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Prognostic Role of Epithelial-Mesenchymal Transition (EMT) Markers SNAIL-1 & Eph A2 Expressions in Breast Carcinoma

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Abstract: ***Background: The breast cancer is one of the commonest cancers worldwide and the commonest malignancy of females in Egypt. The understanding of the molecular pathways of its carcinogenesis is important to discover recent therapeutic targets. Epithelial-mesenchymal transition (EMT) is a recent explanation of cancer progression, metastases and the resistance to chemotherapy in breast cancer. EMT process is controlled by many pathways and markers and understanding their functions, role in metastases is needed to modify the treatment strategies for breast cancer patients. A recently discovered EMT-inducing transcription factors is snail-1, that was involved in cell proliferation, apoptosis, metastasis, drugs resistance, and several aspects of the carcinogenic process. EphA2 protein is an essential component of RTK/Ras-signaling that are involved in EMT and cooperates with Wnt/b-catenin-pathway. Aim of the work: was to evaluate EMT markers Snail1 & Eph A2 expressions in breast cancer patients correlating their expressions with clinicopathological parameters and patients outcome. Methods: EMT markers Snail-1&Eph A2 expressions were evaluated in sections from 60 paraffin blocks of breast cancer using immunohistochemistry. We analyzed correlations between the levels of their expressions, clinical, pathological parameters of the patients and patient's outcome. Results: Snail1 over expression and Eph A2 positive expression in breast cancer was associated with higher grade (<0.03 for both markers), higher stage of the tumor, presence of lymph node metastases, distant metastasis, high KI67 labeling index, negative ER& PR hormonal receptors, high Her2 neu expression and aggressive molecular subtype like triple negative, luminal B and her2 neu amplified (p<0.001 for all of them). The expression of both markers was significantly positively correlated with each other (p<0.001). The 3-year overall survival rate was 86.5% with a mean of 35.2 ± 0.22 months (95% CI; 35.2 – 36.1 months). There was a significant correlation between the progression and Snail-1 (P<0.02) and there were highly significant correlations between combination of both markers expression and overall survival and progression free survival (P<0.001). Conclusion: Snail1&Eph A2 are markers of poor prognosis of breast cancer patients with an impact on survival.***

Keywords: breast carcinoma, Snail-1&Eph A2, EMT, survival

1. Introduction

Breast cancer forms one-quarter of all female malignancies worldwide **(Lakhani et al., 2012).** In Egypt it is the commonest malignancy of females and forms 34.26% of women malignancies according to the Egyptian National Cancer Institute registry **(Mokhtar et al., 2007).** The understanding of the molecular pathways of its carcinogenesis is important to discover recent therapeutic targets. Epithelial mesenchymal transition (EMT) is the process that is characterized by epithelial cell to cell adhesion loss and mesenchymal criteria gain **[Thiery et al., 2009],** it has become acceptable explanation of breast cancer progression and distant metastases occurrence [**Thiery et al., 2013**]. EMT process is controlled by many pathways and markers and understanding their functions, role in metastases is needed to modify the treatment strategies for breast cancer patients**.** Many EMT-inducing transcription factors (EMT-TFs) have been discovered such as snail1 **(Khan, M. A. et al 2015).** Snail is a member of conserved Snail super family of TFs that were expressed during different tissues development (**Seki ET AL., 2013)** Snail has roles in many human processes like cell proliferation, apoptosis, metastasis, drug resistance and many steps of the carcinogenisis (**Martin ET AL., 2005**). The RTK/Ras pathway **is one of** signaling pathways that had an important role in EMT **(Kalluri and Weinberg., 2009), T**he RTK/Ras/MAPK- pathway cooperated with Wnt/b-catenin-pathway controling cell homeostasis, mainly by up regulation of many genes such as Hox genes **(Sundaram et al. 2009).** EphA2 is a receptor that is one of the RTK/Ras signaling components (**Yuan et al., 2009a).** Eph receptors form the family of receptor tyrosine kinases that are divided into two subclasses ephrin-A or ephrin-B according to the type of interaction with their ligands (**Pasquale et al., 2005, Landen et al., 2005).** There are many roles of EphA2 in carcinogenic process include regulation of cancer cell growth, invasion, survival, and angiogenesis **(Thaker et al., 2004) (Kinch and Carles-Kinch., 2003).** That was the first study to clarify roles and mechanisms of expressions of EMT markers Snail1& Eph A2 together in breast carcinoma as it is still uncertain through which pathways they promote EMT in cancer breast.

Aim of the work; was to evaluate EMT markers Snail1 & Eph A2 expressions in breast cancer patients correlating their expressions with clinicopathological parameters and patients outcome.

2. Patients and Methods

This retrospective study was carried out at Clinical Oncology and nuclear medicine , Pathology, General surgery departments, Faculty of Medicine, Zagazig University ,Egypt in the period from December 2013 to November 2016.We

included 60 patients who were diagnosed as breast carcinoma by routine H&E histopathological examination which was done in addition to ER, PR hormonal receptors & Her2 neu expressions and Ki67 labeling index in the Pathology Department, Faculty of Medicine, Zagazig University . We identified patient age, tumor size, stage, grade, lymph node metastasis, distant metastases, ER ,PR ,Her2-neu , ki67 labelling index, capsular invasion ,type of treatment received (surgery, chemotherapy , radiotherapy , with or without hormonal treatment - trastuzumab), by retrospective examination of the patient's records at the involved departments. Local Research Ethics Committee approval of the study was obtained. The American-Joint Committee-on-Cancer staging system classification (7th edition) was used for cancer staging **(Edge and Compton, 2010)** and we used, for cancer grading, the Nottingham (Elston–Ellis) modification of the Scarff– Bloom–Richardson grading system **(Elston, 2002).**

The technique of immunohistochemical staining

It was done using the streptavidin–biotin immunoperoxidase technique **(Hsu et al., 1981).** Sections cut from formalin fixed and paraffin-embedded blocks of about 4 µm thick for all cases, put on the positively charged slides, we did deparaffinization and rehydration of them in xylene and graded alcohol, respectively. We boiled the sections in citrated buffer of (pH6.0) for twenty minutes then washed them in phosphate buffered saline of (pH 7.3), then we blocked the endogenous peroxidase activities by six percent of H2O2 in methanol. In the next step we incubated the slides overnight with primary rabbit poly-clonal anti- snail1 antibody (clone ab180714) (Abcam, Cambridge, UK) and primary rabbit mono-clonal anti-EphA2 antibody (D4A2) XP® (Cell Signaling Technology) both antibodies were used in 1:200 dilution at room temperature for four hours. We counterstained all sections with hematoxylin, dehydrated them then applied cover glasses after rinsing them in distilled water. Sections of mammary and gastric carcinomas were used as positive controls for Snail-1 and Eph A2 respectively. For negative control omission of both of primary antibodies and then replace those with a phosphate-buffered saline buffer.

