

## Research Article

# Nuclear Expression of Snail Is an Independent Negative Prognostic Factor in Human Breast Cancer

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**Background.** Snail is a key regulator of epithelial-mesenchymal transition of tumor cells. Several studies have shown nuclear Snail expression to be a negative prognostic factor in human cancer, where it is generally associated with more aggressive tumor behavior and worse survival. **Objectives and Methods.** To further explore the role of Snail expression in breast cancer, we conducted a study on a tissue microarray, encompassing 1043 breast cancer cases. **Results.** A total of 265 (25.4%) breast cancers were positive for Snail. Snail expression was significantly associated with greater tumor size, higher tumor stage and grade, positive lymph node status, and hormone receptor negative status and was differently expressed in the intrinsic subtypes of breast cancer, being the highest in the basal-like subtype and the lowest in the luminal A subtype. In multivariate analysis, Snail proved to be an independent negative prognostic factor for OS. In the intrinsic subtypes, Snail expression was a negative prognostic factor for OS in the luminal B HER2<sup>-</sup>, the luminal B HER2<sup>+</sup>, and the basal-like subtype. **Conclusions.** This is the first study demonstrating that nuclear Snail expression is an independent negative predictor of prognosis in breast cancer, thus suggesting that it may represent a potential therapeutic target.

## 1. Introduction

Snail, a zinc finger transcription factor, is a key regulator of epithelial-mesenchymal transition (EMT) of epithelial tumor cells [1]. It directly represses transcription of the cell adhesion molecule E-cadherin while inducing transcription of mesenchymal genes [2, 3]. EMT leads to a loss of epithelial features and a gain of mesenchymal features and cell motility and allows the tumor to progress to invasive cancer and metastatic disease [2, 4].

Snail messenger RNA (mRNA) expression has been detected in breast cancer, gastric cancer, hepatocellular carcinomas, and ovarian carcinomas [2, 5–7]. However, since Snail is subject to posttranslational modifications, mRNA expression does not necessarily correlate with the amount of active Snail protein [8]. Certain cell lines express both Snail mRNA and cytoplasmic Snail but lack nuclear Snail [9]. Importantly, these cell lines show no detectable Snail activity,

suggesting that nuclear Snail expression is a better predictor of Snail activity than analysis of Snail mRNA [8, 9].

Several studies have shown nuclear Snail expression to be a negative prognostic factor in human cancer. Shin et al. found that nuclear Snail expression is significantly associated with tumor progression, lymph node metastases, and shorter survival in gastric carcinomas [10]. Similarly, nuclear Snail expression is associated with higher tumor stage and grade and constitutes an independent prognostic predictor of recurrence-free survival and cancer-specific survival in urothelial carcinoma [11] and is associated with aggressive tumor behavior and worse OS in hepatocellular carcinoma [12]. Blechschmidt et al. showed that nuclear Snail expression in ovarian cancer metastases is associated with lower overall survival [8].

In breast cancer, several immunohistochemical studies have investigated the role of nuclear Snail expression [9, 13, 14]. Geradts et al. and Lundgren et al. found an association

between Snail and higher tumor grade and proliferation rate as well as an association between Snail and estrogen receptor (ER) negativity [3, 14]. A recent study by ElMoneim and Zaghoul showed that nuclear expression of Snail is associated with higher tumor grade and greater tumor size and stage as well as positive lymph node status in breast cancer [13]. The authors also confirmed an association between Snail expression and progesterone receptor (PR) [15] and ER negative breast cancer cases [13]. Looking at the impact of Snail expression on prognosis in human breast cancer, some studies found no significant correlation with survival [14], while others report that nuclear Snail expression significantly predicts poorer disease free survival in patients with positive lymph node status, and ER<sup>-</sup> as well as PR<sup>-</sup> breast cancers [4]. Additionally, Snail has been shown to be required for tumor growth and lymph node metastasis in a heterotransplanted human breast cancer cell line [16].

