RESEARCH ARTICLE

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Clinicopathologic Impact of NANOG, ZEB1, and EpCAM Biomarkers on Prognosis of Serous Ovarian Carcinoma

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Abstract

Objectives: Serous ovarian carcinoma (SOC) is a biologically heterogeneous with different genomic and molecular profiles, beside clinical response to the chemotherapy with subsequent in obstacles in starting unified, acceptable treatments and so we assess immunoexpression of Nanog, ZEB1, and EpCAM in SOC. **Methods:** In this study, the immunoexpression of Nanog, ZEB1, and EpCAM was studied in 60 cases of SOC. Overall survival (OS), disease-free survival (DFS) data and response to chemotherapy were analyzed. **Results:** NANOG was immunostained in 65% of the cases with a significant association with tumor grade, lymph node metastasis, and FIGO stage ($p < 0.001$ for each). ZEB1 showed moderate- high expression in 58.3% of the cases with significant up-regulation of ZEB1 expression with SOC grade, nodal metastasis, and SOC FIGO stage ($p<0.001$). EpCAM revealed high expression in 60% of the cases with significant association with higher grade, nodal metastasis, and advanced stage ($p < 0.001$ for each). Up-regulation of Nanog was significantly associated with response to chemotherapy, relapse, shorter OS and DFS ($p < 0.001$ for each). ZEB1 overexpression exhibited a significant association with response to chemotherapy ($p= 0.012$), relapse, shorter OS and DFS ($p<0.001$ for each). Moreover, the high EpCAM had a significant association with response to chemotherapy ($p= 0.043$), relapse ($p < 0.001$) shorter OS ($p=0.006$) and DFS ($p< 0.001$). **Conclusions:** Up-regulation of Nanog and ZEB-1 and EpCAM perhaps promote an aggressive SOC with a high risk of relapse and unfavorable response to standard chemotherapy regimen.

Keywords: Serous ovarian carcinoma- Nanog- ZEB-1- EpCAM- Prognosis

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Introduction

Ovarian cancer is the most fatal gynecologic tumor. Epithelial ovarian carcinoma (EOC) is the most common pathological subtype. EOCs are divided into low-grade serous carcinomas (LGSC) and high-grade serous carcinoma (HGSC), mucinous carcinoma, clear-cell carcinoma, and endometrioid carcinomas where SOC is the most frequent type (Farrag et al., 2021). Cytoreductive debulking, and platinum-based chemotherapy are the basic therapeutic regimens in the advanced stages. The main goal of surgical treatment is tumor debulking due to the diffuse nature of tumor and the invasion of the surrounding organs or peritoneal cavity via the peritoneal fluid. Although most tumors initially respond to the primary chemotherapy, chemo-resistant recurrent tumors are the leading cause of death in most patients (Torre et al., 2018). Pathogenesis of SOC recurrence is blurred but expected to involve a fairly undifferentiated tumor-initiating cells (TICs) that can struggle the chemotherapy and reinitiate aggressive neoplasms; It is thought that cancer stem cells (CSCs) never eradicated leading to chemotherapy resistance and their ablilty to refill their population, leading to recurrence of the neoplasm, which is usually more aggressive (Cho et al., 2019). Accordingly, innovative therapeutic drugs that eliminate the resistance to the therapy, are the key target for cancer investigations.

Epithelial–mesenchymal transition (EMT) is critical for the induction of metastasis and tumor advancement (Davidson et al., 2015). Various studies have emphasized a link between EMT and cancer stem cells (CSCs). As a fundamental stemness-associated transcription factor, Nanog (homeodomain-containing transcription factor) controls the ultimate properties of CSCs, as cell proliferation, cell cycle, EMT, self-renewal, tumorigenicity, and chemotherapy resistance (Wang et al., 2013). In SOC, metastatic foci and ovarian cancer cell lines with metastasis features have high Nanog expression

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(Siu et al., 2013). Nanog messenger RNA is detected in stem cell lines but it is not present in differentiated cells, but Nanog overexpression was also noted in germ cell tumors, beside others, including ovarian cancer (Amsterdam et al., 2013).

Zinc finger E-box binding homeobox 1 (ZEB-1), is a 190 210 kD transcriptional factor, inhibitor of adhesion molecules as well as cell polarity genes. Abnormal ZEB 1 expression in several neoplasms was associated with aggressive prognosis, poor differentiation, and metastases. Additionally, ZEB1 overexpression promotes the progression of gynecological tumors (Feng et al, 2014). Nevertheless, ZEB1 expression in ovarian serous carcinoma and its role in clinical consequences needs further clarification.