Evaluation of immunohistochemical expression of SNAIL-1

We consider nuclear staining as positive for Snail 1; we evaluated staining by calculating stained cells extent and stain intensity. The extent was scored as: zero (negative), one (till 25 percent of positive cells), two (26 to 50 percent), three (51to 75 percent) and four (76 percent). The stain intensity was scored as; zero (negative), one (weakly positive), two (moderately positive) and three (strongly positive). We calculated the final scores by multiplication of the stain intensity and the extent, scores; zero (negative), + (1–4), ++ (5–8) and +++ (9–12) **(Zhang et al., 2010).** Our cutoff value was 5, high expression if values were more than 5 and low expression if values were less than or equal to 5.

Evaluation of immunohistochemical expression of Eph A2

We consider cytoplasmic expression as positive for Eph A2, selected 10 fields randomly from all sections, assessed, graded them and evaluated the extent of stain and gave it scores from zero to 3 (0 = 0–6%; 1= 7–26%; 2 = 27–50%; 3 = more than 50%) and assessed intensity of stain and scored it from zero to three (0= negative; 1 = weak; 2 =moderate; 3 = strong). We summate scores of both the stain intensity and extent to reach final scores from zero to 6. Our cutoff value was three above which was considered as positive expression and below which was considered as negative expression **(Yuan et al., 2009b).**

3. Statistical Analysis

All statistics were made by SPSS 22.0, windows (USA, SPSS Inc., Chicago) and MedCalc windows (MedCalc Software bvba 13, Ostend, Belgium).Percent of categorical variables were compared using Pearson's Chi-square test or Fisher's exact test when was appropriate. Trend of change in distribution of relative frequencies between ordinal data were compared using Chi-square test for trend. All tests were two sided. A p-value <0.05 was considered significant.

Kaplan and Meier method used to estimate overall and event free survival and log rank test compared survival curves. Overall survival (OS): was calculated as the interval between the date of diagnosis till date of death and date of last follow up or end of the study. Progression -free survival (PFS): was calculated from the treatment initiation date till the date of documented disease progression

4. Results

Sixty females patients were included in our study, with age ranged from 32-65 years (Mean ± SD: 56.35 ±10.99). The majority of our patients were postmenopausal (44 patients out of sixty). Fory nine (81.7%) patients had IDC while other histopathological types were presented in 11 (18.3%) patients.Grade III and II were the most common which were present in 23 (38.3%) and 22 (36.6%) patients. Twenty four patients (40%) had negative ER &PR receptor while 35 (58.3%) patients had negative Her2-neu expression. 61.7% of our patients had high ki 67. Luminal A molecular subtype was the most common type which was present in 25 (41.7%) patients followed by Her2-neu amplified subtype which was present in 15 (25%) patients ,while only 10 (16.7%) patients had both Luminal B and triple negative subtype. Nineteen patients (31.7%) had capsular invasion. Eighteen patients had stage III which was the most common stage while only twelve patients (20%) had stage I breast cancer .High expression of Snail -1 and Eph A2 were present in 32 (53.3%) and 30 (50%) patients respectively. Demographic data of all patients were detailed in table (1).

Table 1: Clinicopathological features, snail1& Eph A2 immunohistochemical expressions in our patients

Characteristics	*Number*	*Percent*		*Characteristics*	*Number*	*Percent*
Age (years)				T		
Mean ± SD	56.35	±10.99		T1	15	25%
Median Range	57	(32-65)		T2	23	38.3%
≤ 55 years	24	40%		T3	15	25%
> 55 years	36	60%		T4	7	11.7%
Menopause Premenopause postmenopause	 16 44	 26.6% 73.3%				
Pathological				Capsular		

type				invasion		
IDC	49	81.7%		Absent	41	68.3%
Other	11	18.3%		Present	19	31.7%
Grade				N		
Grade I	15	25%		N0	19	31.7%
Grade II	22	36.6%		N1	11	18.3%
Grade III	23	38.3%		N2	19	31.7%
				N3	11	18.3%
ER				M		
Negative	24	40%		M0	47	78.3%
Positive	36	60%		M1	13	21.7%
PR				AJCC Stage group		
Negative	24	40%		Stage I	12	20%
Positive	36	60%		Stage II	17	28.3%
HER2/neu				Stage III	18	30%
Negative	35	58.3%		Stage IV	13	21.7%
Positive	25	41.7%				
Ki-67				Snail-1		
Low	23	38.3%		Low	28	46.7%
High	37	61.7%		High	32	53.3%
ER/PR				Eph A2		
Positive/ Positive	32	53.3%		Negative	30	50%
Positive/ Negative	4	6.7%		Positive	30	50%
Negative/ Positive	4	6.7%				
Negative/ Negative	20	33.3%				
Molecular type				Snail/Eph A2		
Luminal A	25	41.7%		Low/ Negative	26	43.3%
Luminal B	10	16.7%		Low/Positive	2	3.3%
HER2 amplified	15	25%		High/ Negative	4	6.7%
Triple -ve	10	16.7%		High/ Positive	28	46.7%

SNAIL1 expression in relation to clinicopathological features (Tables 1& 2; fig 1)

Snail1 overexpression in breast carcinoma was significantly correlated with larger tumor size (p= 0.002), higher grade (P<0.03), older age of the patients, higher stage of the tumor, aggressive molecular type, presence of lymph node metastases, high KI67 index, negative ER& PR hormonal receptors, high Her2 neu expression (p<0.001 for all of them), presence of distant metastasis (p=0.011) and presence of capsular invasion (p=0.002). But it had no significant correlation with histop-athological subtype of breast cancer.

