11%, respectively) (Figure 1). For patients receiving no systemic treatment, expression of SNAIL, SLUG, and TWIST was available in 370 (88%), 370 (89%), and 370 (88%) of cases, respectively. No significant differences were observed between cases with data available for

Table 1 Association between the transcription factors and well-established prognostic factors.

	SNAIL				SLUG				TWIST			
	Low		High		Low		High		Low		High	
	n	%	n	%	n	%	n	%	n	%	n	%
Age (years)												
Median (range)	58	23-91	57	27-96	56	28-89	59	23-96	57	27-90	58	23-96
Mean (SD)	58	14	58	14	57	14	59	15	57	14	59	15
<40	17	7	25	10	20	7	25	10	18	7	24	9
40-50	70	28	58	22	75	29	53	20	73	29	58	23
51-60	57	22	64	25	63	24	58	22	55	22	63	25
>60	111	44	113	446	105	40	125	48	108	43	111	43
Extent of disease												
Early breast cancer	209	82	205	79	215	82	207	79	207	82	205	80
Locally advanced	46	18	55	21	48	18	54	21	47	19	51	20
Tumour stage												
pT1	99	39	87	34	81	31	104	40	105	41	79	31
pT2	115	45	131	50	146	56	108	41	106	42	139	54
pT3/4	32	13	35	14	29	11	38	15	30	12	36	14
missing	9	4	7	3	7	3	11	4	13	5	2	1
Grade												
I	39	15	30	12	31	12	39	15	41	16	26	10
II	117	46	131	50	116	44	140	54	133	52	117	46
III	92	36	98	38	111	42	79	30	75	30	110	43
Missing	7	3	1	0	5	2	3	1	5	2	3	1
Histological type												
Ductal	233	91	228	88	244	93	224	86	226	89	232	91
Other	15	6	31	12	14	5	34	13	23	9	21	8
Missing	7	3	1	0	5	2	3	1	5	2	3	1
Nodal stage												
pN0	131	51	140	54	134	51	140	54	136	54	136	53
pN+	124	49	120	46	129	49	121	46	118	47	120	47
Estrogen receptor												
Negative	104	41	91	35	136	52	62	24	89	35	103	40
Positive	143	56	167	64	123	47	1094	74	158	62	151	59
Missing	8	3	2	1	4	2	5	2	7	3	2	1
Progesterone receptor												
Negative	115	45	95	37	132	50	85	33	104	41	111	43
Positive	133	52	162	62	130	49	170	65	145	57	143	56
Missing	7	3	3	1	1	0	6	2	5	2	2	1
Human epidermal growth factor receptor 2												
0+/1+/2+	176	69	195	75	184	70	188	72	183	72	184	72
3+	28	11	15	6	32	12	12	5	17	7	27	11
Missing	51	20	50	19	47	18	61	23	54	21	45	18
Total	**255**	**100**	**260**	**100**	**263**	**100**	**261**	**100**	**254**	**100**	**256**	**100**

SNAIL, SLUG, or TWIST expression and those for whom data were not available with respect to conventional prognostic markers (data not shown). SNAIL, SLUG, and TWIST expression were categorized into low versus high expression based on the median histoscore (SNAIL 106.67, range 3–250; SLUG 95, range 0–287; and TWIST 142.50, range 0–293). With this cut-off, high expression of SNAIL was seen in 54% and high expression of SLUG and TWIST in 50%. All three transcription factors showed positive statistically significant associations with each other: SNAIL and SLUG expression, $p < 0.001$; SNAIL and TWIST expression, $p = 0.002$; and SLUG and TWIST expression, $p = 0.035$.

Association with various parameters

High SNAIL expression was inversely associated with ductal histology ($p = 0.015$), PgR positivity ($p = 0.020$), and HER2-negative disease ($p = 0.021$). High SLUG expression was associated with a lower tumor stage ($p = 0.013$), lower tumor grade ($p = 0.014$), positive hormone receptor expression (both $p < 0.001$), and HER2 overexpression ($p = 0.003$) and was inversely associated with ductal histology ($p = 0.002$). High TWIST expression was associated with a higher tumor stage ($p = 0.003$) and a higher tumor grade ($p = 0.004$) (Table 1).