To further explore the role of nuclear Snail expression in human breast cancer, we conducted an immunohistochemical study on a breast cancer tissue microarray [17] encompassing a total of 1043 formalin fixed breast cancer cases with detailed clinical annotation and outcomes data. The aim of our study was to investigate the association between nuclear Snail expression and other clinicopathological parameters in breast cancer and to study the impact of Snail on prognosis.

## 2. Materials and Methods

**2.1. Tissue Microarray.** We used a TMA encompassing 1402 breast cancer tissue punches of formalin-fixed and paraffin-embedded tumor samples collected from patients diagnosed with primary breast cancer between 1985 and 2007 at the Institute for Pathology, University of Basel, and the Viollier Institute in Basel, Switzerland. Of these 1402 tissue punches, a total of 1043 were evaluable for our study. The tissue samples were brought into a TMA format as previously described [18]. Briefly, 0.6 mm tissue cylinders were punched out of donor tumor tissue blocks and transferred into a recipient paraffin block using a semiautomated tissue arrayer. Each TMA contained a number of tumor punches ranging from 159 to 522. Histopathological data was obtained from the pathology reports, and raw patient survival data was obtained from the Cancer Registry of Basel or from the patient's attending physician. Retrieval of tissue and clinical data was performed according to the regulations of the local institutional review boards and data safety laws with specific regard to ethical standards and patient confidentiality. The mean follow-up time of the patients was 69.9 months (range 1 to 174 months), and the median age was 63.1 years (range 27 to 101 years). Demographic information of the patients can be found in Table 1.

**2.2. Immunohistochemistry.** For immunohistochemical staining, 4  $\mu$ m sections of the TMA blocks were incubated overnight with the polyclonal anti-Snail antibody (Clone RB1400, Abgent, San Diego, CA, USA) in a dilution of 1:100 after heat induced antigen retrieval with citrate buffer at pH 6. Standard DAB technique (Dako EnVision+ System-labelled Polymer Anti-rabbit followed by Liquid DAB+ Substrate

Chromogen System) was employed for immunostaining. Counterstaining was performed with hematoxylin solution. The percentage of cells with a distinctive strong nuclear staining was estimated (Figure 1). Snail expression was only evaluated in tumor cells, and all cases with a nuclear Snail expression of 5% of tumor cells were considered positive. Our decision to set the threshold at  $\geq 5\%$  was based primarily on a distribution analysis of the cases in this study. Since 42.6% of cases with nuclear Snail expression were in the 5% and 10% categories, we decided that a cutoff set at  $\geq 5\%$  represents the most appropriate threshold. The staining intensity of ER, PR, and human epidermal growth factor receptor 2 (HER2) was scored as described previously [19].

**2.3. Statistical Analysis.** The distributions of patient and clinical characteristics between Snail positive and negative tumors were compared using Chi-square test, Wilcoxon rank sum test, or two-sample *t*-test as appropriate. Overall survival (OS) was defined as the time from the first operation to death due to any cause. Survivors were censored at the date of last contact. Survival curves by Snail status were estimated using the Kaplan-Meier product-limit method and compared by log-rank test. Univariate Cox proportional hazard models were fit to identify factors significantly related to overall survival. To assess whether Snail was an independent predictor of survival, a multivariate Cox model was constructed to adjust other patient/clinical characteristics that were significant in the univariate analyses. Two-way interaction terms between Snail and other factors in the multivariate Cox model were also assessed. All analyses were two-sided and significance was set at a *P* value of 0.05. Statistical analyses were performed using SAS (SAS Institutes, Cary, NC).