Eph A2 expression and its correlation clinic-pathological features (Tables 1& 2; fig 2)

The positive expression of Eph A2 in our breast cancer patients was significantly correlated with higher grade (P<0.03), older age of the patients, larger tumor size, higher stage of the tumor, aggressive molecular type, presence of lymph node metastases, high KI67 index, negative ER& PR hormonal receptors, high Her2 neu expression, presence of distant metastasis and presence of capsular invasion (p<0.001 for all of them). But it had no significant correlation with histo-pathological subtype of breast cancer.

The expression of Snail and Eph-A2 in patients with breast cancer was significantly positively correlated with each other (p<0.001). Snail1 over expression& Eph-A2 positive expression was associated with aggressive molecular subtype like triple negative, luminal B and her2 neu amplified (p<0.001).

Table 2: correlation between clinic-pathological features, snail1& Eph A2 immuno-histochemical expressions in our patients

	All	Snail-1			Eph A2		
	(N=60)	Low (N=28)	High (N=32)	p-value	Negative (N=30)	Positive (N=30)	p-value
Characteristics	No. (%)	No. (%)	No. (%)		No. (%)	No. (%)	
Age (years)							
Mean ± SD	56.35 ±10.99	51.60 ±9.01	60.50 ±11.01	0.002	49.56 ±6.87	63.13 ±10.17	<0.001•
Median (Range)	57 (39-87)	50 (40-76)	60 (39-87)		48.50 (39-60)	62.50 (40-87)	
≤ 55 years	24 (40%)	18 (75%)	6 (25%)	<0.001‡	21 (87.5%)	3 (12.5%)	<0.001‡
> 55 years	36 (60%)	10 (27.8%)	26 (72.2%)		9 (25%)	27 (75%)	
Menopause							
Premenopausal	16(26.6	9 (32.1%)	7(21.8	0.054	6(20)	10(33.32	0.095
Postmenopausal	44(73.3	19 (67.8%)	25(78.2		24(80)	0(66.6	
Pathological type							
IDC	49 (81.7%)	24 (49%)	25 (51%)	0.448‡	24 (49%)	25 (51%)	1.000‡
Other	11 (18.3%)	4 (36.4%)	7 (63.6%)		6 (54.5%)	5 (45.5%)	
Grade							
Grade I	15 (25%)	11 73.3%)	4 (26.6%)		10 (66.6%)	5 (33.3%)	
Grade II	22 (36.6%)	12 (54.5%)	10 (45.4%)	<0.03	14 (63.6%)	8 (36.3%)	P<0.03
Grade III	23 (38.3%)	5 (21.7%)	18 (78.2%)		6 (26.1%)	17 (73.9%)	
ER							
Negative	24 (40%)	1 (4.2%)	23 (95.8%)	<0.001‡	2 (8.3%)	22 (91.7%)	<0.001‡
Positive	36 (60%)	27 (75%)	9 (25%)		28 (77.8%)	8 (22.2%)	

ER/PR							
Positive/Positive	32 (53.3%)	27 (84.4%)	5 (15.6%)	<0.001§	28 (87.5%)	4 (12.5%)	<0.001§
Positive/Negative	4 (6.7%)	0 (0%)	4 (100%)		0 (0%)	4 (100%)	
Negative/Positive	4 (6.7%)	0 (0%)	4 (100%)		0 (0%)	4 (100%)	
Negative/Negative	20 (33.3%)	1 (5%)	19 (95%)		2 (10%)	18 (90%)	
HER2/neu							
Negative	35 (58.3%)	27 (77.1%)	8 (22.9%)	<0.001‡	27 (77.1%)	8 (22.9%)	<0.001‡
Positive	25 (41.7%)	1 (4%)	24 (96%)		3 (12%)	22 (88%)	
Ki-67							
Low	23 (38.3%)	20 (87%)	3 (13%)	<0.001‡	20 (87%)	3 (13%)	<0.001‡
High	37 (61.7%)	8 (21.6%)	29 (78.4%)		10 (27%)	27 (73%)	
Molecular type							
Luminal A	25 (41.7%)	25 (100%)	0 (0%)	<0.001‡	25 (100%)	0 (0%)	<0.001‡
Luminal B	10 (16.7%)	1 (10%)	9 (90%)		3 (30%)	7 (70%)	
HER2 amplified	15 (25%)	0 (0%)	15 (100%)		0 (0%)	15 (100%)	
Triple –ve	10 (16.7%)	2 (20%)	8 (80%)		2 (20%)	8 (80%)	
T							
T1	15 (25%)	9 (60%)	6 (40%)	0.002§	12 (80%)	3 (20%)	<0.001§
T2	23 (38.3%)	15 (65.2%)	8 (34.8%)		14 (60.9%)	9 (39.1%)	
T3	15 (25%)	4 (26.7%)	11 (73.3%)		4 (26.7%)	11 (73.3%)	
T4	7 (11.7%)	0 (0%)	7 (100%)		0 (0%)	7 (100%)	
N							
N0	19 (31.7%)	16 (84.2%)	3 (15.8%)	<0.001§	18 (94.7%)	1 (5.3%)	<0.001§
N1	11 (18.3%)	6 (54.5%)	5 (45.5%)		6 (54.5%)	5 (45.5%)	
N2	19 (31.7%)	6 (31.6%)	13 (68.4%)		6 (31.6%)	13 (68.4%)	
N3	11 (18.3%)	0 (0%)	11 (100%)		0 (0%)	11 (100%)	
Capsular invasion							
Absent	41 (68.3%)	25 (89.3%)	16 (50%)	<0.002	28 (93.3%)	13 (43.3%)	<0.001‡
Present	19 (31.7%)	3 (10.7%)	16 (50%)		2 (6.6%)	17 (56.6%)	
M							
M0	47 (78.3%)	26 (55.3%)	21 (44.7%)	0.011‡	30 (63.8%)	17 (36.2%)	<0.001‡
M1	13 (21.7%)	2 (15.4%)	11 (48.6%)		0 (0%)	13 (100%)	
AJCC Stage group							
Stage I	12 (20%)	9 (75%)	3 (25%)	<0.001§	11 (91.7%)	1 (8.3%)	<0.001§
Stage II	17 (28.3%)	13 (76.5%)	4 (23.5%)		14 (82.4%)	3 (17.6%)	
Stage III	18 (30%)	4 (22.2%)	14 (77.8%)		5 (27.8%)	13 (72.2%)	
Stage IV	13 (21.7%)	2 (15.4%)	11 (84.6%)		0 (0%)	13 (100%)	
Snail-1							
Low	28 (46.7%)				26 (92.9%)	2 (7.1%)	<0.001‡
High	32 (53.3%)				4 (12.5%)	28 (87.5%)	
Eph A2							
Negative	30 (50%)	26 (86.7%)	4 (13.3%)	<0.001‡			
Positive	30 (50%)	2 (6.7%)	28 (93.3%)				