Epithelial cell adhesion molecule (EpCAM) is a 3942kDa calcium-independent transmembrane glycoprotein that was recognized as a CSC marker. EpCAM signaling pathway is participated in multiple cellular functions such as cell adhesion, migration, and differentiation. High expression of EpCAM enhances carcinogenesis via stimulation of reprogramming factors as Oct-4, Nanog, and SOX2 whereas its downregulation inhibited them, thus suppressing tumor initiation, and progression. Specific ablation of EpCAM expressing CSCs could be a novel cancer therapeutic strategy (Mohtar et al, 2020).

SOC is a biologically heterogeneous tumor with diverse genomic backgrounds, molecular profiles, and response to the therapy leading to difficulties in proving unified, suitable treatment (Wang et al., 2014). The shortage of approaches to deal with biological complexity is a leading cause of failure to improve the patient prognosis. So, it is important to explore precise and effective protocols from a biological point of view. Even though several prognostic parameters have been explored, they remain insufficient to classify the high-risk patients prone for chemo-resistant, recurrent SOC (Rampes and Choy, 2022). Consequently, there is an actual requirement to detect new markers for SOC agressiveness and therapy- resistance. Thus, the current work evaluated the prognostic significance of Nanog, ZEB1, and EpCAM in SOC patients.

Materials and Methods

Patients' selection

This is a prospective cohort study where 60 patients with SOC, who underwent debulking surgery (optimal or suboptimal) at Gynecology and Obstetrics Department were enrolled. None of patients received preoperative chemotherapy or radiotherapy. The specimens were assessed for grading and staging at Pathology department according to FIGO criteria. Prior to patients signing informed consent this study was approved by the local Ethics Committee.

Follow-up was done at Clinical oncology department - zagazig university hospitals, patients had taken chemotherapy; including carboplatin (AUC 6) with paclitaxel (175 mg/m^2), administered each 3 weeks for six cycles. The response was evaluated by both RECIST (Response evaluation criteria in solid tumors) criteria (by radiology) and serum CA-125. Disease-Free Survival (DFS) was the time from optimal surgical procedure or complete clinical response to the time of relapse or the last relapse-free time. Overall Survival (OS) was from the initial diagnosis to death or the last contact of patient.

Immunohistochemistry

It was held by polymer Envision detection system; (Dako EnVision™ kit) (Dako, Copenhagen, Denmark). Nearly 3–5 µm tissue sections were deparaffinized in xylene then rehydrated in the down-graded alcohol. The slides were incubated for 10 min in 3% H_2O_2 to block endogenous peroxidase. Then after, antigen retrieval solution (pH 6.0) was applied for 20 min. Lastly, the slides were incubated for 60 min at 37°C with monoclonal rabbit ZEB-1 antibody (dilution 1:100; clone EPR17375; cat. no. ab203829; Abcam), monoclonal EpCAM antibody (1:1,000 dilution, ab124825; Abcam, UK) and rabbit monoclonal Nanog antibody (ab109250; Abcam, Cambridge, MA). The reaction was envisioned by incubating the slides with DAB for fifteen min and then Mayer's hematoxylin was used.

Interpretation

Nanog scoring

The intensity (0–3) and pattern (1–4) of cytoplasmic expression were evaluated. The intensity score was summed to its parallel pattern score: 0 (0-4%), 1 (5%-49%), 2 (50%-74%), and 3 (75%-100%) to get the final score: Negative Nanog (0), weak Nanog (1, 2), moderate Nanog (3, 4), and strong Nanog (5, 6) (Šuster et al., 2017).

ZEB1 scoring

The nuclear staining intensity was recorded as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). The staining extension was counted as 0 (0%), 1 (1 10%), 2 (10 50%), and 3 (>51%). The sum of both scores was computed as (0 6) for ZEB1. Tumors were classified as negative, weak, moderate, and high expression if having a final score of 0, 1 2, 3 4, or 5 6, respectively (Sakata et al, 2017).

EpCAM scoring

The intensity (0–3) and pattern (1–6) of membranous expression were evaluated. The intensity score was multiplied by its parallel pattern score (1 = 1-25% of positive cells; 2 = 26-50%; 3 = 51-75%; 4 >75% to attain the final score. EpCAM expression was categorized as low (0–4) and high (5–12) (Tayama et al, 2017).

Statistics

Continuous variables were expressed as the mean ± SD and median (range), and the categorical variables were expressed as a number (percentage). Percent of categorical variables were compared by Fisher's exact test or Pearson's Chi-square test. Stratification of DFS and OS was organized in relation to the markers. These time-to-event distributions were assessed by Kaplan-Meier plot and compared by the two-sided exact log-rank test. All used tests were two sided. Statistics were accomplished using

SPSS 22.0 for windows (IBM Inc., Chicago, IL, USA) and MedCalc windows (MedCalc Software bvba 13, Ostend, Belgium).

Results

Patients' characteristics

The mean age of the studied cases at the initial diagnosis was 50.27±7.60 years (range 32–66 years). Optimal surgery was performed for 46.7% of the cases, while in 53.3% of the cases suboptimal surgery was done. Lymph node showed that 37 patients (61.7%) have nodal malignant metastasis. Most of the studied cases (50%) were presented at advanced FIGO stages (III-IV). A two-tier grading system was used in this study, where low-grade (n=37) and high-grade (n=33) were defined (Figures 1–2).

