Research Article

High Snail 1 Expression is a Poor Prognostic Factor in Patients with Invasive Ductal Carcinoma of the Breast

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Abstract
Background: Breast carcinoma is the most common cancer in Egyptian women. Developing metastasis is the leading cause of death in patients with invasive ductal carcinoma of the breast. The epithelial–mesenchymal transition (EMT) plays an important role in breast cancer metastasis. Snail 1 is a key regulator of the EMT of tumor cells. **Aim:** To study the immunohistochemical expression of Snail 1 in invasive ductal carcinoma NOS of the breast and their association with different clinicopathological features of breast IDC. **Material and methods**: This study comprised 70 cases of IDC-NOS of the breast. Formalin fixed and paraffin-embedded tissue sections from cases under investigation were subjected to haematoxylin and eosin staining and immunohistochemical staining for Snail 1 using the avidin biotin-peroxidase complex method. **Results:** Snail 1 immunostaining was nuclear. High snail 1 expression was detected in 52.86% of cases. High snail 1 expression was significantly associated with larger tumor size, higher tumor grade, higher lymph node stage, higher LNR, advanced tumor stage, poor Nottingham prognostic index (NPI), high Ki-67 PIs, high Her-2 neu expression, negative ER hormonal receptors, negative PR hormonal receptors and aggressive molecular subtypes being highest in triple negative and her-2 enriched types (P= 0.028, 0.003, 0.024, 0.002, 0.001, 0.001, 0.001, 0.011, 0.002, 0.001, 0.002 respectively). High Snail 1 expression had significantly shorter OS (p <0.001) and poor DFS (p< 0.001). Snail 1 was independent prognostic indicators for OS (P= 0.03) and DFS (p<0.001). **Conclusions:** High snail 1 expression is associated with poor clinicopathological features of IDC of the breast, aggressive behavior of the tumor, shorter Os and Poor DFS.
Keywords: Snail 1, EMT, IDC, DFS.

Introduction
Breast cancer has been recognized as a major health problem worldwide and its incidence has increased in the last decades. It is the second most common cancer in the world and the most commonly occurring cancer among women[1]. In Egypt, breast cancer is the most common malignant tumor among women, accounting for 35.1% of all female carcinomas and is the second-leading cause of cancer death in Egyptian women[2]. IDC is the most common type of invasive carcinoma of the breast; representing 60–75% of all mammary invasive carcinomas[3].

Developing metastasis is the main cause of death in breast cancer patients. The epithelial mesenchymal transition "EMT" phenomenon has been the preferential explanation of distant metastases for epithelial cancers[4]. Many EMT-inducing transcription factors have been discovered such as snail 1[5]. Snail-1 is an important regulator of EMT. It acts by repressing the expression of E-cadherin, resulting in the up-regulation of N-cadherin protein[6]. Snail also has roles in many human processes like cell proliferation, apoptosis, metastasis, drug resistance and many steps of the carcinogenesis[7].

In the present study we evaluated the immunohistochemical expression of snail 1 in IDC of the breast and analyzed its association with different clinicopathological features of tumor in an attempt to elucidate their possible role in breast carcinoma progression, lymph node metastasis and prognosis.

Material and Methods
Patients and tissue specimens
This retrospective study was performed on archival material of 70 formalin-fixed paraffin-

embedded samples of IDC of the breast from female patients. All material was retrieved from the archives and databases of pathology laboratories of Minia Oncology Center and Pathology Department, Faculty of Medicine, Minia University.

Immunohistochemistry

Four µm sections were prepared from on positively charged slides subjected to snail 1 immunohistochemical staining. Sections were heated at 60ºC for 10 minutes, deparaffinized in xylene, rehydrated in descending graded alcohol. Then the rehydrated sections were immersed in a 3% solution of hydrogen peroxide in methanol and incubated for 30 minutes at room temperature to block endogenous peroxidase activity, and then slides were rinsed in buffer solution. For purpose of antigen retrieval, sections were treated in microwave by immersion of the slides in citrate buffer solution (pH 6) for 20 minutes, then slides were allowed to cool for 20 minutes to reach room temperature then washed with PBS buffer for 5 minutes. Protein block was done. Next, slides were incubated overnight with the primary antibodies at room temperature using snail 1 antibody (Polycolonal goat antibody 0.1ml concentrated, ab53519; Abcam, Cambridge, UK) at 1:100 concentration, followed by rinsing in PBS (pH7.4). This was followed by incubation with the Secondary antibody for 30 minutes at room temperature. After that, slides were rinsed in buffer solution for 5 minutes; and streptavidin reagent was then applied for 30 minutes at room temperature. Then slides were rinsed gently and placed in PBS for 5 minutes. Diaminobenzidine tetrachloride (DAB) substrate was applied on sections, and then slides were left till brown color appears or until 15 minutes at room temperature pass, then slides were rinsed with distilled water.