Survival and patient's outcome and their relation to the markers (Table 3, figure 3& 4):-

After a follow-up period of 36 months, No patients lost during follow up period .13.3 % of patients died (8/60 patients). The 3-year overall survival rate was 86.5% with a mean of 35.2 ± 0.22 months (95% CI; 35.2 – 36.1 months) while the median OS was not reached. There was 20% of patients [12/60 patients] developed progression of the disease. There was a significant correlation between the progression and Snail-1 (P<0.02) .The 3-year PFS rate was 79.4% with a mean of 33.9± 0.63 months (95% CI; 32.7-35.2 months); however the median PFS was not reached. The 3-y PFS had significant correlation with Snail-1 (P<0.025) and the combination between both markers (P<0.001). There was no significant correlation between 3y OS and each one of both markers but the high significance appeared between it and the combination of the two markers (P<0.001).

There was a significant correlation between the progression and Snail-1 (P<0.02) There is no significant correlation between progression free survival and Eph A2 expression but there were highly significant correlations between both markers expression and overall survival and progression free survival(P<0.001).

Table 3: Correlation between snail1& Eph A2 immunohistochemical expressions, survival and patients,s outcome:-

Variable		3-year overall survival Rate (%)	p-value	3-year PFS Rate (%)	P-value	Progression No(%)	P-value	Mortality No(%)	P-value
Snail-1	Low (28 pts)	92.9%	0.185	92.9%	0.025	2(7.1%)	0.02	2(7.1%)	0.18
	High (32 pts)	80.8%		68.1%		10(31.1%)		6(18.6%)	
Eph A2	Negative(30pts)	93.3%	0.134	86.7%	0.188	4(13.3%)	0.17	2(6.6%)	0.13
	Positive (30pts)	79.5%		72.8%		8(26.6%)		6(19.8%)	
Snail-1/	Low/Negative (26 pts)	100%	<0.001	100%	<0.001	0(0%)	<0.001	0(0%)	<0.001
Eph A2	Low/Positive (2pts)	50%		50%		2(100%)		2(100%)	
	High/Negative (4pts)	50%		25%		4(100%)		2(50%)	
	High/Positive (28 pts)	85.3%		78%		6(21.3%)		4(14.2%)	