After the suboptimal surgery, sixteen cases (50%) had an overall clinical response (OAR) to the chemotherapy, while the other 16 cases (50%) had no response (NR). The median follow-up was 24 months (range 9–36), during which twenty-two cases (36.7%) died. The clinicopathological parameters of the cases were evaluated and presented in Table 1.

Association of Nanog, ZEB1, and EpCAM expression with clinicopathological parameters:

Cytoplasmic NANOG was expressed in 65% of the cases. A significant association of Nanog with higher grade, nodal metastasis, and FIGO stage ($p < 0.001$ for each). The nuclear ZEB1 showed moderate- high expression in 58.3% of the cases. A highly significant overexpression of ZEB1 with tumor grade, nodal metastasis, and FIGO stage ($p<0.001$ for each) was detected (Table 3), (Figure 4). The membranous EpCAM revealed high expression in 60% of the cases. A significant association of EpCAM expression with higher grade, nodal metastasis, and advanced FIGO stage ($p < 0.001$ for each) was noted. (Table 3), (Fig. 5). Furthermore, co-expression of Nanog/ZEB1/EpCAM IHC staining showed a significant association with higher tumor grade, nodal metastasis, and FIGO stage ($p < 0.001$ for each) (Table 3).

Table 1. Clinicopathological Parameters and Outcome among 60 Ovarian Carcinoma Patients

Clinicopathological parameters	All studied ovarian carcinoma patients (N=60)		Clinicopathological parameters	All studied ovarian carcinoma patients (N=60)	
	No.	%		No.	%
Age (years)			EpCAM IHC staining		
Mean±SD	50.27	±7.60	Low	24	40
Median (Range)	52	(32 – 66)	High	36	60
≤50 years	27	45			
>50 years	33	55			
Surgery			NANOG/ZEB1/EpCAM	(N=49)	
Optimal	28	46.7	Negtaive/Negative/Low	18	36.7
Suboptiomal	32	53.3	Strong/High/High	31	63.3
Grade			Response	(N=32)	
Low grade	27	45	CR	10	31.2
High grade	33	55	PR	6	18.8
Lymph node			SD	2	6.2
Negative	23	38.3	PD	14	43.8
Positive	37	61.7	OAR	16	50
			NR	16	50
FIGO stage			Follow-up duration (months)		
Stage IC	14	23.3	Mean±SD	22.25	±8.73
Stage II	16	26.7	Median (Range)	24	(9 – 36)
Stage III	21	35			
Stage IV	9	15			
NANOG IHC staining			Relapse	(N=36)	
Negative	21	35	Absent	17	47.2
Weak	7	11.7	Present	19	52.8
Moderate	10	16.7			
Strong	22	36.7			
ZEB1 IHC staining			Mortality	(N=60)	
Negative-Low	25	41.7	Alive	38	63.3
Moderate-High	35	58.3	Died	22	36.7

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

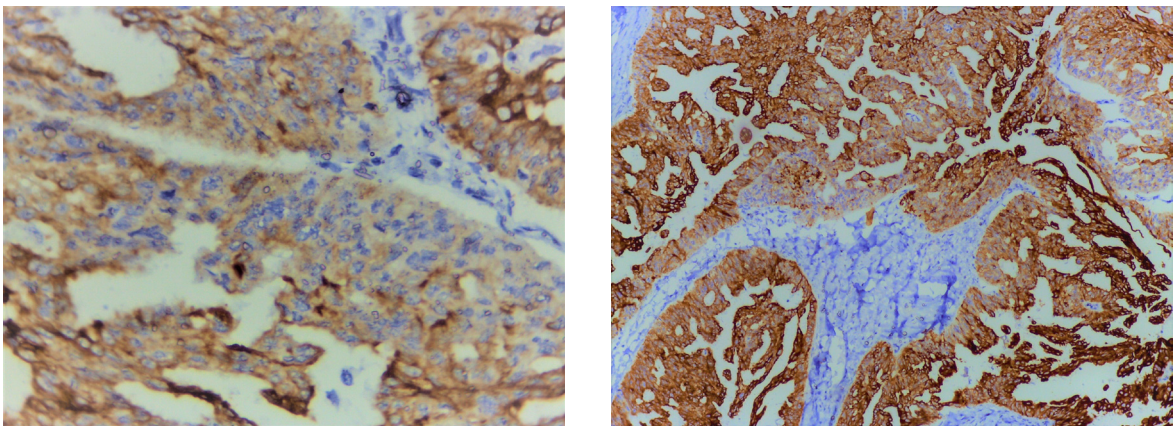

Figure 1. Nanog Immunoexpression in Serous Ovarian Carcinoma: a, Low-grade SOC shows a moderate Nanog immunostaining in tumor cells (IHC×400); b, High-grade SOC shows strong Nanog immunostaining in tumor cells (IHC×200).