Lastly, sections were counterstained in Harris hematoxylin, rinsed gently in distilled water, dehydrated in ascending grades of alcohols (70%, 95% and 100% alcohol), then cleared in xylene and, mounted using an aqueous-based mounting medium, Disterene plasticizer xylene (DPX) and covered slips. Tissue sections from kidney tissue were included in each run as a positive control. Negative controls were obtained by omission of primary antibody and its replacement with a BPS buffer.

Evaluation of immunohistochemical staining

Snail 1 expression was detected as a nuclear staining in beast carcinoma cells. Snail 1 was scored by multiplying the percentage of positive tumor cells and the staining intensity. The extent of positivity was scored as 0 when the percentage of positive cells was <5%; 1 when it was 5–25%; 2 when it was 26–50%; 3 when it was 51–75%; and 4 when it was >75%. The staining intensity was scored as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3 when strong staining was identified. The extent and intensity scores were multiplied to obtain a total score, which ranged from 0 to 12. Snail 1 expression was dichotomized as negative/low expression if total score ≤4 and high expression if total score > 4[8].

Statistical Analysis

Data was checked, coded and analyzed using computer based SPSS (Statistical Package for Social Science), Version 16.0 software. The Pearson Chi-square test and Fisher's exact test were used to evaluate comparisons of clinicopathologic characteristics. Results were considered statistically significant when p-value ≤ 0.05 for any relationship being considered. In univariate survival analysis, overall survival (OS) and disease/relapse free survival (DFS/ RFS) were estimated. Kaplan-Meier curves were used for plotting of patients' survival data. Differences between survival curves were tested using Log-Rank test. Cox multivariate regression analysis was used to analyze the hazard ratio and the prognostic value of clinical as well as other examined variables. P-values ≤ 0.05 were regarded as statistically significant.

Results

Clinicopathological data

This study included 70 cases of IDC of female breast. The mean age of the studied cases was 49.67 (ranged from 25-74y). The clinicopathological data of the patients are summarized in Table (1).

Immunohistochemical results of snail 1 expression and its relationship with different clinicopathological features of breast IDC patients

High nuclear snail 1 expression was detected in 37(52.86%) cases. Thirty seven (47.14%) cases showed negative/low expression (figure 1,2,3).

A statistically significant association was found between snail 1 expression and larger tumor size (T) (P= 0.028), higher tumor grade (P= 0.003), higher lymph node stage (p value = 0.024), advanced tumor stage (p= 0.001), Nottingham prognostic index (NPI) (p=0.001), high Ki-67 PI (p=0.001), high Her-2 neu expression (p= 0.011), negative ER& PR hormonal receptors (p= 0.002 and 0.001) respectively. No significant relationship was found between snail 1 and patient age (p= 0.469) and tumor laterality (p=0.146). The association between snail 1 expression and different clinicopathological features was summarized in Table (2).

Snail 1 expression in relation to OS&DFS/ RFS

Univariate analysis also revealed that patients exhibited high Snail 1, had significantly shorter survival and worse outcome, when compared with patients that had -ve/low Snail 1 (Log Rank (p <0.001)). Regarding DFS/RFS, univariate analyses; significant associations were found between adverse DFS/RFS and high snail 1 expression (p< 0.001). Cox multivariate regression analysis has been done, to evaluate the prognostic significance of Snail 1 immunoexpressions. In such analysis, the procedure has selected Snail 1(P=0.03) as independent prognostic indicators for OS, and Snail 1 (p<0.001) as independent prognostic indicators for DFS/RFS while the other variables included in the model did not reach significance.