## 3. Results

Using a threshold of  $\geq 5\%$  of cancer cell nuclei staining positive for Snail, a total of 265 (25.4%) of the 1043 evaluable primary breast cancers were positive. Snail expression was significantly associated with greater tumor size, higher tumor stage and grade, positive lymph node status, higher Ki-67 expression and ER<sup>-</sup> and PR<sup>-</sup> status (Table 2), and histological subtype, being the highest in the medullary subtype (71%) and lowest in the cribriform subtype (9%) (data not shown). Of note, there was a significant association between Snail expression and ER<sup>-</sup> status (Snail expression 13.0% in ER<sup>+</sup> cancers versus 64.4% in ER<sup>-</sup> cancers, *P* value < 0.0001) as well as PR<sup>-</sup> status (Snail expression 15.0% in PR<sup>+</sup> cancers versus 29.9% in PR<sup>-</sup> cancers, *P* value < 0.0001) (Table 2). No significant association between Snail expression and HER2 status was found (Table 2). In addition, Snail expression was associated with the intrinsic subtypes of breast cancer, as defined by the St. Gallen consensus conference. The breast cancer intrinsic subtypes were originally defined by gene expression profiling [20, 21] but can be approximated using immunohistochemistry for ER, PR, Ki-67, and HER2 [22, 23]. These subtypes are known to have differing epidemiological risk factors, prognosis, and response to therapy [23]. Snail expression was the highest in the basal-like subtype (73.8%)

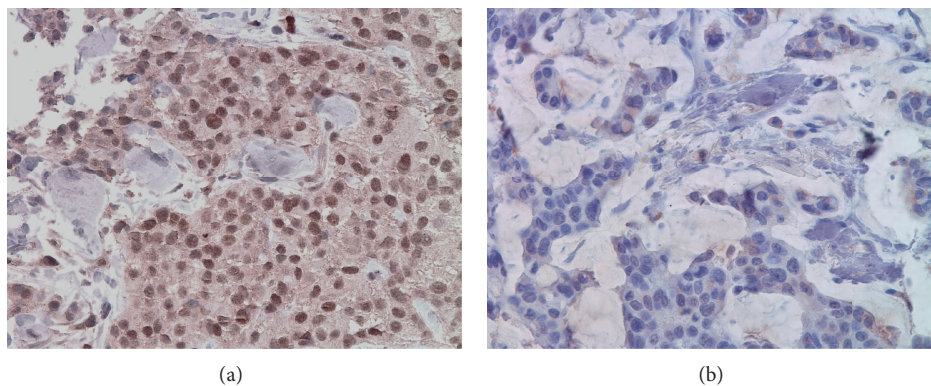


FIGURE 1: Representative photographs of nuclear Snail expression in breast cancer with (a) invasive ductal carcinoma with nuclear Snail staining. Magnification 400x. (b) Invasive ductal carcinoma negative for Snail staining. Magnification 400x.

TABLE 1: Basic demographic data for 1043 evaluable breast cancer cases.

	Number (n)	Percent (%)
Tumor stage		
pT1	307	29.4
pT2	544	52.2
pT3	71	6.8
pT4	121	11.6
Lymph node involvement		
pN0	555	53.4
pN1	398	38.3
pN2	87	8.3
Tumor grade		
1	254	24.4
2	439	42.1
3	350	33.5
Histologic subtype		
Invasive ductal	735	70.5
Invasive lobular	152	14.6
Mucinous	30	2.9
Apocrine	11	1.1
Cribriform	33	3.2
Papillary	15	1.4
Medullary	31	3.0
Other	35	3.3
Intrinsic subtype		
Luminal A (ER <sup>+</sup> and/or PR <sup>+</sup> , HER2 <sup>-</sup> , Ki-67 < 14%)	176	16.9
Luminal B (HER2 <sup>-</sup> ) ER <sup>+</sup> and/or PR <sup>+</sup> , HER2 <sup>-</sup> , Ki-67 ≥ 14%)	507	48.7
Luminal B (HER2 <sup>+</sup> ) (ER <sup>+</sup> and/or PR <sup>+</sup> , HER2 <sup>+</sup> )	120	11.4
HER2 type (ER <sup>-</sup> or PR <sup>-</sup> , HER2 <sup>+</sup> )	79	7.6
Basal-like (ER <sup>-</sup> , PR <sup>-</sup> , HER2 <sup>-</sup> )	160	15.4
Mean tumor size (mm) ± standard deviation (SD)		31.5 ± 16.8
Mean age at diagnosis (years) ± standard deviation (SD)		63.1 ± 13.7

TABLE 2: Association between Snail expression and clinicopathological parameters.