Prognostic relevance of Nanog, ZEB1, and EpCAM immunoexpression in SOC cases

17 patients revealed a relapsed cancer after the initial response and 22 patients (36.7%) were died due to SOC. Nanog overexpression was associated significantly with chemotherapy response (p< 0.001), relapse (p < 0.001), shorter OS (p < 0.001) and DFS (p < 0.001, Table 5). ZEB1 overexpression exhibited a significant association with response to chemotherapy (p= 0.012), relapse (p< 0.001) shorter OS (p<0.001) and DFS (p<0.001) (Table 6). Additionally, the high EpCAM was associated significantly with chemotherapy response (p= 0.043), relapse (p < 0.001) shorter OS (p=0.006) and DFS (p< 0.001) (Table 6). Kaplan-Meier plot curves that

Table 2. Relation between Clinicopathological Parameters and NANOG IHC Staining among 60 Ovarian Carcinoma Patients

	All studied ovarian carcinoma patients (N=60)		NANOG IHC staining								p-value[a]
			Negative (N=21)		Weak (N=7)		Moderate (N=10)		Strong (N=22)		
	No.	%	No.	%	No.	%	No.	%	No.	%	
Age group											
≤50 years	27	45	10	37	3	11.1	4	14.8	10	37	0.982
>50 years	33	55	11	33.3	4	18.2	6	18.2	12	36.4	
Surgery											
Optimal	28	46.7	15	53.6	5	17.9	4	14.3	4	14.3	0.003
Suboptiomal	32	53.3	6	18.8	2	6.2	6	18.8	18	56.2	
Grade											
Low grade	27	45	20	74.1	2	7.4	3	11.1	2	7.4	<0.001
High grade	33	55	1	3	5	15.2	7	21.2	20	60.6	
Lymph node											
Negative	23	38.3	16	69.6	2	8.7	3	13	2	8.7	<0.001
Positive	37	61.7	5	13.5	5	13.5	7	18.9	20	54.1	
FIGO stage											
Stage IC	14	23.3	10	71.4	2	14.3	1	7.1	1	7.1	<0.001
Stage II	16	26.7	6	37.5	2	12.5	5	31.2	3	18.8	
Stage III	21	35	5	23.8	3	14.3	4	19	9	42.9	
Stage IV	9	15	0	0	0	0	0	0	9	100	
ZEB1 IHC staining											
Negative-Low positive	25	41.7	20	80	3	12	0	0	2	8	<0.001
Moderate-High positive	35	58.3	1	2.90%	4	11.4	10	28.6	20	57.1	
EpCAM IHC staining											
Low	24	40	18	75	2	8.3	3	12.5	1	4.2	<0.001
High	36	60	3	8.3	5	13.9	7	19.4	21	58.3	

Categorical variables were expressed as number (percentage); a, Chi-square test; p<0.05 is significant.

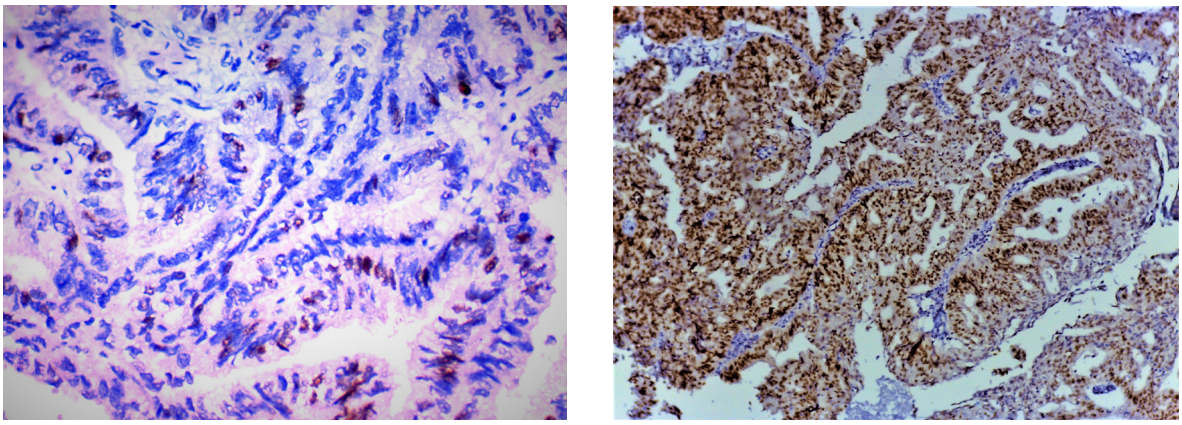

Figure 2. ZEB1 Immunoexpression in Serous Ovarian Carcinoma. a, Low-grade SOC shows low ZEB1 immunostaining in tumor cells (IHC×400); b, High-grade SOC shows high ZEB1 immunostaining in tumor cells (IHC×200).