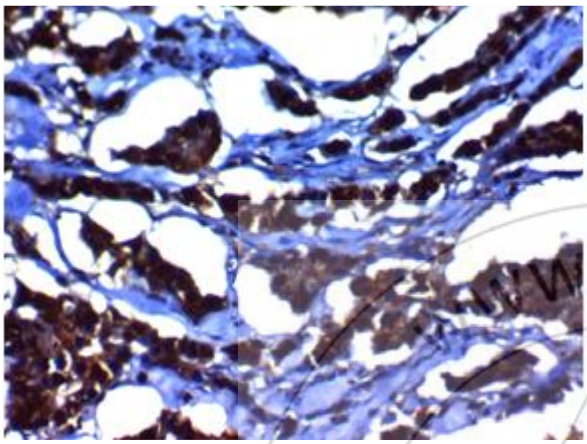


Figure1 A

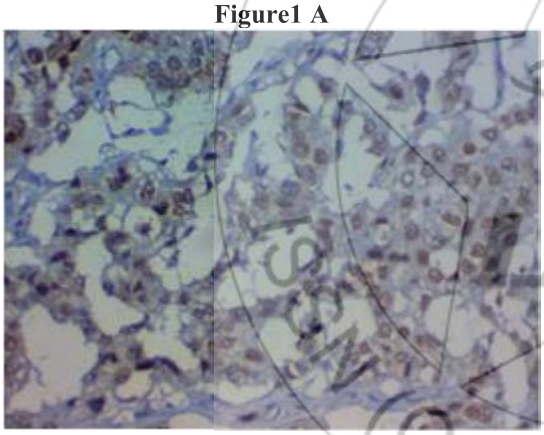


Figure1B

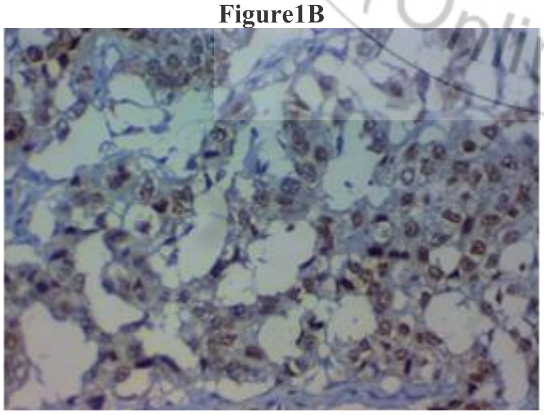


Figure1 C

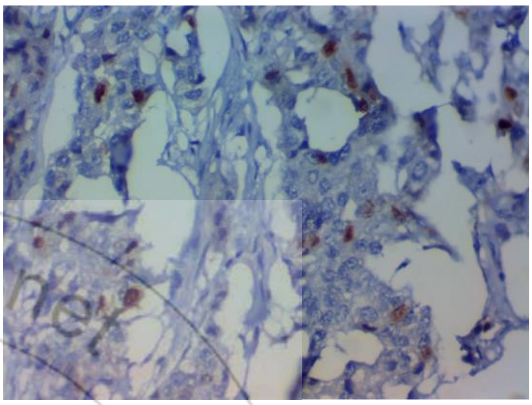


Figure1 D

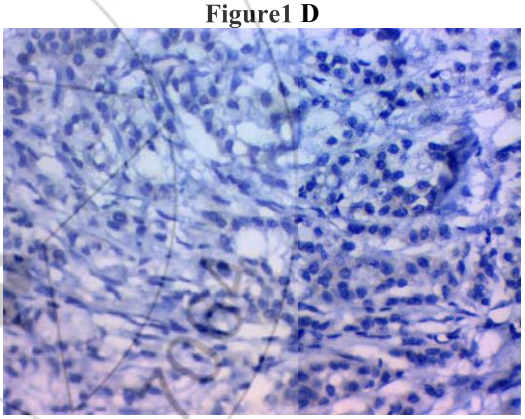


Figure1E

Figure 1: Immunohistochemical expepression of Snail-1 in carcinoma of the breast: (A) High expression in the nucleus of high grade infiltrating lobular carcinoma of the breast stage IV x400. (B) High expression in the nucleus of high grade infiltrating duct carcinoma of the breast stage IV x400 ;((C) High expression in the nucleus of high grade infiltrating duct carcinoma of the breast stage III x400. D) Low expression in the nucleus of low grade infiltrating duct carcinoma of the breast stage I x400. E) Low expression in the nucleus of low grade infiltrating lobular carcinoma of the breast stage II x400.

Note: High Snail-1immunohistochemical expression in high grade& stage carcinoma of the breast and low expression in low grade& stage carcinoma of the breast.

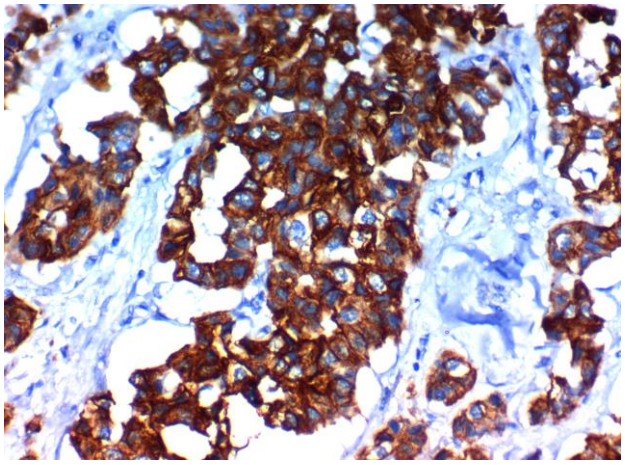


Figure 2A

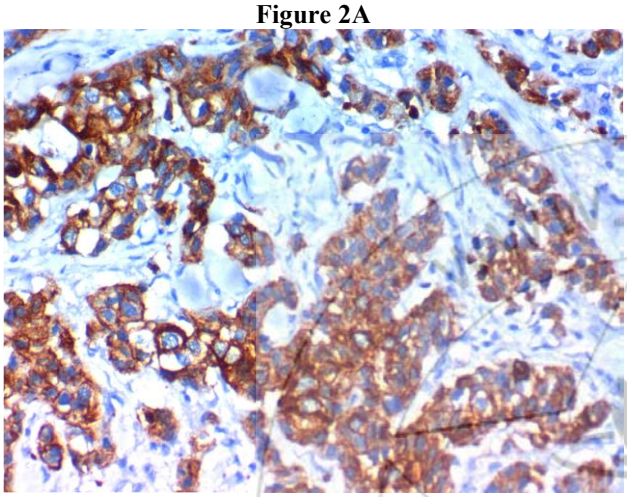
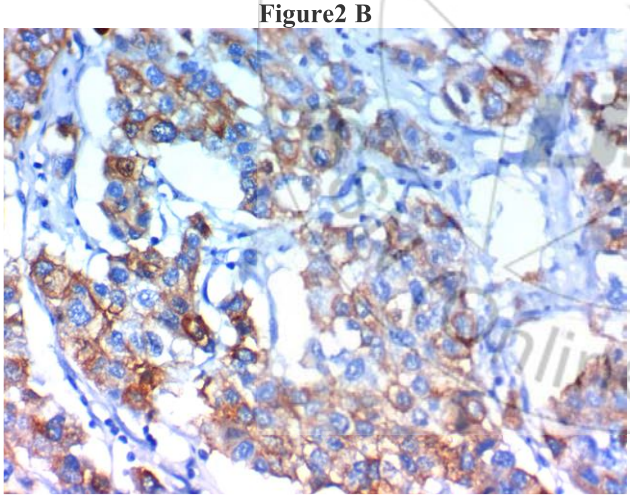


Figure2 B



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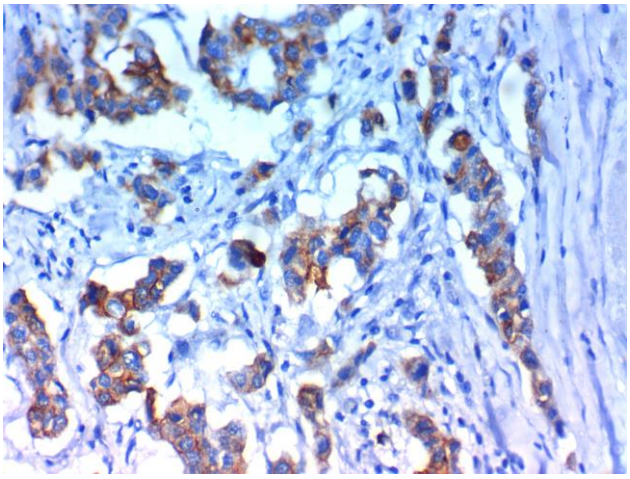


