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Clinico-Pathological Significances of Snail 1& Cox2 Co-Expression in Serous Ovarian Carcinoma

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Abstract: ***Background: The aggressive phenotype and dismal prognosis of serous ovarian carcinoma (SOC) could be due to the presence of more genetic changes than in any other types of ovarian cancers. Epithelial mesenchymal transition (EMT) that became a logic explanation of cancer invasion, progression and spread is a process in which epithelial cell to cell contact loss and then a gain of mesenchymal characters occur. It is controlled by several pathways and EMT-stimulating transcription factors e.g. snail1. Snail has many essential roles in human cell proliferation, apoptosis and several steps of oncogenesis. Cyclooxygenase-2(COX-2) is an important enzyme in the production of the inflammatory mediators' prostaglandins (PGs) that had many roles in a number of biologic processes. COX-2 overexpression had been found to be a linking molecule in inflammatory changes that are associated with neoplastic changes. The role of the EMT marker Snail1, inflammatory mediators, prostaglandins and their synthesizing enzyme Cox 2 in SOC oncogenesis had not been adequately clarified. The aim of the work; to explore Snail 1& Cox2 co-expression in SOC of different grades and stages correlation their expression with clincopathological criteria of the patients. Patients and Methods: We assesses Snail 1& Cox2 co-expression by using immunohistochemistry in sections from 40 SOC paraffin blocks then correlating such expression with clincopathological parameters. Results: High expression of Snail 1 was significantly positively correlated with advanced age of the patient (p=0.01), higher grade (p=0.03), advanced FIGO stage (p<0.001), presence of L.N (p=0.005), and distant metastases (p=0.006). High expression of Cox 2 was significantly positively correlated with advanced age of the patient, higher grade,, advanced FIGO stage, presence of L.N (p<0.001) and distant metastases (p=0.005). Both Snail1 and Cox-2 were positively correlated with each other.***
Conclusion: Snail 1& Cox2 are markers of poor prognosis in SOC

Keywords: Snail 1; Cox2; SOC; immunohistochemistry

1. Introduction

Epithelial ovarian carcinoma is the 4th commonest female cancer worldwide and serous subtype forms about fifty percent of all cases, and it has the worst prognosis of all subtypes [**Konstantinopoulos and Matulonis, 2013].** The aggressive phenotype and dismal prognosis of serous ovarian carcinoma (SOC) could be due to the presence of more genetic changes than in any other types of ovarian cancers **(Taetle et al., 1999)**. Epithelial mesenchymal transition (EMT) that became a logic explanation of cancer invasion, progression and spread is a process in which epithelial cell to cell contact loss and then a gain of mesenchymal characters occur [**Thiery and Lim, 2013**]. It is controlled by several pathways **and** EMT-stimulating transcription factors e.g. snail1 **(Khan et al., 2015).** Snail is a conserved super family of TFs member that were expressed during different tissues development (**Seki et al., 2013).** It has many essential roles in human cell proliferation, apoptosis and several steps of oncogenesis (**Martin et al., 2005**). The exact pathogenesis of SOCs is not fully explained but, it was found that chronic inflammation and the inflammatory mediators could participate in SOC carcinogenesis [**Ness and Cottreau, 1999]**. Cyclooxygenase-2(COX-2) is an important enzyme in the production of the inflammatory mediators' prostaglandins (PGs) that had many roles in a number of biologic processes [**Williams and DuBois, 1996].** COX-2 overexpression had been found to be a linking molecule in inflammatory changes that are associated with neoplastic changes [**Riman et al., 2004**].The role of the EMT marker Snail1, inflammatory mediators, prostaglandins and their synthesizing enzyme Cox 2 in SOC oncogenesis had not been adequately clarified.

The aim of the work; to explore Snail **1& Cox-2** co-expression in SOC of different grades and stages correlation their expression with clincopathological criteria of the patients.

2. Patients and Methods

We assessed Snail**1& Cox2** co-expression by using immunohistochemistry in sections from 40 SOC paraffin blocks in Pathology department, Faculty of medicine, Zagazig University.