Prognostic value of transcription markers

Patients who were treated with only local therapy and did not receive any systemic treatment were selected to analyze the prognostic effect of SNAIL, SLUG, and TWIST expression (Table 2; Figures 2 and 3). Univariate analyses did not show an association between high versus low expression of SNAIL and RFP (hazard ratio (HR) 1.24, 95%CI 0.89–1.72; $p = 0.20$, Figure 2a). HR of RFP for high versus low expression of SLUG and high versus low expression of TWIST were 1.03 (95%CI 0.74–1.42; $p = 0.87$) and 1.26 (95%CI 0.91–1.75; $p = 0.16$), respectively (Figure 2b, c). Independent statistically significant prognostic factors for RFP were nodal stage and tumor grade (Table 2).

ER status and transcription markers expression

In all patients with ER-negative tumors (n = 204), SNAIL, SLUG, and TWIST expression also had no significant influence on the RFP (Cox proportional hazards univariate analyses $p = 0.61$, 0.20, and 0.63, respectively, data not shown). In Cox proportional hazards univariate analyses of all patients with ER-positive tumors, high SNAIL expression resulted in a worse RFP (HR 1.51, 95%CI 1.09–2.11; $p = 0.01$) (Figure 3a). A similar pattern was observed for TWIST expression (HR 1.57, 95%CI 1.14–2.17; $p = 0.006$) (Figure 3b). High SLUG expression also resulted in a worse patient RFP within the ER-positive group, although not statistically significant (HR 1.09, 95%CI 0.79–1.52; $p = 0.60$). Subsequently, we combined SNAIL and TWIST expression into three groups: (1) both SNAIL and TWIST low expression, (2) either SNAIL or TWIST high expression, and (3) both SNAIL and TWIST high expression. This variable again showed no statistically significant differences in outcome concerning RFP in patients with ER-negative tumors (Cox proportional hazards univariate analysis: both low versus either low: HR 1.05, 95%CI 0.63–1.75; both low versus both high: HR 0.88, 95%CI 0.49–1.56; overall $p = 0.80$). In patients with ER-positive tumors, higher expression of SNAIL and TWIST tumor expression was associated with increased relapse rates compared with low SNAIL and TWIST tumor expression in both univariate and multivariable analyses (Cox proportional hazards multivariable analysis: both low versus either low: HR 1.15, 95%CI 0.74–1.77; both low versus both high: HR 1.84, 95%CI 1.18–2.86; overall $p = 0.010$, Table 3; Figure 3). Statistical interaction between SNAIL and TWIST expression with ER status on RFP was tested in Cox proportional hazards analysis, which revealed that the prognostic effect of SNAIL and TWIST differed statistically significantly between ER-positive and -negative disease (interaction $p = 0.039$).

Cadherin switch and transcription markers

Tumor expression of E-cadherin and N-cadherin was seen in 49 and 47% of patients, respectively (data not shown). Statistically significant associations were found between high SNAIL expression with low E-cadherin expression ($p = 0.01$) and high TWIST expression with high N-cadherin expression ($p = 0.002$). SLUG expression showed similar, although not statistically significant, associations with E-cadherin and N-cadherin expression ($p = 0.32$ and 0.08, respectively).

Subsequently, combined SNAIL and TWIST expression was related to combined E-cadherin and N-cadherin expression. Expression of E-cadherin and N-cadherin was combined in a new variable, representing tumors with: (1) both low or both high expression of E-cadherin and N-cadherin, (2) high expression of E-cadherin and low expression of N-cadherin, and (3) low expression of E-cadherin and high expression of N-cadherin. The two combined variables, SNAIL–TWIST expression and E-cadherin–N-cadherin expression, showed a statistically significant correlation with each other ($p = 0.04$). Higher expression levels of SNAIL and TWIST were associated with lower expression of E-cadherin and higher expression of N-cadherin and vice versa: lower expression levels of SNAIL and TWIST were associated with higher expression of E-cadherin and lower expression of N-cadherin

Table 2 Univariate and multivariable analysis of relapse free period.