Clinicopathologic parameter	Snail positive		Snail negative		P value
Mean tumor size (mm) $\pm$ SD	35.1 $\pm$ 20.4		27.9 $\pm$ 15.6		<0.0001
Mean age at diagnosis (years) $\pm$ SD	63.2 $\pm$ 14.9		63.1 $\pm$ 13.3		0.9164
	(n)				
Tumor stage					<0.0001
pT1	47	15.3	260	84.7	
pT2	128	23.5	416	76.5	
pT3	20	28.2	51	71.8	
pT4	70	57.8	51	42.2	
Lymph node involvement					<0.0001
pN0	115	20.7	440	79.3	
pN1	103	25.9	295	74.1	
pN2	47	54.0	40	46.0	
Tumor grade					<0.0001
1	28	11.0	226	89.0	
2	59	13.4	380	86.6	
3	178	50.9	172	49.1	
Estrogen receptor					<0.0001
ER <sup>+</sup>	102	13.0	685	87.0	
ER <sup>-</sup>	163	64.4	90	35.6	
HER2					0.1266
HER2 <sup>+</sup>	59	29.6	140	70.4	
HER2 <sup>-</sup>	206	24.4	638	75.6	
Ki67					<0.0001
Ki67 <sup>+</sup>	229	28.5	574	71.5	
Ki67 <sup>-</sup>	35	15.0	198	85.0	

and the lowest in the luminal A subtype (11.9%,  $P < 0.001$ ) (Table 3).

Studying the impact of Snail expression on survival, we found that in univariate survival analyses, breast cancer cases with positive Snail expression had a significantly worse OS (hazard ratio (HR) = 2.843,  $P < 0.001$ ) (Table 4 and Figure 2). In multivariate analysis, after adjusting for age, grade, tumor size, lymph node status, and intrinsic subtype, Snail remained an independent negative prognostic factor for OS (HR = 1.930,  $P < 0.001$ ) (Table 5). In subset univariate analyses of the specific intrinsic subtypes, Snail expression proved to be a negative prognostic factor for OS in the luminal B HER2<sup>-</sup> type (HR = 3.674,  $P < 0.0001$ ), the luminal B HER2<sup>+</sup> type (HR = 3.692,  $P < 0.0001$ ), and the basal-like subtype (HR = 3.610,  $P = 0.0006$ ) (Table 4 and Figure 2). Of note, the negative prognostic effect of Snail expression was independent of ER (Table 6), PR, and HER2 status (data not shown).

#### 4. Discussion

In our study, we evaluated the nuclear expression of Snail in a large cohort of functionally annotated primary breast cancer specimens. We observed that Snail is expressed in 25.4% of primary breast cancers. This is practically identical to the results of Geradts et al. as well as Lundgren et al., who used the same scoring system as we did and found nuclear

Snail expression in 23.7% of their breast cancer specimens ( $n = 58$  and  $n = 384$ , resp.) [3, 14]. Similarly, Becker et al. reported 33% of breast cancers as being positive for nuclear Snail staining [24]. Taking a cutoff of >10% nuclear Snail expression in tumor cells, ElMoneim and Zaghoul found a significantly higher Snail expression in breast cancers (40.9%,  $n = 132$ ) [13]. Other studies that have used a weighted histoscore, multiplying the proportion of stained cells by the intensity of staining, have also found significantly higher nuclear Snail expression in breast cancer (42.6% and 54%, resp.) [4, 13, 25]. Since all of the breast cancers in our collective with nuclear Snail expression showed a strong staining intensity, we assessed the percentage of positive nuclei in each tumor but did not integrate staining intensity into a combined score. Our decision to set the threshold at  $\geq 5\%$  was based primarily on a distribution analysis of the cases in this study. Since 42.6% of cases with nuclear Snail expression were in the 5% and 10% categories, we decided that a cutoff set at  $\geq 5\%$  represents the most appropriate threshold. The differences seen with some of the previously mentioned studies could also be due to different antibodies used for the immunohistochemical staining.