were stratified according to Nanog, ZEB1, and EpCAM immunoexpression were presented in Figure 6. Likewise, co-expression of NANOG/ZEB1/EpCAM IHC staining had a significant relation with response to chemotherapy,

Table 3. Relation between Clinicopathological Parameters and ZEB1/EpCAM IHC Staining among 60 Ovarian Carcinoma Patients

	All studied ovarian carcinoma patients. (N=60)		ZEB1 IHC staining				p-value[a]	EpCAM IHC staining				p-value[a]
			Negative-Low (N=25)		Moderate-High (N=35)			Low (N=24)		High (N=36)		
	No.	%	No.	%	No.	%		No.	%	No.	%	
Age group												
≤50 years	27	45	13	48.1	14	51.9	0.357	10	37	17	63	0.672
>50 years	33	55	12	36.4	21	63.6		14	42.4	19	57.6	
Surgery												
Optimal	28	46.7	17	60.7	11	39.3	0.005	21	75	7	25	<0.001
Suboptiomal	32	53.3	8	25	24	75		3	9.4	29	90.6	
Grade												
Low grade	27	45	22	81.5	5	18.5	<0.001	24	88.9	3	11.1	<0.001
High grade	33	55	3	9.1	30	90.9		0	0	33	100	
Lymph node												
Negative	23	38.3	18	78.3	5	21.7	<0.001	21	91.3	2	8.7	<0.001
Positive	37	61.7	7	18.9	30	81.1		3	8.1	34	91.9	
FIGO stage												
Stage IC	14	23.3	12	85.7	2	14.3	<0.001	12	85.7	2	14.3	<0.001
Stage II	16	26.7	5	31.2	11	68.8		9	56.2	7	43.8	
Stage III	21	35	8	38.1	13	61.9		3	14.3	18	85.7	
Stage IV	9	15	0	0	9	100		0	0	9	100	
NANOG IHC staining												
Negative	21	35	20	95.2	1	4.8	<0.001	18	85.7	3	14.3	<0.001
Weak	7	11.7	3	42.9	4	57.1		2	28.6	5	71.4	
Moderate	10	16.7	0	0	10	100		3	30	7	70	
Strong	22	36.7	2	9.1	20	90.9		1	4.5	21	95.5	
ZEB1 IHC staining												
Negtaive-Low	25	41.7						20	80	5	20	<0.001
Moderate-High	35	58.3						4	11.4	31	88.6	
EpCAM IHC staining												
Low	24	40	20	83.3	4	16.7	<0.001					
High	36	60	5	13.9	31	86.1						

Categorical variables were expressed as number (percentage); a, Chi-square test; p<0.05 is significant.

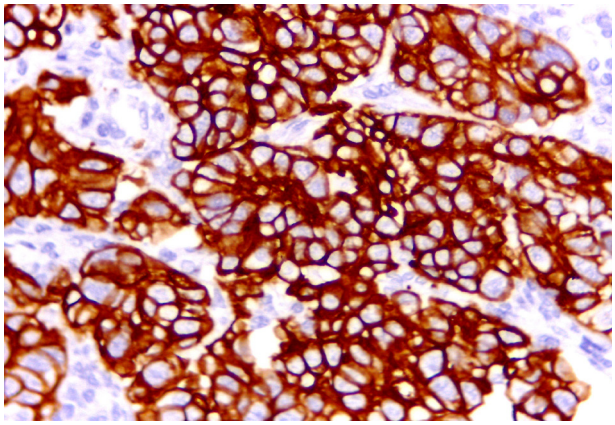

Figure 3. High-Grade SOC Shows a High EpCAM Immunoexpression in Serous Ovarian Carcinoma (IHC×400).

relapse, shorter OS, and DFS (p< 0.001 for each) (Table 7).

Discussion

Serous ovarian carcinoma is the most aggressive ovarian cancer that's presented at advanced stage due to lack of symptoms in the early stages. Cytoreductive surgery is usually not a radical intervention owing to its diffuse nature. Unfortunately, development of a chemo-resistant phenotype finally leads to recurrent incurable neoplasm. Understanding the molecular basis of relapsed tumor is still the principal challenge in cancer research (Torre et al., 2018). Currently, research interest is focusing on molecular features, and tumor biology to individualize treatment of SOC patients. As existing treatments for ovarian SOC are still far from optimal, there is a definite need to discover innovative therapeutic targets (Bast et al., 2009).