We have collected all patients' data as age, cancer size, grade and stage from the files of pathology department. For staging of SOC cases we used the TNM [tumor-node-metastasis] and FIGO [International Federation of Gynecology and Obstetrics] classifications **[Part, 2014],** and for pathological grading of SOC we used the WHO grading system **[Kurman et al., 2014].** We analyzed correlations between the levels of Snail 1& Cox2 co-expression with each other and all the clinicopathological parameters.

Immunohistochemical staining:

Streptavidine-biotin technique was used for immune-staining **[Hsu et al., 1981],** where we incubated the positively charged slides with primary rabbit poly clonal anti- snail1 antibody (clone ab180714) (Abcam, Cambridge, UK) & with COX-2-specific antihuman monoclonal antibody (160112; Cayman Chemical Co., Ann Arbor, MI) in a dilution of 1:200**.** Sections from breast and SOC were used as positive controls for Snail-1 and cox 2 respectively

Evaluation of immunohistochemical expression of SNAIL-1 & COX-2:-

We have considered only nuclear staining as positive for Snail 1 and cytoplasmic stain as positive for COX-2; we evaluated staining by calculating stained cells extent and stain intensity. Intensity of the stain was scored as; 0 (negative), 1 (weakly positive), 2(moderately positive) and 3 (strongly positive). The stain extent was scored as: 0 (negative), 1 (till 25 percent of positive cells), 2 (26 to 50 percent), 3 (51to 75 percent) and 4 (76 percent). We calculated the final staining scores by multiplying the stain intensity and the extent, scores; 0 (negative), + (one to four), ++ (five to eight) and +++ (nine to twelve) **(Zhang et al., 2010, Erkinheimo et al., 2004). We used the** cutoff value 5, above which is considered high expression, equal or below which was considered low expression.

3. Statistical Analysis

We performed the statistics by using SPSS 22.0 (SPSS Inc., Chicago, IL, USA) and (MedCalc Software 13, Belgium). The percent of categorical variables had been compared using Chi-square or Fisher's exact tests when were appropriate. Independent sample Student's t-test was used to compare between two groups of variables. Kruskal Wallis H test was used to compare between more than two groups of non-normally distributed variables. A p-value <0.05 is considered significant.

4. Results

Patient Characteristics
The clinical characteristics of the 40 patients with SOC that were included in our study are summarized in **Table (1)** with age ranged from (30-77) years& the Mean age is: 58.48 ± 12.97 years. 25 cases have high grade and 15 cases with low grade SOC distant metastases are present in 10 of our cases.

Immunohistochemical results
Snail 1 results Table 2, Fig 1
High expression of **Snail 1** was detected in 28 out of 40 (70%) cases of SOC and it was significantly positively correlated with advanced age of the patient (p=0.01), higher grade(p=0.03),, advanced FIGO stage (p<0.001), presence of L.N (p=0.005), and distant metastases (p=0.006),

Cox 2 expression Table 3, Fig 2
High expression f **Cox 2** was detected in 30 out of 50 (75%) cases of SOC and it was significantly positively correlated with advanced age of the patient, higher grade,, advanced FIGO stage, presence of L.N (p<0.001) and distant metastases (p=0.005),
Expression of **Snail 1** was significantly positively correlated with that of **Cox 2**. r (correlation coefficient) +0.438(p= 0.002).

5. Discussion

SOC cells could invade and spread in the pelvic cavity that is the cause of high fatality of such type of cancer more than any gynecological cancer [**Xu et al., 2014**]. The process of EMT occurs early in the course of malignant invasion and spread [**Thietry et al., 2013**]. The induction of EMT is caused by many internal and external criteria of cancer cells and needs many molecules involving many signaling pathways and transcription factors that have participated. Snail is an important transcription factor and a regulator in EMT occurrence. Snail has become a point of the cancer research to detect its role in invasion and spread of SOC cells and whether the process of EMT occurs in such type of carcinoma.