In accordance with previous reports of nuclear Snail expression in human breast cancers, we found a significant association between Snail and higher tumor grade and proliferation rate as well as with greater tumor size and stage

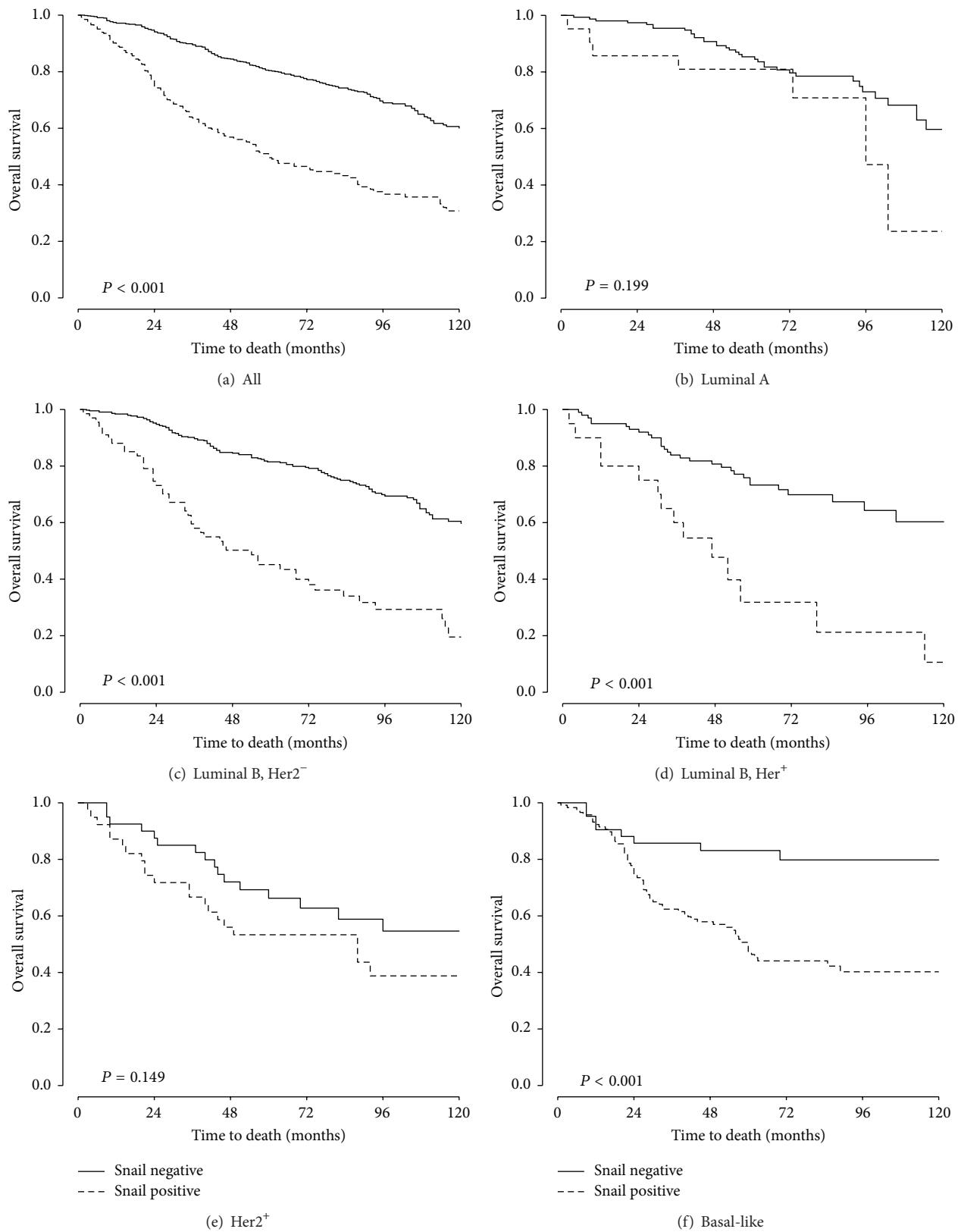


FIGURE 2: Kaplan-Meier survival curve for (a) overall survival depending on Snail expression (univariate analysis). (b)–(f) Kaplan-Meier survival curve for overall survival depending on Snail expression for the intrinsic breast cancer subtype.

TABLE 3: Association between Snail expression and breast cancer intrinsic subtype.