			Univariate analyses			Multivariable analysis		
Characteristic		**n**	**HR**	**95% CI**	***p***	**HR**	**95% CI**	***p***
Age	<60	192	1.00		0.803			
	≥60	178	0.96	0.70-1.31				
Tumour stage	pT1	158	1.00		<0.001	1.00		0.259
	pT2,3,4	203	1.87	1.36-2.58		1.24	0.86-1.79	
Nodal stage	pN0	266	1.00		<0.001	1.00		<0.001
	pN+	104	4.33	3.18-5.91		4.00	2.89-5.53	
Grade	I/II	244	1.00		<0.001	1.00		0.019
	III	117	1.86	1.36-2.54		1.47	1.07-2.03	
Morphology	Ductal	329	1.00		0.857			
	Other	33	1.05	0.62-1.79				
ER	Negative	127	1.00		0.851			
	Positive	221	1.03	0.74-1.43				
PgR	Negative	139	1.00		0.925			
	Positive	204	0.99	0.71-1.36				
HER2	Negative	248	1.00		0.695			
	Positive	23	1.13	0.61-2.10				
Surgery	MST	207	1.00		0.001	1.00		0.079
	BCS	163	0.58	0.42-0.80		0.73	0.51-1.04	
Radiotherapy	No	152	1.00		0.640			
	Yes	218	1.08	0.79-1.48				
SNAIL	Low	150	1.00		0.198			
	High	177	1.24	0.89-1.72				
SLUG	Low	164	1.00		0.874			
	High	166	1.03	0.74-1.42				
TWIST	Low	160	1.00		0.163			
	High	164	1.26	0.91-1.75				

Abbreviations BCS: breast conserving surgery; ER: estrogen receptor; HER2: human epidermal growth factor receptor 2; MST: mastectomy; PgR: progesterone receptor.

(Table 4A), suggesting that SNAIL and TWIST expression may regulate an E- to N-cadherin switch.

Again, we stratified analyses based on ER expression to observe whether ER expression again would interplay in SNAIL and TWIST expression and their association with E-cadherin and N-cadherin expression. This revealed that the correlation described above between expression of SNAIL and TWIST with expression of E-cadherin and N-cadherin was specifically pronounced in patients with ER-positive tumors ($p = 0.009$, Table 4B), whereas statistical significance of this correlation was lost in ER-negative tumors ($p = 0.66$, Table 4C).

Discussion

We have quantified expression of the transcription factors SNAIL, SLUG, and TWIST in a cohort of breast cancer patients and found that their expression levels are statistically significantly associated with each other, indicating their frequent co-expression. A statistically significant decreased RFP was seen for patients with tumor expression of SNAIL and TWIST, exclusively in ER-positive tumors. SLUG expression showed a similar effect on RFP. However, it did not reach statistical significance. In the subpopulation of ER-positive patients, combined expression of SNAIL and TWIST resulted in a statistically significant independent prognostic parameter for RFP. Interaction analyses for RFP revealed statistically significant interaction between SNAIL/TWIST expression and ER expression, supporting the hypothesis that these factors interplay in their role on disease progression. Moreover, we found high expression of SNAIL and TWIST to be statistically significantly associated with low E-cadherin and high N-cadherin expression, which is considered as a hallmark of EMT. Again, this correlation was exclusively found in patients with ER-positive tumors.