We found in this study that high expression of **Snail 1** was significantly positively correlated with higher grade, advanced FIGO stage, presence of L.N and distant metastases. that was similar to results of **Wang et al., 2015** who demonstrated that the expression levels of Snail 1, in FIGO III and IV were higher than that in FIGO stages I and II, and result of **Jin et al., 2009** who demonstrated that the Snail expression was significantly correlated with the advanced stage and metastatic liability in SOC. Our results demonstrated many novel roles of Snail in controlling SOC metastases, which could be explained by that Snail regulates many genes that were associated with metastasis e.g. MMPs. MMPs activity is essential for Snail related SOC metastasis. Malignant cells ability to invade and metastasize needed tissue architecture disruption these events required many extracellular proteinases e.g. MMPs, for this purpose. Snail which is a potent mediator of EMT could control the MMPs proteolytic activity that contributed to phenotypic aberrations that are associated with EMT related [**Zha et al., 2007, Sun et al., 2008].** Another explanation of how Snail control SOC growth, invasion and spread is that it could suppress the expression of E-cadherin which is a cell adhesion molecule which subsequently decreased cell to cell contact, increased malignant invasion and spread [**Yang et al., 2004**] Interestingly, **Yu-Lou Wang et al., 2015** found that knockdown of Snail expression not only suppressed ovarian cancer metastasis but also inhibited primary tumor growth, as Snail, in addition to inducing EMT, also functions as a survival factor that can block cancer cells from undergoing apoptosis. So, as our results proved that Snail plays many roles in SOC proliferation, invasion and spread, discovering new therapeutic targets that could block its action could decrease ovarian cancer invasion and spread which subsequently improve the dismal outcome of such type of cancer. We show that both Snail expression level and nuclear localization could predict SOC patients' prognosis that was similar to results of **Wang et al., 2015**. Also, Snail contributed to the acquisition of stem like features in ovarian carcinoma cells [**Kurrey et al., 2009**]. And other previous studies had found that Snail was highly expressed in advanced stage SOC [**Yoshida, et al., 2009 and Jin et al., 2010**]. Snail overexpression was related to poor clinicopathological criteria, progression and dismal outcome in cancers of many organs, that was nearly similar to our results in SOC [**Chen et al., 2016).**

Our results were slightly different from results of **Kim, et al, 2014** that, demonstrated that Snail was highly expressed in the serous carcinomas, but they found no predictive or prognostic relations between Snail expression and clinicopatholgical criteria. In addition, they found that Snail

high expression in the early stage SOCs proved the important role of Snail in carcinogenesis early phase.

Moreover, some reserchers had detected cytoplasmic rather than nuclear staining for Snail which is considered to be an active form [**Yoshida, et al., 2009, H. Jin, et al., 2010**]. These discrepant results could be due to the different antibodies clone used and different method of evaluation of immune-staining. Our finding of diffuse strong nuclear staining of Snail is similar to **Wang et al., 2015** study which demonstrated that Snail mRNA and its nuclear protein expression were found in all primary ovarian carcinoma cells.

There are many contradictory results regarding, inflammatory mediators' prostaglandins and their forming enzyme COX-2, role in SOC pathogenesis. **Yoshida et al., 2009** suggested that SOC proliferative activity is unrelated to expression of COX-2, however, **Gu et al., 2008& Bijman et al., 2008** have detected essential roles of COX-2 in SOC carcinogenesis**. So** we decided to explore the expression of SOX-2 protein in SOC and then explore its clinicopathological significance to detect the possible roles of COX- 2 in the pathogenesis of such type of cancer.