Table (1): Clinicopathological features for patients with IDC (n=70)

Clinicopathological features	No. (%)
Age at Surgery, (years)	
≤50	40 (57.14%)
>50	30 (42.86%)
Laterality	
Right breast	28 (40%)
Left breast	42 (60%)
Size	
T1	8 (11.43%)
T2	50 (71.43%)
T3	12 (17.14%)
Lymph node stage	
N0	31 (44.29%)
N1	21 (30%)
N2	11 (15.71%)
N3	7 (10%)
AJCC Stage	
I	6 (8.58%)
II	46 (65.71%)
III	18 (25.71%)
Tumor grade	
Grade I	5 (7.14%)
Grade II	33 (47.14%)
Grade III	32 (45.72%)
Nottingham prognostic index (NPI)	
Good prognosis (≤3.4)	11 (15.71%)
Moderate prognosis (3.41-5.4)	33 (47.14%)
Poor prognosis (>5.4)	26 (37.15%)
Estrogen Receptor	
Negative	24 (34.29%)
Positive	46 (65.71%)
Progesteron Receptor	
Negative	30 (42.86%)
Positive	40 (57.14%)
Her 2 neu	
Negative	46 (65.71%)
Positive	24 (34.39%)
KI67	
<14%	29 (41.43%)
>14%	41 (58.57%)
Molecular classification	
Luminal A	29 (41.43%)
Luminal B	17 (24.28%)
Her 2 type	14 (20%)
Triple negative	10 (14.29%)

Table (2): Association between snail 1 expression and clinicopathological features for patients with IDC (n=70)

Clinicopathological features	No	Snail 1 expression		*P* value
		-ve/low expression No. (%)	high expression No. (%)	
Age at Surgery, y				
≤50	40	17 (42.5%)	23 (57.5%)	0.469
>50	30	16 (53.33%)	14 (46.67%)	
Laterality				
RT	28	10 (35.71%)	18 (64.29%)	0.146
LT	42	23 (54.76%)	19 (45.24%)	
Size				
T1	8	6 (75%)	2 (25%)	0.028*
T2	50	25 (50%)	25 (50%)	
T3	12	2 (16.67%)	10 (83.33%)	
Lymph node stage				
N0	31	19 (61.29%)	12 (38.71%)	
N1	21	11 (52.38%)	10 (47.62%)	0.024*
N2	11	2 (18.18%)	9 (81.82%)	
N3	7	1 (14.29%)	6 (85.71%)	
AJCC Stage				
I	6	6 (100%)	0 (0%)	
II	46	24 (52.17%)	22 (47.83%)	0.001*
III	18	3 (16.67%)	15 (83.33%)	
Tumor grade				
Grade I	5	5 (100%)	0 (0%)	0.003*
Grade II	33	19 (57.58%)	14 (42.42%)	
Grade III	32	9 (28.13%)	23 (71.87%)	
Nottingham prognostic index				
Good prognosis ≤3.4	11	10 (90.91%)	1 (9.09%)	
Moderate prognosis 3.41-5.4	33	17 (51.52%)	16 (48.48%)	0.001*
Poor prognosis >5.4	26	6 (23.08%)	20 (76.92)	
Estrogen Receptor				
Negative	24	5 (20.83%)	19 (79.17%)	0.002*
Positive	46	28 (60.87%)	18 (39.13%)	
Progesteron Receptor				
Negative	30	7 (23.33%)	23 (76.67%)	0.001*
Positive	40	26 (65%)	14 (35%)	
Her 2 neu				
Negative	46	27 (58.7%)	19 (41.3%)	0.011*
positive	24	6 (25%)	18 (75%)	
KI67 PI				
<14%	29	21 (72.41%)	8 (27.59%)	0.001*
>14%	41	12 (29.27%)	29 (70.73%)	
Molecular classification				
Luminal A	29	21 (72.41%)	8 (27.59%)	
Luminal B	17	7 (41.18%)	10 (58.82%)	0.002*
Her 2 type	14	3 (21.43%)	11 (78.57%)	
Triple negative	10	2 (20%)	8 (80%)	

Test of significance: Chi-Square and Fisher's exact tests. * P value < 0.05 is considered statistically significant.