Figure2 D

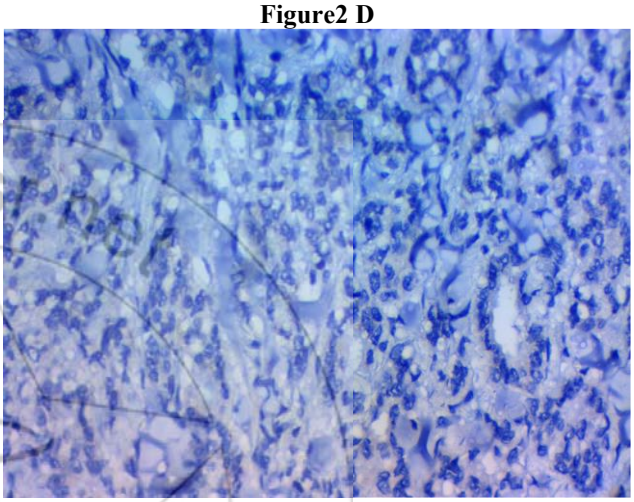
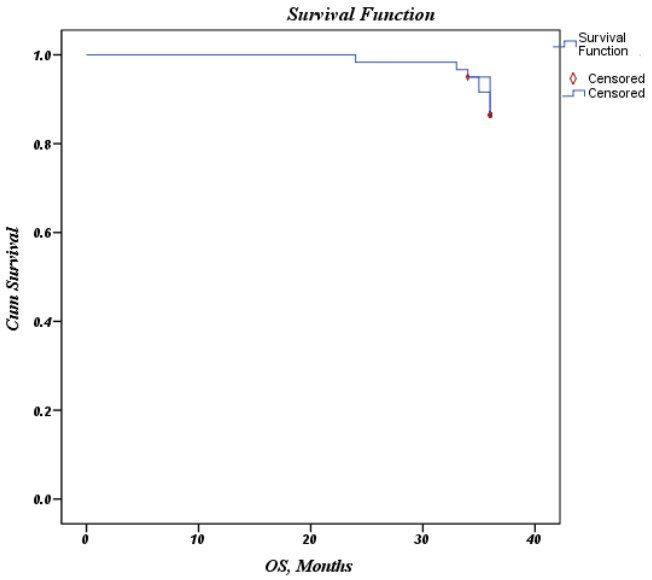


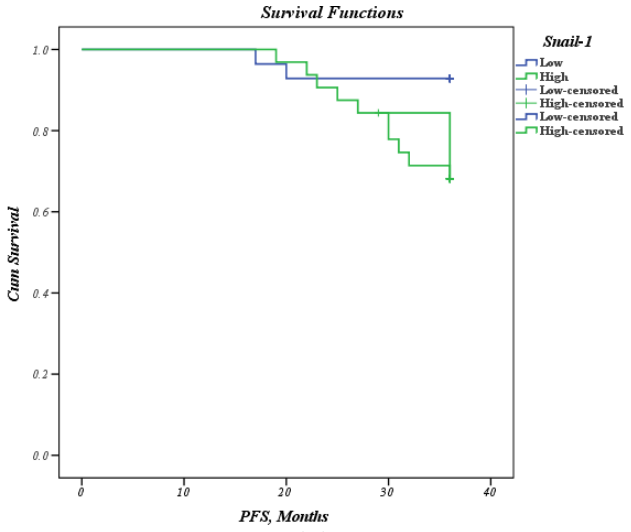
Figure 2 E

Figure 2: Immunohistochemical staining of Eph-A2 in carcinoma of the breast: (A) High expression in the cytoplasm of High grade infiltrating duct carcinoma of the breast stage IV x400. (A) High expression in the cytoplasm of high grade infiltrating duct carcinoma of the breast stage III x400 (C) Low expression in the cytoplasm of Low grade infiltrating duct carcinoma of the breast x400;((D) Low expression in the cytoplasm of Low grade infiltrating duct carcinoma of the breast stage IIx400. (E) Low expression in the cytoplasm of Low grade infiltrating duct carcinoma of the breast stageI x400. (E) Low expression in the cytoplasm of Low grade infiltrating lobular carcinoma of the breast x400.

Note: High Eph A2 immunohistochemical expression in high grade& stage carcinoma of the breast and low expression in low grade& stage carcinoma of the breast.

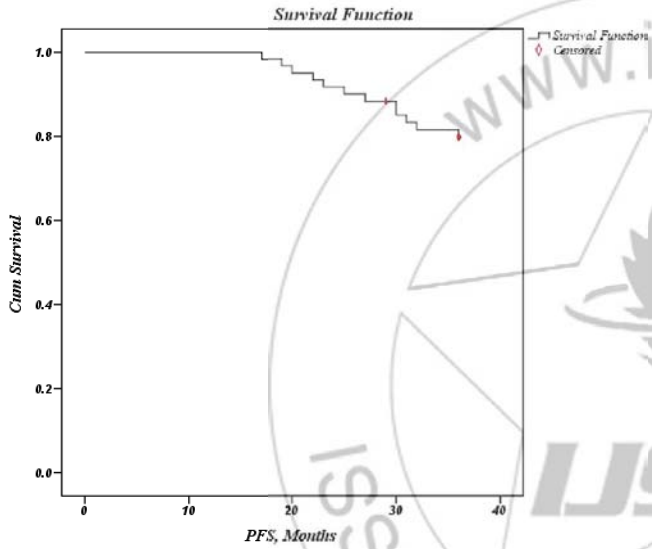


(A)

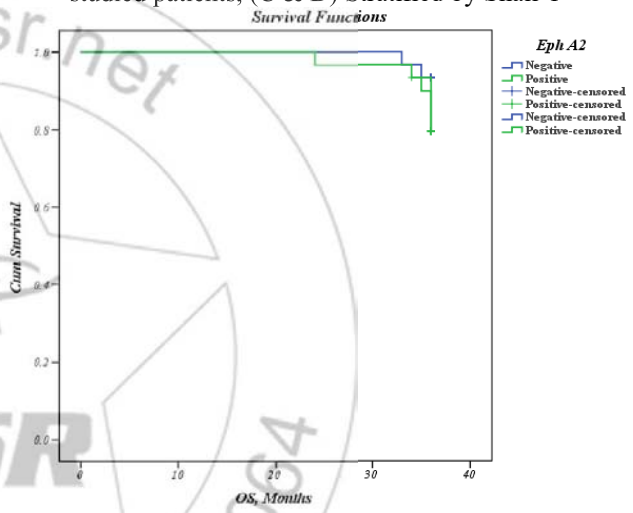


(D)