We found in this study that high expression of COX-2 was significantly positively correlated with higher grade, advanced FIGO stage, presence of L.N and distant metastases that was similar to results of **Shahab Uddin, et al., 2009 and Erkinheimo et al., 2004** who proved the same in ovarian cancer. The importance of our study regarding the role of COX 2 expression in SOC and relation to higher grade and advanced stage allow the possible use of COX-2 inhibitors for prevention and/or treatment of SOC, which was in line with results of several studies that have found that COX-2 inhibitors could be used as therapeutic agents during the course of management of several malignancies [**Sun et al., 2008 and Alam et al., 2007]**. Also many previous studies had found that usage of aspirin and other NSAIDs regularly could inhibit cancer colon development. In addition, **Bertagnolli et al., 2006** have stated that celecoxib which is a COX-2 inhibitor could suppress colon adenomas development**.** The underlying molecular mechanisms of that anticancer role are still not fully understood. COX-2 inhibition could induce apoptosis by pAKT inactivation, resulting eventually in apoptosis of cancer cells. So that COX-2 might play a role in cancer growth by regulating PI3K/AKT oncogenic pathways on which malignant cells depend for growth, invasion and survival. In line with all these previous reports **Shahab Uddin, et al., 2009** results have supported that COX-2 inhibition might have therapeutic role in SOC and, COX-2 over expression had been found in ovarian cancer [**Denkert et al., 2002 and Shigemasa, et al., 2003].** Prostaglandin E2 (PGE2), that is the main COX-2 metabolite, had been found to stimulate cancer cell survival, invasion, proliferation, and inhibit apoptosis, that's all could influence cancer development **[Gierse et al., 1995].** Another role of COX-2 in SOC is that its overexpression was linking factor of chronic inflammation that was detected with neoplastic changes [**Colby et al., 2008]**.A high variation of levels of COX-2 expression among different studies, which may depend on the use of variable antibodies clones, different scoring methods, and variable number of patients. Similarly to our data, **Denkert et al.2002)** reported recently that COX-2 over expression is a marker of poor prognosis invasive ovarian carcinoma patients of different histopathological subtypes but they failed to prove any clinicopathological correlation between such expression and tumor grade. **Shahab Uddin et al., 2009** found similar results to us that found highly significant relations between COX-2 expression and high histological grade of ovarian carcinoma. As there are no single accepted grading system for ovarian cancer as the same characters are not applied to all histological types, many studies have discordant results from us. We used the FIGO grading system, that is the most widely accepted and that has been found to be a powerful patient outcome determinant, especially in SOC (**Friedlander, 1998**).

Our results are slightly different from results of **Magnowska et al., 2014** who found that COX-2 over-expression was found in well differentiated low grade ovarian cancer, was not associated with advanced age of the patient, CA125 level or any special histological type. But they found that **COX**-2 over-expression was an unfavorable prognostic marker for overall survival and progression-free survival rates.

In our study we found that the expression of the EMT marker **Snail 1** was significantly positively correlated with that of inflammation related enzyme **Cox 2**, it was the first study that could find a relation between EMT and chronic inflammation in SOC.

Summary, we have provided recent insights into Snail 1 role and regulation in SOC metastasis, and have demonstrated that Snail 1 is critical for SOC growth, differentiation and metastasis, also we have spotted light on the role of COX-2 expression in SOC progression, invasion and spread, the functional importance of Snail 1& COX-2 expressions in SOC suggests that their inhibition may serve as therapeutic targets for better SOC managing.

In conclusion, we have detected the correlation between Snail & COX-2 roles in differentiation, invasion and metastasis of SOC, epithelial-mesenchymal transition and chronic inflammation based on detection of protein expression levels, and also we have explored the molecular mechanism of EMT and inflammatory processes mediated by Snail& COX-2. But, our study have some limitations, as small number of patients, inclusion of only SOC in our study and studying protein expression of both markers by immunohistochemistry only. So we recommend to do further researches on of patients with different types of ovarian carcinoma, expansion of the sample size and studying levels of markers by other methods like DNA microarray are needed to reach more accurate results about the roles of both markers in ovarian cancer progression and spread.