Intrinsic subtype	Snail positive		Snail negative		P value
	(n)	(%)	(n)	(%)	
Luminal A (ER <sup>+</sup> and/or PR <sup>+</sup> , HER2 <sup>-</sup> , Ki-67 < 14%)	21	11.9	155	88.1	<0.0001
Luminal B (HER2-negative) ER <sup>+</sup> and/or PR <sup>+</sup> , HER2 <sup>-</sup> , Ki-67 ≥ 14%)	67	13.2	440	86.8	
Luminal B (HER2-positive) (ER <sup>+</sup> and/or PR <sup>+</sup> , HER2 <sup>+</sup> )	20	16.7	100	83.3	
HER2 type (ER <sup>-</sup> , PR <sup>-</sup> , HER2 <sup>+</sup> )	39	49.4	40	50.6	
Basal-like (ER <sup>-</sup> , PR <sup>-</sup> , HER2 <sup>-</sup> )	118	73.7	42	26.3	

TABLE 4: Univariate analyses for all cases, by intrinsic subtype, for the effect of Snail expression on overall survival.

Snail expression, all cases	Hazard ratio (95% CI)	P value
Snail positive	1.702 (0.755–3.834)	<0.0001
Snail expression, by intrinsic subtype		
Luminal A	1.702 (0.755–3.834)	0.1994
Luminal B (HER2 <sup>-</sup> )	3.674 (2.637–5.119)	<0.0001
Luminal B (HER2 <sup>+</sup> )	3.692 (1.971–6.915)	<0.0001
HER2 type	1.616 (0.843–3.101)	0.1485
Basal-like	3.610 (1.728–7.541)	0.0006

[3, 13, 14]. In compliance with the notion that Snail expression leads to a more mobile and aggressive cancer cell type, nuclear Snail expression was also significantly associated with lymph node metastases in our collective, a finding concordant with the results of previous studies [3, 13, 14].

Furthermore, we could confirm the association between nuclear Snail expression and ER<sup>-</sup> as well as PR<sup>-</sup> status described in previous immunohistochemical studies [3, 13, 14] and by microarray analysis of primary human breast cancers [26]. The inverse association between Snail expression and expression of ER has also been described for breast cancer cell lines [26] and is in line with a current model depicting transcriptional repression of Snail as a secondary biological effect of the ER [27, 28]. Additionally, it has also been shown that Snail is in turn able to directly downregulate ER [26]. Taken together, this data suggests a crucial role for the ER in EMT-dependent tumor progression. A study by Lundgren et al. reports that breast cancer patients with nuclear Snail expression have a better response to tamoxifen [14], but the underlying mechanism of this effect is yet to be elucidated. Speculatively, tamoxifen might inhibit the function of Snail, as described previously for the ER antagonist ICI [14]. This in turn may lead to a less invasive tumor phenotype, since EMT is reverted [14]. Interestingly, another study found that acquisition of hormonal resistance in breast cancer correlates with increase in Snail expression and activity while inhibition of Snail partially restores the sensitivity of the resistant cancer cells to tamoxifen [29]. Evidently, further studies including large randomized clinical cohorts are needed to investigate the role of Snail as a treatment predictive marker in breast cancer.

In our collective, Snail expression was differently associated with the intrinsic breast cancer subtypes, being the

highest in the basal-like subtype and the lowest in the luminal A subtype. In the only study to date looking at nuclear Snail expression in breast cancer subtypes, Geradts et al. found nuclear Snail protein in 75% of triple-negative breast cancers [3], which is almost identical to our findings in the basal-like intrinsic subtype (73.8%). Of note, the St. Gallen consensus conference uses the triple-negative phenotype as an approximation for basal-like breast cancer [23].