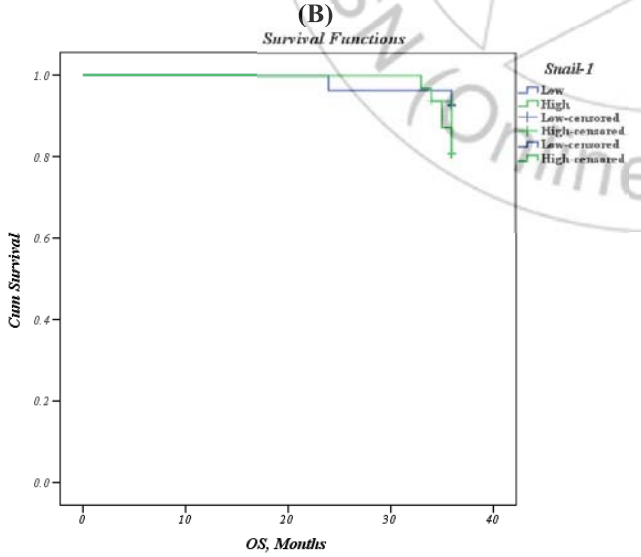
Figure 3: Kaplan Meier plot, Right panel: 3-yProgression Free Survival, Left panel: 3-y Overall Survival; (A & B) All studied patients, (C & D) Stratified by Snail-1



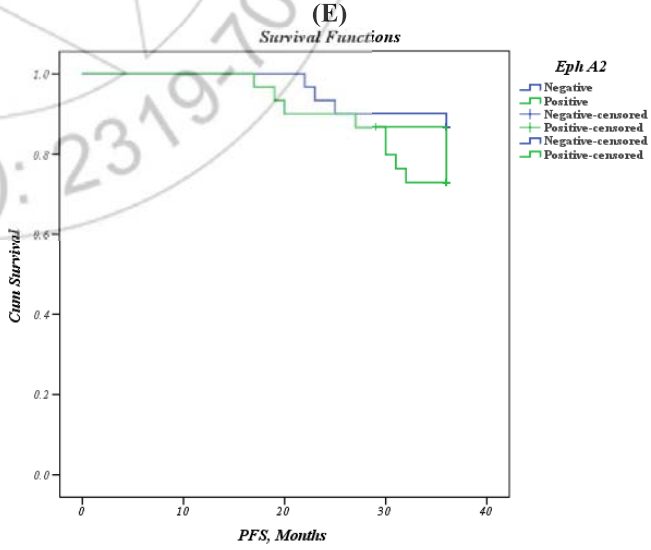
(B)



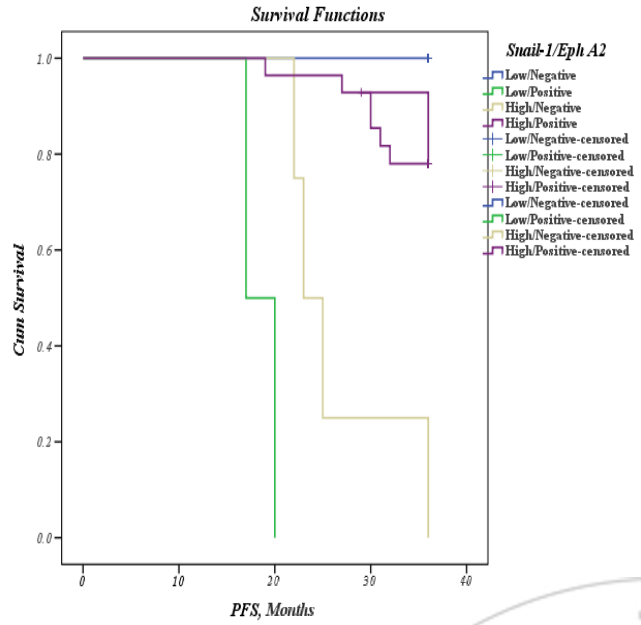
(E)



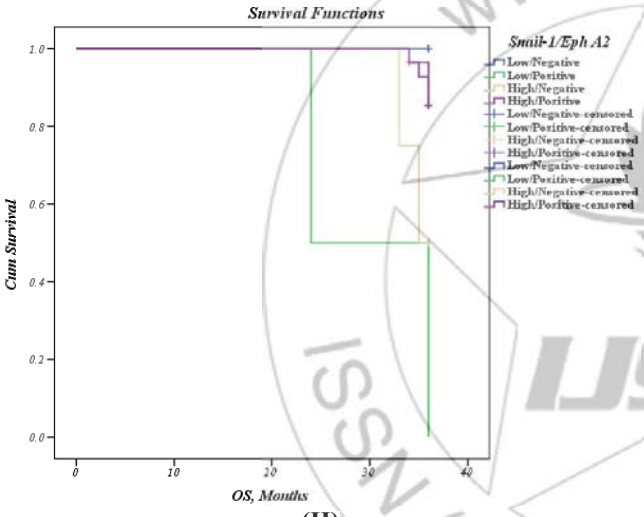
(C)



(F)



(G)



(H)

Figure 4: Kaplan Meier plot, Right panel: Progression Free Survival, Left panel: Overall Survival; (E & F) Stratified by Eph A2, (G & H) Stratified by both Snail-1 and Eph A2

5. Discussion

There are many nuclear TFs that participated in EMT, metastasis and role in patient outcome that has been studied for many years but results of most of them were not conclusive uptill now.

In our study we detected that the snail1 overexpression in breast cancer patients was associated with older age of the patients, high grade and stage, aggressive molecular subtype of the tumor, presence of lymph node metastases, high KI67 index , capsular invasion, negative ER& PR hormonal receptors and high Her2 neu expression this is in agreement with previous results, which showed that Snail expression correlates with poor clinicopathological parameters of breast cancer (**Muenst, S.** ***et al.2013,*** **Zhang, A.** ***et al.2013*** **Mimoto, R.** ***et al,2013,*** **Abd ElMoneim and Zaghloul, 2011),** all these similar results to us pointed to that snail 1 had a role in the breast cancer progression and could be a marker of metastatic liability . In contrast to us **Logullo et al., 2010** found that there is no association between Snail and any poor prognostic factors. These contradictory results could be due to the use of different antibody clone and different technique of staining or method of stain interpretation

In our study, the 3-year overall survival rate was 86.5% with a mean of 35.2 ± 0.22 months (95% CI; 35.2 – 36.1 months). There was a significant correlation between the progression and Snail-1 (P<0.02) and combination of both markers (P<0.001). The 3-year PFS rate was 79.4% with a mean of 33.9± 0.63 months (95% CI; 32.7- 35.2 months); the 3-y PFS had significant correlation with Snail-1 (P<0.025) and the combination between both markers (P<0.001).