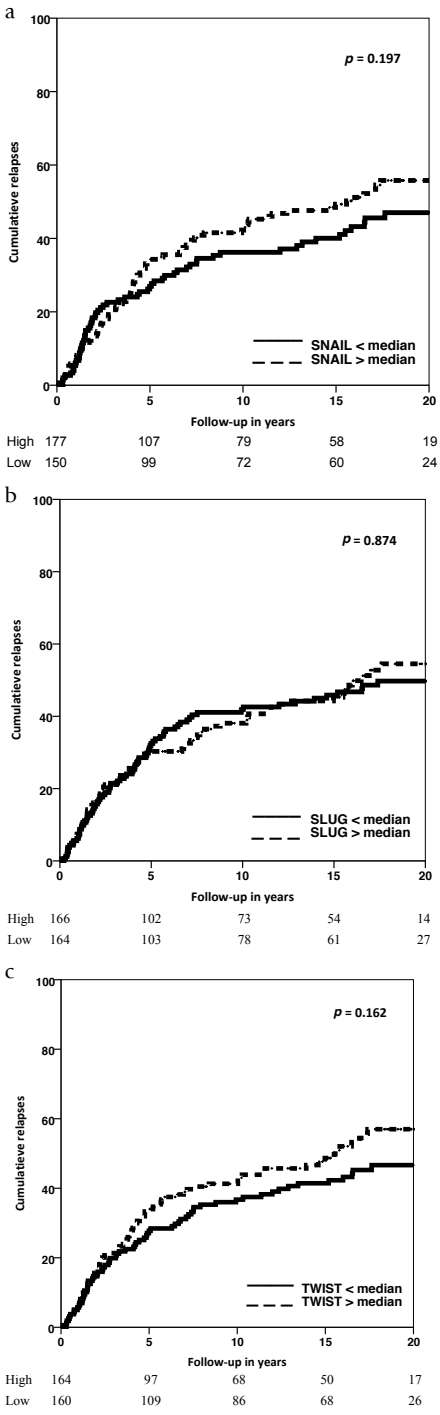

Figure 2 Cumulative incidence of relapse for SNAIL (a), SLUG (b), and TWIST (c). The expression is divided into two groups based on the median histoscore for SNAIL 106.67, SLUG 95.00, and TWIST 142.50.

Elevated expression of SNAIL, SLUG, and TWIST has been described in previous studies of multiple tumor types, including gastric cancer, non-small cell lung cancer (NSCLC), prostate cancer, breast cancer hepatocellular cancer (HCC), head and neck cancer, ovarian cancer, colorectal cancer, esophageal squamous cell carcinoma (ESCC), bladder cancer, upper-tract urothelial carcinoma (UTUC), and cervical cancer. [8-21] High expression of these markers has been found to be associated with unfavorable clinicopathological parameters in almost all these studies.[9-21] Several studies investigated the effect of these transcription markers on patient outcome.[9-11,13-21] The results seen in these studies are in concordance with the prognostic effects for SNAIL, SLUG, and TWIST found in our analyses. High expression of SNAIL is associated with an unfavorable prognosis in different types of invasive carcinomas.[10,11,13-15,21] High SLUG expression is linked to poor progression-free and overall survival in colorectal cancer and ESCC.[16,17] Expression of TWIST was a statistically significant prognostic factor in several types of cancer, where high expression of TWIST resulted in a worse patient outcome.[9-11,13-15,17,20] In a breast cancer study, TWIST expression was positively correlated to a positive nodal status and was associated with tumors from patients who had died from breast cancer.[12]

In our study, we found that specifically in ER-positive tumors, expression of both SNAIL and TWIST identified a patient population with markedly increased risk of recurrence. SLUG expression showed a similar association with RFP, although not statistically significant. A combined variable of SNAIL and TWIST showed a synergistic prognostic effect of expression of both factors, which is consistent with previous research demonstrating the additive effect of SNAIL and TWIST expression on prognosis in NSCLC, HCC, and head and neck squamous cell carcinomas.[10,13,14] In breast cancer, co-expression of SNAIL–TWIST results in EMT and increased mammosphere-forming capability.[27] Functional studies in Drosophila suggest that TWIST can induce SNAIL expression, which would make SNAIL expression a marker of functional TWIST.[28] This was also the case in rat epithelial cells; it was suggested that TWIST acts upstream from SNAIL and that their collaborative functioning is able to downregulate E-cadherin expression

Table 3 Univariate and multivariable analysis of relapse free period in patients with estrogen receptor positive breast cancer.