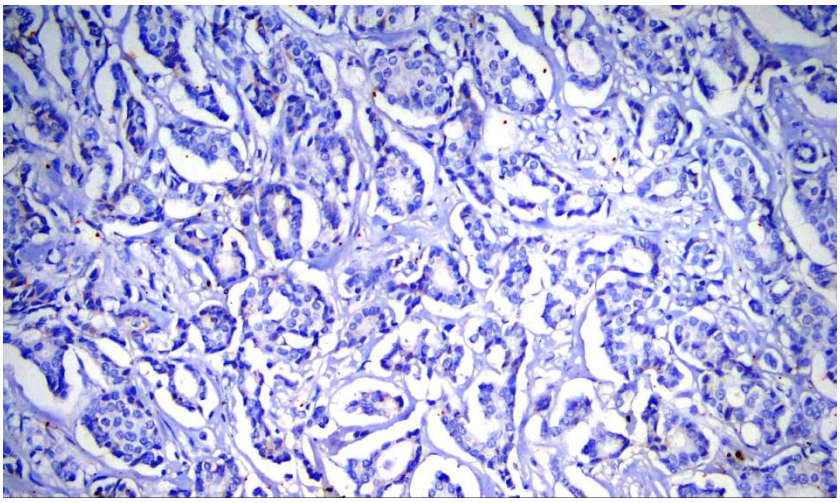

Figure 1: Negative snail1 expression in grade I IDC of the breast

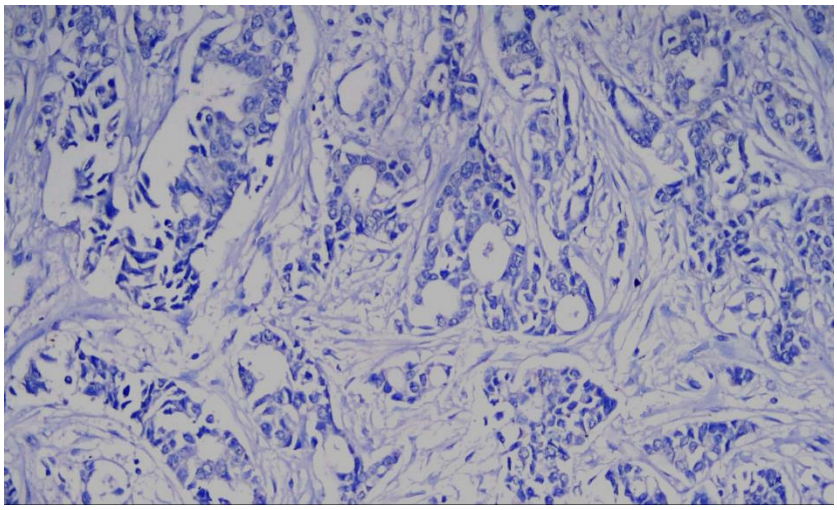

Figure 2: Negative Snail 1 expression in grade II IDC of the breast

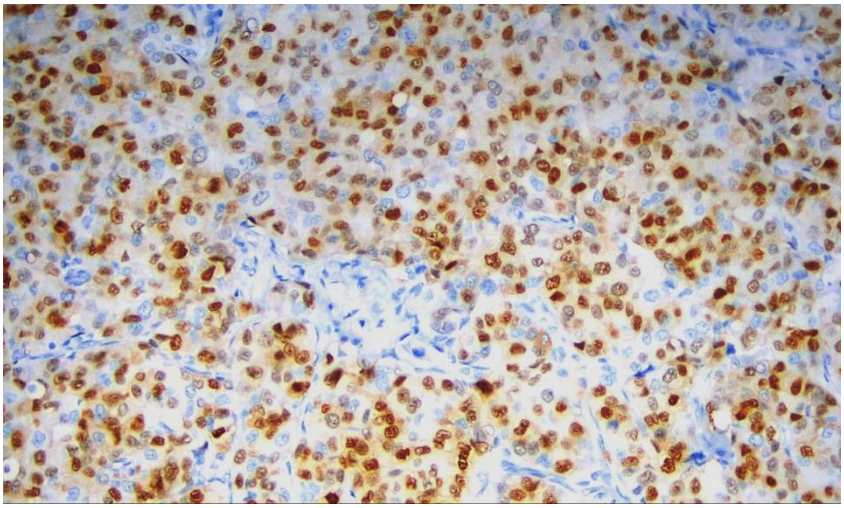

Figure 3: High Snail 1 expression in grade III IDC of the breast

Discussion

Developing metastasis is the main cause of death in breast cancer patients. To date, the EMT phenomenon has been the favored explanation of distant metastases for epithelial cancers including breast cancer. EMT is a complex biological process defining the change of epithelial cells into mesenchymal phenotype. During EMT, the epithelial cells gain mesenchymal properties resulting in increased motility and invasiveness[9].