In the existing study, we assessed Nanog, ZEB1 and EpCAM in SOC and relation to survival of patients , recurrence, and response to the therapy protocol. Since SOC is the commonest type, we focused on it at Zagazig Hospitals with analogous chemotherapeutic lines to reduce any bias. In this study, we investigated the clinical relevance of deregulated Nanog immunoexpression in SOC and the molecular mechanism by which Nanog affects EMT process and tumor recurrence. In the present study, Nanog immunoexpression was predominantly cytoplasmic in contrast to other studies that revealed nuclear and cytoplasmic immunostaining (Noh et al., 2012: Kenda et al., 2017). This can be related to Nanog role in transcriptional regulation of cytoplasmic

Table 4. Relation between Clinicopathological Parameters and Co-Expression of NANOG/ZEB1/EpCAM IHC Staining among 60 Ovarian Carcinoma Patients

	All studied ovarian carcinoma patients (N=49)		NANOG/ZEB1/EpCAM IHC staining				p-v
			Negative/Negative/Low (N=18)		Strong/High/High (N=31)		
	No.	%	No.	%	No.	%	
Age group							
≤50 years	19	38.8	7	36.8	12	63.2	0.99
>50 years	30	61.2	11	36.7	19	63.3	
Surgery							
Optimal	22	44.9	15	68.2	7	31.8	<0.001
Suboptiomal	27	55.1	3	11.1	24	88.9	
Grade							
Low grade	19	38.8	18	94.7	1	5.3	<0.001
High grade	30	61.2	0	0	30	100	
Lymph node							
Negative	18	36.7	16	88.9	2	11.1	<0.001
Positive	31	63.3	2	6.5	29	93.5	
FIGO stage							
Stage IC	12	24.5	10	83.3	2	16.7	<0.001
Stage II	12	24.5	5	41.7	7	58.3	
Stage III	16	32.7	3	18.8	13	81.2	
Stage IV	9	18.4	0	0	9	100	

Categorical variables were expressed as number (percentage); a, Chi-square test; p<0.05 is significant.