			Univariate analyses			Multivariable analysis		
Characteristic		**n**	**HR**	**95% CI**	***p***	**HR**	**95% CI**	***p***
Age	<60	170	1.00		0.676			
	≥60	167	0.94	0.68-1.28				
Tumour stage	pT1	135	1.00		0.002	1.00		0.903
	pT2,3,4	191	1.67	1.20-2.32		0.98	0.66-1.44	
Nodal stage	pN0	189	1.00		<0.001	1.00		<0.001
	pN+	148	3.12	2.27-4.29		3.06	2.15-4.36	
Grade	I/II	239	1.00		<0.001	1.00		0.048
	III	91	1.86	1.35-2.58		1.42	1.00-2.00	
Morphology	Ductal	298	1.00		0.332			
	Other	33	1.28	0.78-2.08				
PgR	Negative	77	1.00		0.468			
	Positive	253	0.87	0.61-1.26				
HER2	Negative	252	1.00		0.505			
	Positive	7	1.40	0.52-3.80				
Surgery	MST	182	1.00		<0.001	1.00		0.027
	BCS	155	0.57	0.42-0.78		0.66	0.46-0.95	
Radiotherapy	No	135	1.00		0.515			
	Yes	202	1.11	0.81-1.54				
SNAIL&TWIST	Both low	81	1.00		0.001	1.00		0.010
	Either high	129	1.16	0.76-1.79		1.15	0.74-1.77	
	Both high	90	2.10	1.36-3.24		1.84	1.18-2.86	

Abbreviations BCS: breast conserving surgery; ER: estrogen receptor; HER2: human epidermal growth factor receptor 2; MST: mastectomy; PgR: progesterone receptor.

Table 4 Relation between co expression of SNAIL and TWIST and expression of the combination of E-cadherin and N-cadherin.

SNAIL and TWIST	**n**	**E-cadherin high**	**Both low or both high**	**N-cadherin high**
A No stratification				
Both low	116	42 (36%)	54 (47%)	20 (17%)
Either high	187	44 (24%)	107 (57%)	36 (19%)
Both high	433	119 (25%)	223 (48%)	91 (27%)
B In ER-negative tumours				
Both low	43	15 (35%)	18 (42%)	10 (23%)
Either high	72	17 (24%)	37 (51%)	18 (25%)
Both high	50	17 (34%)	21 (42%)	12 (24%)
C In ER-positive tumour				
Both low	69	27 (39%)	33 (48%)	9 (13%)
Either high	113	27 (24%)	68 (60%)	18 (16%)
Both high	79	15 (19%)	41 52%)	23 (29%)

ER estrogen receptor.

and promote EMT.[29] We observed that SNAIL and TWIST expression, separately or combined, was not statistically significant prognostic factors for RFP in ER-negative tumors. Interaction analyses provided statistical proof that SNAIL and TWIST were exclusively of influence on RFP in patients