Many EMT-inducing transcription factors have been discovered such as snail1[5]. Snail has roles in many human processes like cell proliferation, apoptosis, metastasis, drug resistance and many steps of the carcinogenesis[7]. Snail1 is also expressed in many types of cancer. Snail1 over-expression usually correlates with increased migration, invasion, and metastasis[10]. Our study showed snail expression correlates with poor clinic-pathological parameters of IDC of the breast.

In the current study high nuclear snail 1 expression was detected in 52.9% of IDC cases, this was identical to the results of previous study by Megahed et al., who used the same scoring system as we did and found high nuclear snail expression in 53.3% of IDC cases[11]. On the other hand, other studies reported much less positive expression rate of snail 1 in IDC cases[12, 13]. These differences could be due to different scoring system and different antibodies used for the immune-histochemical staining.

No significant relationship between snail 1 immunostaining and patient age, similarly to the previous studies[13-16]. However one previous study detected that the snail1 over-expression in breast cancer patients was associated with older age of the patients[11].

This study showed that snail 1 over-expression was significantly associated with larger tumor size, this was in concordance with multiple precious studies[12-14,17]. In contrast to our result Patel et al., found no significant difference between snail 1 expression and tumor size[15].

A significant relationship was found between snail immunostaining and higher histological grades of the tumor implying that snail 1 over-expression is associated with more aggressive disease. Our results were in concordance with previous studies[11-13].

In the current study, we found a significant association between snail 1 expression levels and an increase in the number of invaded lymph nodes. These observations were similar to previous results[11,12,14,17,18]. These results emphasize the role of Snail in overall tumor invasiveness.

Furthermore we detected that the snail1 over-expression in IDC patients was associated with advanced tumor stage. Our results were in agreement with several previous studies[13, 14, 19]. These similar results pointed to that **s**nail 1 had a role in the breast cancer progression and could be a marker of metastatic liability. In contrast to us, Lugullo et al., didn't found significant association between Snail 1 expression and any poor prognostic factors[20]. These contradictory results could be due to the use of different antibody clone and different technique of staining.

In the current study, a significant positive association was found between snail 1 and NPI, where most of poor NPI cases displayed high snail 1 expression scores, suggesting the association of snail 1 with poor prognostic factors. Similarly, a previous study found that snail 1 expression is significantly increased in poor prognostic index cases[14].

In the present study, snail1 over-expression was associated with negative ER& PR hormonal receptors and high Her2 neu expression this is in agreement with previous results by Megahed et al., who reported that over-expression of snail1 was associated with hormonal receptor down-regulation and HER2 neu over-expression[11]. Additionally, it has also been shown that Snail is in turn able to directly down-regulate ER[21] and Scherbakov et al., in his study proposed that Snail1 may be considered as one of the negative regulators of ER in breast tumors[22]. In contrast to our results, some studies found no significant associations between the expression of Snail 1 and ER, PR or HER2 status[15, 18] while another study reported that Snail expression is associated with ER-positive and PR positive[23]. A statistically significant association was found

between snail 1 expression and Ki-67 PI, this was in agreement with previous studies[13, 17, 24]. On analyzing the relationship between snail 1 expression and molecular subtypes of tumor, a statistically significant association was found between snail1 expression and molecular type being highest in triple negative and lowest in luminal type A. Our results was in agreement with previous studies that found that the highest snail 1 expression was associated with aggressive molecular type whereas it was highest in triple negative[12, 13]. On the other hand; one previous study indicated that Snail expression was not associated with any of the above molecular subtype markers[20]. The differences between our findings and other studies could be due to a number of factors such as different sample type/size included, clones of antibodies used, and scoring systems.

Conclusion

Our results showed that high snail expression correlates with poor clinicopathological features of IDC of the breast as high snail 1 expression showed a statistically significant association with larger tumor size, higher tumor grade, higher lymph node stage, advanced tumor stage, Nottingham prognostic index (NPI), high Ki-67 PI, high Her-2 neu expression, negative ER& PR hormonal receptors respectively, Shorter OS and Poor DFS.

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