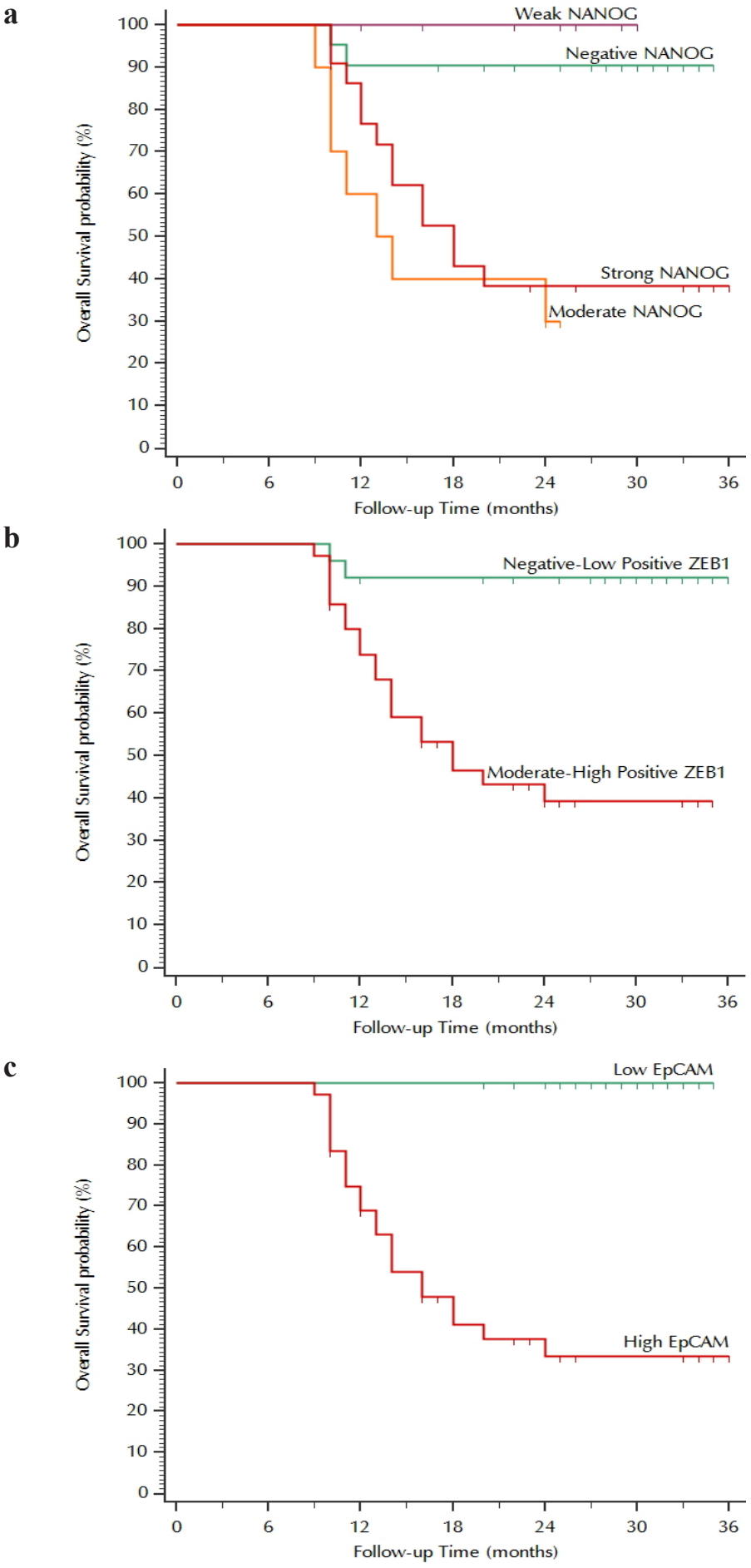

Figure 4. Kaplan Meier Plot for Overall Survival (OS): a, Stratified by Nanog IHC staining; b, Stratified by ZEB1 IHC staining; c, Stratified by EpCAM IHC staining.

Table 5. Relation between NANOG IHC Staining and Outcome among 60 Ovarian Carcinoma Patients

	All studied ovarian carcinoma patients		NANOG IHC staining								p-value
			Negative		Weak		Moderate		Strong		
	No.	%	No.	%	No.	%	No.	%	No.	%	
Response	(N=32)		(N=6)		(N=2)		(N=6)		(N=18)		
CR	10	31.2	3	50	0	0	0	0	7	38.9	<0.001[a]
PR	6	18.8	3	50	0	0	0	0	3	16.7	
SD	2	6.2	0	0	2	100	0	0	0	0	
PD	14	43.8	0	0	0	0	6	100	8	44.4	
OAR	16	50	6	100	0	0	0	0	10	55.6	0.007[a]
NR	16	50	0	0	2	100	6	100	8	44.4	
Relapse	(N=36)		(N=18)		(N=5)		(N=4)		(N=9)		
Absent	17	47.2	15	83.3	2	40	0	0	0	0	<0.001[a]
Present	19	52.8	3	16.7	3	60	4	100	9	100	
Disease Free Survival											
Mean DFS (months)	24.44 months		31.66 months		17.40 months		12.50 months		16.67 months		<0.001[b]
(95%CI)	(20.68 – 28.68)		(29.23 – 34.09)		(9.09 – 25.70)		(9.56 – 15.44)		(10.14 – 23.19)		
12-month DFS	69.4		100		40		50		33.3		
18-month DFS	58.3		94.4		40		0		22.2		
24-month DFS	52.8		83.3		40		0		22.2		
30-month DFS	52.8		83.3		40		0		22.2		
Mortality	(N=60)		(N=21)		(N=7)		(N=10)		(N=22)		
Alive	38	63.3	19	90.5	7	100%	3	30	9	40.9	<0.001[a]
Died	22	36.7	2	9.5	0	0%	7	70	13	59.1	
Overall Survival											
Mean OS (months)	27.46 months		32.67 months		30 months		16.60 months		22.54 months		<0.001[b]
(95%CI)	(24.59 – 30.33)		(29.59 – 35.74)				(12.38 – 20.82)		(17.89 – 27.19)		
12-month OS	81.5		90.5		100		60		76.6		
18-month OS	65.7		90.5		100		40		43.1		
24-month OS	61.8		90.5		100		30		38.3		
30-month OS	61.8		90.5		100		------		38.3		

Categorical variables were expressed as number (percentage); a, Chi-square test; b, Log-rank test; p<0.05 is significant.

mitochondrial DNA, with the nuclear transcriptional regulation leading to Nanog translocation between the nucleus and the cytoplasm by dynamic processes via growth factors.

Kenda et al showed that though Nanog is detected in the nucleus in several neoplasms, it is expressed in the cytoplasm in major gynecological tumors (breast and ovary) (Kenda et al., 2017). We thought that phenomenon is triggered by various protein function in tumor cells that is associated with sex hormones. Furthermore, different anti-Nanog antibodies with diverse sensitivities and specificities and used in different searches, leading to a diverse expression (nuclear or cytoplasmic pattern).

Nanog immunoexpression was noted in 65% of cases with a significant overexpression with SOC grade where 97% of Nanog-positive cases were high-grade, nodal metastasis, and tumor stage confirming the previous findings (Pan et al., 2010; Lee et al., 2012; Siu et al., 2013). Centered on these results, we suggest that Nanog expression may affect ovarian tumorigenesis, spread, and tumor cells survival which may be related to the ability of Nanog to regulate relation between tumor cells and host immune cells. Thus, it enables tumor cells to avoid immune system attack. Amsterdam et al.. (2013) reported that Nanog-mediated triggering of the Akt pathway helps in adapting tumor cells to host immune system and escaping immune-mediated clearance. Moreover, it was reported that Nanog attributes to self-renewal of embryonic stem cells, preserving the undifferentiated state and enhancing cell proliferation (Clark et al, 2004). Unlike these data, Kenda et al., (2017) did not find any significant association between Nanog staining and clinical criteria, including patient survival. We supposed this due to the difference between anti-Nanog antibodies and also owing to diverse composition of the study groups. Furthermore, we observed that Nanog up-regulation had a significantly relation with poor OS and DFS. Our statistics confirmed the former studies (Siu et al., 2013; Amsterdam et al., 2013); where Siu et al., (2013), found that Nanog is an independent prognostic factor for OS and DFS in SOC group.