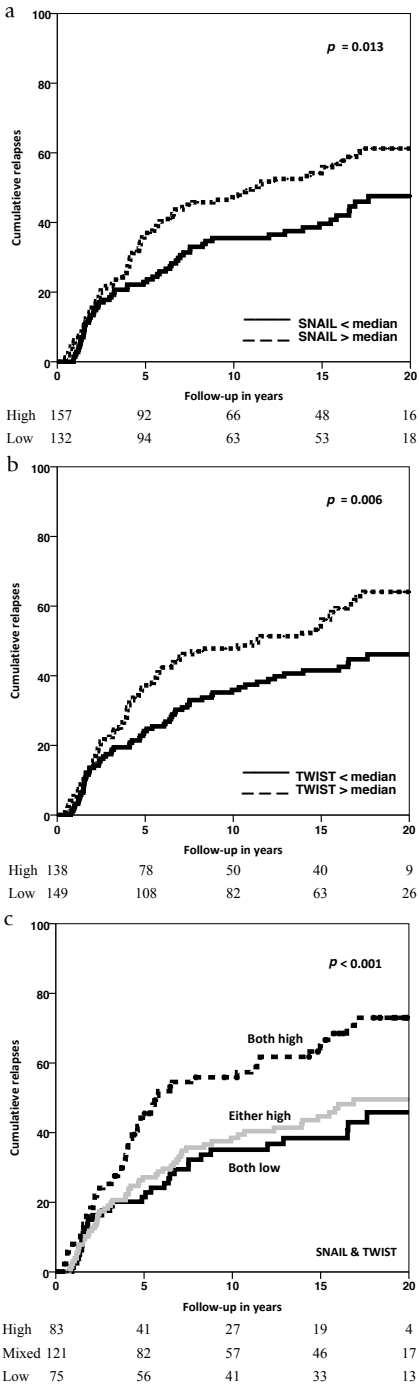

Figure 3 Cumulative incidence of relapse for patients with estrogen-positive tumors for SNAIL (a), TWIST (b), and the combination (c). The expression is divided into two groups based on the median histoscore for SNAIL 106.67 and TWIST 142.50.

ER-positive tumors. Supportive for these findings is the fact that the mechanisms by which these transcription factors regulate tumor progression and invasiveness interplay with a variety of pathways, including the estrogen receptor pathway.[15,30,31] The relation between estrogen signaling and transcription factors SNAIL and SLUG has been previously highlighted in ovarian cancer.[15] In this study, expression and promoter activity of these transcription factors in ovarian cancer cell lines was specifically enhanced by estrogen stimulation, resulting in downregulation of E-cadherin and induction of EMT. They provided supportive evidence that the ER is a critical link in mediating the induction of EMT by estrogen. The above described is consistent with and may explain the interaction found in our study between transcription factors and ER expression in the prognostic effect on RFP in breast cancer.

SNAIL and TWIST have been described to induce EMT, but their exact functioning is complicated and remains largely unknown. A hallmark of EMT is an E- to N-cadherin switch in cell surface expression. In breast cancer cell lines, loss of E-cadherin and expression of N-cadherin has been shown to promote motility and invasion and to induce EMT.[32] To seek whether the prognostic effect of SNAIL and TWIST expression as found in our study could be linked to EMT, we tested for correlation between SNAIL and TWIST expression with E-cadherin and N-cadherin expression. Low E-cadherin with combined high N-cadherin expression was strongly associated with high co-expression of SNAIL and TWIST. Again, these associations were exclusively found in the subpopulation of ER-positive tumors (which is the specific subpopulation of patients where SNAIL and TWIST had a statistically significant prognostic effect). These associations are indicative for functional interactions between SNAIL, TWIST, E-cadherin, N-cadherin, and ER leading to induction of EMT.

Our study may have some limitations. First, this is a retrospective analysis and not a prospective study. However, data of patients in this cohort (characteristics and follow-up) were well documented and important prognostic variables (ER, PgR, HER, and grade) were determined centrally. Second, we did perform several sub analysis. Nevertheless, interaction analyses revealed statistically

significant interactions for these analyses. Third, we quantified the immunohistochemical staining manually and not using an automated image analysis machine. However, for all markers evaluated, the interobserver variability was above 0.9, which is excellent.[24]

In conclusion, we confirm that SNAIL and TWIST expression are prognostic factors for RFP in breast cancer. In addition, to our knowledge, we are the first to show that this effect is predominantly seen in ER-positive breast cancer patients and that co-expression of SNAIL and TWIST results in a more accurate prognostic patient stratification. Our results suggest that SNAIL and TWIST may act cooperatively to promote tumor relapses in breast cancer, specifically in patients with ER-positive tumors. In addition, we have shown that expression of SNAIL and TWIST is associated with low expression of E-cadherin and high expression of N-cadherin, suggesting a functional role in the process of EMT. Importantly, the findings of our study, may be explained by underlying biology, are supported by previous mechanistic findings and add to the current knowledge of breast cancer progression.

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