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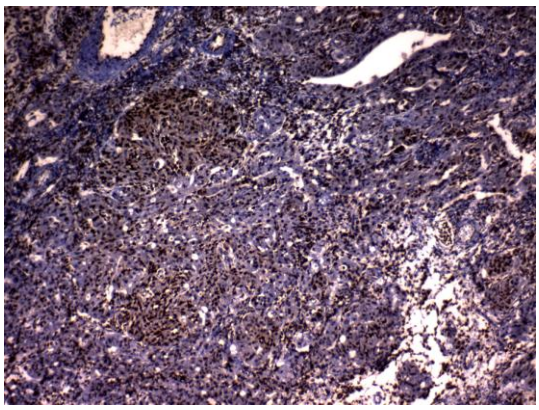


Figure 1 A

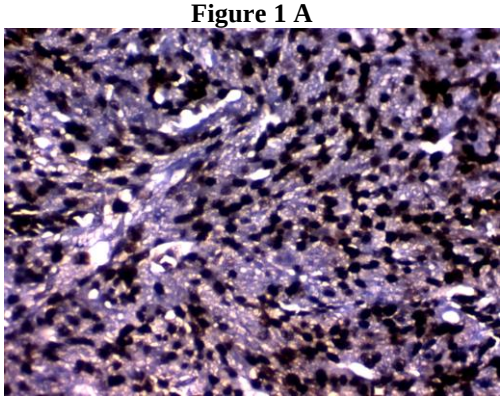


Figure 1 B

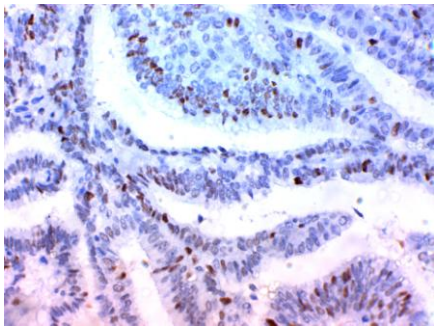


Figure 1 C

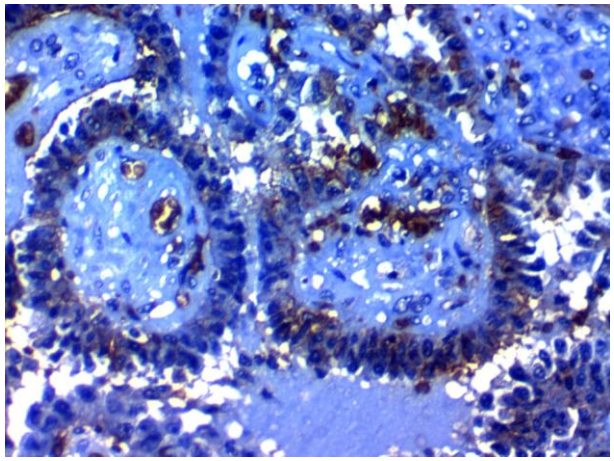


Figure 1 D

Figure1. Immunohistochemical staining of Snail 1 in serous ovarian carcinoma (SOC) :(A) High expression in the nucleus of SOC high grade x100 (B) High expression in the nucleus of SOC high grade x400. (C) Low expression in the nucleus of SOC low grade x400. (D) Negative expression in the nucleus of SOC low grade x400.

Note: High Snail 1immunohistochemical expression in high grade SOC and low expression in low grade SOC: A the original magnification was x100 B, C& D the original magnification was ×400

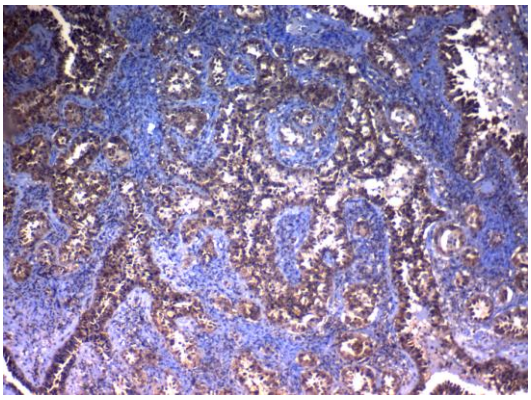
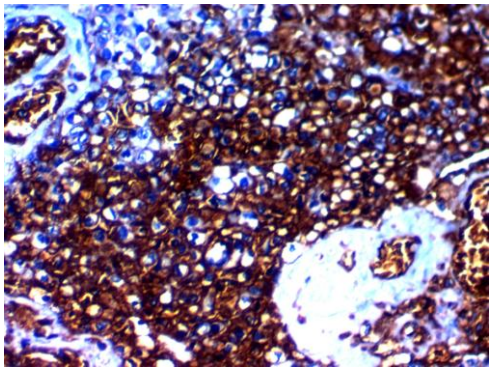