Also nearly similar to our results in carcinoma of the breast other studies proved that snail overexpression was associated with progression and poor prognosis in malignancies of many organs [**Chen et al., 2016).** We can explain our results by that snail 1 have been incriminated in cancer progression by E-cadherin regulation **by** interaction with proximal E-boxes of the E-cadherin promoter inhibiting its expression That transcriptional repression mechanism became one of the essential mechanisms that underlay the E-cadherin expression down-regulation during cancer development and progression (**Sugimachi et al., 2003).**

In addition we correlated snail 1expression with ER, PR hormonal receptors & HER2 neu expressions and we proved that overexpression of snail1 was associated with ER& PR hormonal receptor downregulation and HER2 neu over expression which was in agree with **Abd El-Moneim and Zaghloul 2011** that found inverse correlations between Snail 1 and ER and PR protein expression levels. Our results were matched with **Wang Y et al, 2013, who** stated that expression of Snail has been associated with cancer recurrence, chemotherapy and radiotherapy resistance. This is confirmed by **Moody SE et al., 2005**, who said that Snail is up-regulated in recurrent tumors and that the relapse-free survival is decreased in breast cancer patients with high levels of Snail. Blocking Snail functions prevent tumor cell metastasis by antagonizing EMT, invasion and metastasis, so that prevent cancer recurrence **(Harney AS et al, 2009)**. The studying Snail functions in cancer will allow detecting effective therapeutic strategies for treating metastases **(Wang Y et al, 2013)**. Snail 1transcriptional silencing causes stimulation of EMT and E-cadherin reduced expression which reduces adhesion between malignant cells and allow its spread and metastases**.** The zinc finger transcription factors such as Snail are important for the EMT in embryogenesis, during which the expression of Ecadherin is lost. The Experimental deletion of Ecadherin in cancer breast cell lines has confirmed that E-cadherin is a tumor suppressor as its down regulation increased tumor spread (**Onder et al., 2008).**

EphA2 which is a RTK family member transport extracellular signals to the cell then allow downstream Ras and phosphatidylinositol 30-kinase signaling pathways (**Menges et al., 2008).** EphA2–Ephrin-A1 signaling is a complicated pathway, so to define its accurate role in cancer is confusing. Also, EphA2 is one of the direct transcriptional-targets of Raf-MARK- pathway (**Zhang et al., 2008).**

In the present study, we found that positive expression of EphA2 was associated with poor clinicopathological criteria of the breast cancer as high level of EphA2 protein was more often detected in aged patients, larger tumor, tumors with high grade, stage, tumor with positive lymph nodes and distant metastases.

Similar results were proved by **Youngblood et al., 2016** who found that The EPHA2 RTK is overexpressed in aggressive forms of breast cancer, including the $HER2^{+}$ subtype, and correlates with poor prognosis, they explained their results by proving that dysregulation of receptor tyrosine kinases (RTK) plays a role in cellular transformation and cancer progression by disrupting key metabolic signaling pathways. Also **Huang et al., 2014** in gastric cancer who demonstrates that EphA2 overexpression has promoted carcinogenesis; malignant proliferation, invasion and metastases in stomach cancer cells and its suppression had the opposite effect. We also observed that EphA2 expression positively correlated with the EMT marker snail 1 and metastasic status of breast cancer cells which was in agreement with **Huang et al., 2014. There are many studies** found that over expression of Eph A 2 was associated with poor clinicopathological criteria and poor prognosis in different types of cancer **(Wang et al., 2015, Tatsukawa et al., (2016)**. EphA2 initiate activation of EMT in carcinogenesis and this is the pathogenesis of EphA2-mediated malignancy invasion and metastasis. That was proved by that EphA2-overexpressing cells acquire mesenchymal criteria (E-cadherin-negative) **(Huber et al., 1996).**

Youngblood M et al., 2016 observe that EphA2 elevated-expression stimulated EMT and activated the Wnt/b-catenin signaling pathway which subsequently stimulates EMT markers as snail-1 which was similar to our results. EphA2 activated RTK/Ras signaling pathways; so there is an interaction between Ras& Wnt signaling regarding the role of EphA2 in the EMT **Huang et al., 2014**.

EphA2 is an oncogene and a promising target for cancer therapies [**Tsouko et al., 2015, Guo Z et al., 2015, Charmsaz et al., 2015).** EphA2 may have variable functions as cancer suppressor or promoters [**Kaenel et al., 2012)**. It may play an oncogenic role by malignant transformation and progression. In contrast to our results that high EphA2 expression associated with poor prognosis, other reports found that high EphA2 overexpression is not associated with poor prognosis [**Holm et al., 2006**] and other researchers found that EphA2 overexpression is correlated with decreased survival, but only in univariate analyses [**Miyazaki et al., (2003**]. **Brantley-Sieders D ,et al ,2011** , found significant correlations between ephA2, and other EPh RTKs and overall survival and/or recurrence-free survival in breast cancer, inaddition, co-expression of ephrin-A1 and EphA2 protein was significantly associated with recurrence in Stage I breast cancer which is a valuable observation regarding breast cancer treatment. More recently, EphA2 was reported to cause resistance to trastuzumab therapy , and to affect estrogen dependence and tamoxifen sensitivity , in cell line and xenograft models **(Zhuang G et al ,2010 and Gokmen-Polar Y et al ,2010)** . Also, in stage I lung cancer EphA2 overexpression is related to good clinicopathologic parameters (**Ishikawa et al., 2011**). This discrepancy needed further studies on Eph A 2 expressions in cancers of different types to prove its role as a novel prognostic marker and therapeutic target of breast cancer. The drawbacks of our study are small number of patients and short follow up duration.

In summary, we proved that the EMT markers Snail 1& EphA2 enhanced the Wnt/b-catenin signaling pathway and so they had important roles in breast cancer progression, invasion and metastasis with an impact on survival, so they are excellent targets for breast cancer therapy which could decrease metastasis and kill the tumor cells.Our recommendation is, further studies have to be done on these two markers with larger number of patients and with longer follow up duration.

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