Our results show that nuclear Snail expression is an independent prognostic factor for worse OS in primary breast cancers. This is in contrast with a previous study by Logullo et al. where the authors found no significant correlation between Snail expression and survival. In their study, the authors, however, analyzed only cytoplasmic Snail expression, and their collective of invasive breast cancers was quite small ( $n = 55$ ) [30]. In an independent study, Yuen et al. found no impact of nuclear Snail expression on survival in a collective of 115 breast cancers when looking at all cases but found that nuclear Snail expression significantly predicted poorer disease-free survival in patients with positive lymph node status and ER<sup>-</sup> as well as PR<sup>-</sup> breast cancers [4]. Contrastingly, van Nes et al. found an association between nuclear Snail expression and a decreased relapse-free period in patients with ER<sup>+</sup> breast cancers [25]. Of note, in our collective of 1043 breast cancer patients, the negative prognostic effect of Snail expression was independent of ER status. Since Snail represents an important transcription factor for EMT in carcinomas, thus increasing cell motility and allowing the tumor to metastasize, and it is very feasible that nuclear expression of Snail is associated with a worse prognosis in breast cancer, as it has been shown in various other human cancers [10–12].

TABLE 5: Multivariate analysis for the effect of clinicopathologic parameters and Snail expression on overall survival.

Clinicopathologic parameter	Hazard ratio (95% CI)	P value
Age (per 1 year)	1.037 (1.029–1.046)	<0.0001
Tumor stage		
pT1 (reference)	1	
pT2	1.392 (1.042–1.859)	0.0251
pT3	1.620 (1.047–2.508)	0.0303
pT4	1.619 (1.103–2.377)	0.0140
Lymph node involvement		
pN1 (reference)	1	
pN1	1.446 (1.147–1.822)	0.0018
pN2	2.603 (1.852–3.658)	<0.0001
Tumor grade		
BRE grade 1 (reference)	1	
2	1.593 (1.161–2.186)	0.0039
3	2.123 (1.510–2.986)	<0.0001
Snail expression, all cases		
Snail positive	1.930 (1.476–2.524)	<0.0001
Snail expression, by intrinsic subtype		
Luminal A (reference)	1	
Luminal B (HER2 <sup>-</sup> )	1.012 (0.721–1.414)	0.9458
Luminal B (HER2 <sup>+</sup> )	1.206 (0.788–1.844)	0.3884
Her2	0.999 (0.618–1.614)	0.9958
Basal-like	1.258 (0.819–1.934)	0.2951

TABLE 6: Effect of Snail on overall survival by ER status.

Effects of Snail expression	Hazard ratio (95% CI)	P value
Interaction with ER status		0.2391
ER <sup>+</sup>	3.334 (2.530–4.394)	
ER <sup>-</sup>	2.443 (1.575–3.789)	

When looking at the different intrinsic breast cancer subtypes, we are the first to show that nuclear Snail expression is a negative prognostic factor for OS in the luminal B HER2<sup>-</sup> type, the luminal B HER2<sup>+</sup> type, and the basal like subtype.

Due to its important role in the epithelial-mesenchymal transformation of tumor cells and its correlation with tumor malignancy, Snail is an attractive target for the development of blocking pharmaceutical agents [31]. By blocking Snail, cell migration and invasion, and, consequently, metastasis of tumor cells are diminished, thus improving the clinical outcome. With the Schiff base complex Co(III)-Ebox, a highly selective inhibitor that prevents Snail from binding to its DNA target has recently been identified [32] and has proven to be a potent inhibitor of Snail-mediated transcriptional repression in breast cancer cells [31]. It is thus feasible that this selective inhibitor could be used to therapeutically target Snail and other zinc-finger transcription factors such as Slug in human cancers.

## 5. Conclusions

Our study is the largest TMA study to date to evaluate the role of nuclear Snail expression in breast cancer and is the first to

demonstrate that nuclear Snail expression is an independent predictor of prognosis in breast cancer and is differently expressed in the intrinsic breast cancer subtypes. Our data suggests that Snail represents a potential target in human breast cancer. Of note, targeted inactivation of Snail with the Schiff base complex Co(III)-Ebox has recently been reported in breast cancer cells [31]. Routine staining for Snail in breast cancer could thus be used to identify patients eligible for targeted therapy but could also identify those patients with a more aggressive course of disease. Additional studies to define the functional role of Snail in human breast cancer and assess treatment options are, however, needed.

## Conflict of Interests

The authors declare that they have no conflict of interests.

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