Figure 2 A



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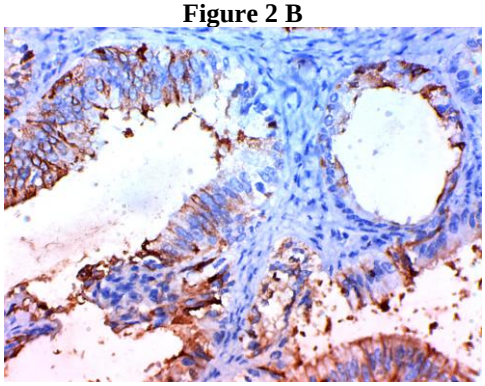

Figure 2 C

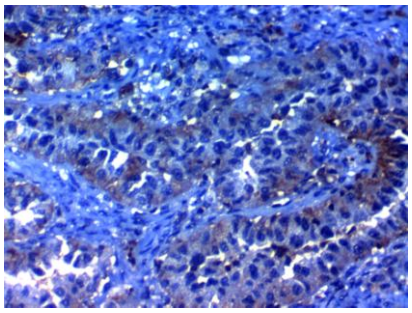

Figure 1 D

Figure2. Immunohistochemical staining of Cox-2 in serous ovarian carcinoma (SOC) :(A) High expression in the cytoplasm of SOC high grade x100 (B) High expression in the cytoplasm of SOC high grade x400. (C& D) Low expression in the cytoplasm of SOC low grade x400.

Note: High Cox2 immunohistochemical expression in high grade SOC and low expression in low grade SOC: A the original magnification wasx100; B, C& D the original magnification was ×400

Tables

Table 1: Demographic data of our patients

Characteristics	Number	Percentage (%)
Age (year)		
Mean ± SD	58.48 ± 12.97	
Median (Range)	60 (30 – 77)	
≤40 years	4	10%
41-49 years	20	50%
> 60 years	16	40%
FIGO stage		
Stage IA	1	2.5%
Stage IB	2	5%
Stage IC	2	5%
Stage IIB	3	7.5%
Stage IIC	4	10%
Stage IIIA	9	8%
Stage IIIB	5	12.5%
Stage IIIC	4	10%
Stage IV	10	20%
Grade		
Low grade	15	37.5%
High grade	25	62.5%
Lymph node		
Negative	12	30%
Positive	28	70%
Distant Metastasis		
Absent	30	75%
Present	10	25%

Continuous variables were expressed as mean ± SD & median (range); categorical variables were expressed as number (percentage).

Table 2: correlation between clinicopathological features and Snail 1 expression in our patients

Characteristics	All (N=40)		Snail 1 Low (N=12)		Snail 1 High (N=28)		p-value
	No.	(%)	No.	(%)	No.	(%)	
Age (years)							
Mean ± SD	58.48	±12.97	48.93	±10.47	55.97	±8.01	<0.01*
Median (Range)	60	(30-77)	43.5	(25-60)	59	(45-75)	
≤40 years	4	-10%	4	-100%	0	0%	0.01‡
41-49 years	20	-50%	7	-35%	13	-65%	
> 60 years	16	-40%	1	-6.25%	15	-93.75%	
Grade							
Low grade	15	-37.50%	9	-60%	6	-409%	0.03‡
High grade	25	-62.50%	3	-12%	22	-88%	
FIGO stage							
Stage IA	1	-2.50%	1	-100%	0	0%	<0.001§
Stage IB	2	-5%	2	-100%	0	0%	
Stage IC	2	-5%	2	-100%	0	0%	
Stage IIB	3	-7.50%	1	-33.30%	2	-66.70%	
Stage IIC	4	-10%	1	-25%	3	-75%	
Stage IIIA	9	-8%	0	0%	9	-100%	

Stage IIIB	5	-12.50%	4	-80%	1	-20%	
Stage IIIC	4	-10%	1	-25%	3	-75%	
Stage IV	10	-25%	0	0%	10	-100%	
Lymph node							
Negative	12	-30%	9	-75%	3	-25%	0.005‡
Positive	28	-70%	3	-10.70%	25	-89.20%	
Distant Metastasis							
Absent	30	-75%	12	-40%	18	-60%	0.006‡
Present	10	-25%	0	0%	10	-100%	
Cox2							
Low grade	15	-42%	9	-60%	3	-40%	0.002‡
High grade	25	-58%	0	0%	25	-100%	

Categorical variables were expressed as number (percentage), continuous variables were expressed as mean ± SD & median (range).
• Mann Whitney U test; ‡ Chi-square test; p<0.05 is significant.

Table 3: correlation between clinicopathological features and Cox 2 expression in our patients

Characteristics	All (N=50)		p-value: Low (N=10)		p-value: High (N=30)		p-value
	No.	(%)	No.	(%)	No.	(%)	
Age (years)							
Mean ± SD	58.48	±12.97	48.93	±11.47	56.97	±8.01	<0.001*
Median (Range)	60	(30-77)	46.5	(28-60)	57	(45-75)	
≤40 years	4	-10%	2	-50%	2	-50%	0.001‡
41-49 years	20	-50%	6	-30%	14	-70%	
> 60 years	16	-40%	2	-12.50%	14	-87.50%	
Grade							
Low grade	15	-37.50%	8	-53.30%	7	-46.70%	0.001‡
High grade	25	-62.50%	2	-8%	23	-92%	
FIGO stage							
Stage IA	1	-2.50%	1	-100%	0	0%	<0.001§
Stage IB	2	-5%	2	-100%	0	0%	
Stage IC	2	-5%	2	-100%	0	0%	
Stage IIB	3	-7.50%	2	-66.70%	1	-33.30%	
Stage IIC	4	-10%	1	-25%	3	-75%	
Stage IIIA	9	-8%	0	0%	9	-100%	
Stage IIIB	5	-12.50%	1	-20%	4	-80%	
Stage IIIC	4	-10%	1	-25%	3	-75%	
Stage IV	10	-25%	0	0%	10	-100%	
Lymph node							
Negative	12	-30%	8	-66.70%	4	-33.30%	<0.001‡
Positive	28	-70%	2	-7%	26	-93%	
Distant Metastasis							
Absent	30	-75%	10	-30%	20	-70%	0.005‡
Present	10	-25%	0	0%	10	-100%	
Snail 1							
Low grade	15	-42%	9	-60%	3	-40%	0.002‡
High grade	25	-58%	0	0%	25	-100%	

Categorical variables were expressed as number (percentage), continuous variables were expressed as mean ± SD & median (range).
• Mann Whitney U test; ‡ Chi-square test; p<0.05 is significant.

Table 4: Association & correlation between Snail-1, cox 2 and study parameters in our patients

	Snail-1 (low, high)		cox 2 (low, high)		Snail-1 / cox 2 (low/low……..high/high)	
	r	p-value	r	p-value	r	p-value
Age (years)	+0.502	<0.001	+0.601	<0.001	+0.718	<0.001
FIGO stage (IA, ……,IV)	+0.473	<0.001	+0.688	<0.001	+0.701	<0.001
Grade (low, high)	+0.367	0.001	+0.628	<0.001	+0.544	<0.001
LN (negative, positive)	+0.440	<0.001	+0.546	<0.001	+0.563	<0.001
DM (negative, positive)	+0.370	0.006	+0.478	0.001	+0.653	<0.001
Snail-1 (low, high)	---	---	+0.438	+0.002	---	---
cox 2 (low, high)	+0.438	0.002	---	---	---	---

r correleation coefficient; p<0.05 is significant.