In this study we provide verification that Nanog

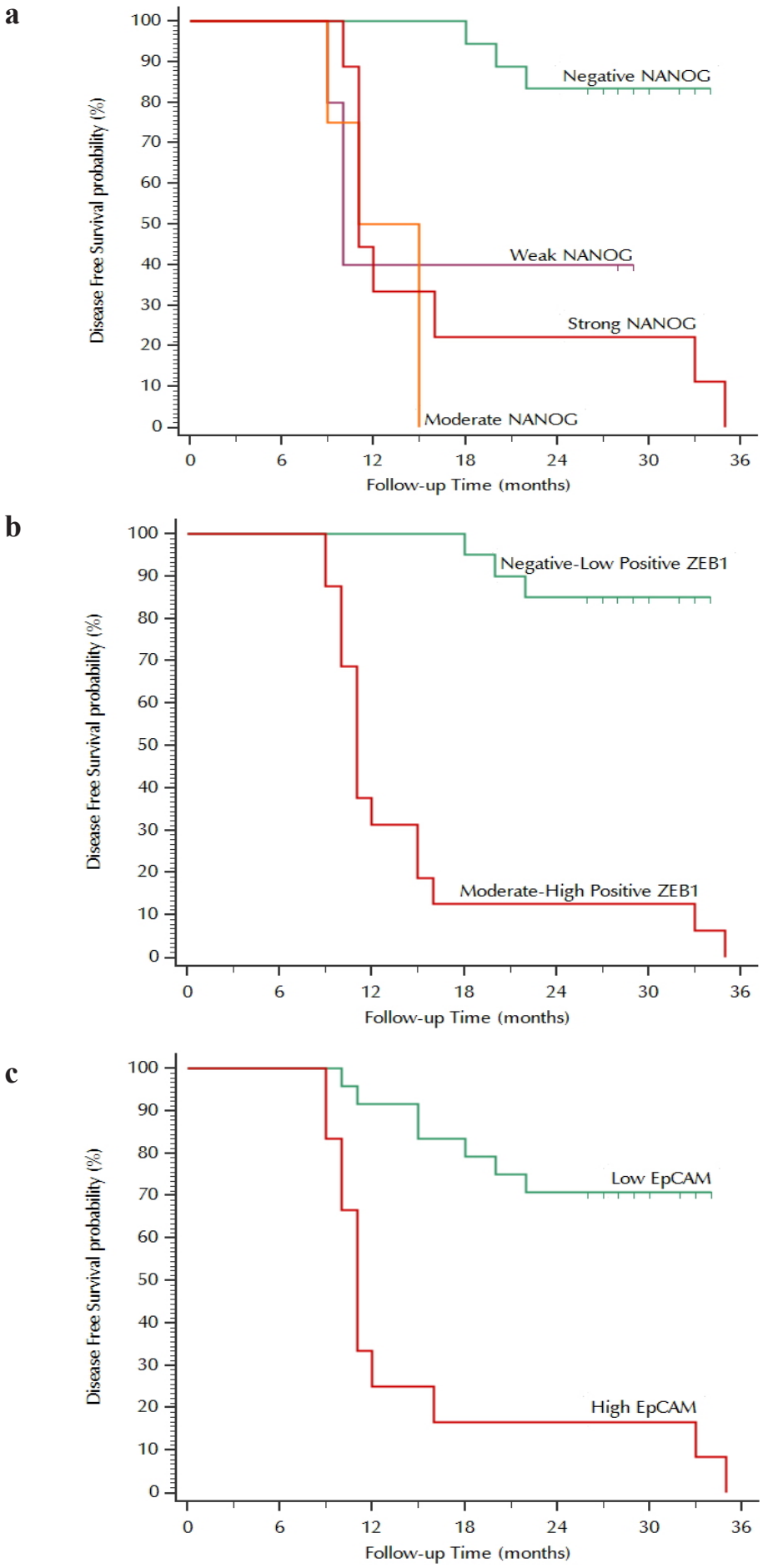

Figure 5. Kaplan Meier Plot for Disease Free Survival (OS): a, Stratified by Nanog IHC staining; b, Stratified by ZEB1 IHC staining; c, Stratified by EpCAM IHC staining.

Table 6. Relation between ZEB1/EpCAM IHC Staining and Outcome among 60 Ovarian Carcinoma Patients

	All studied ovarian carcinoma patients		ZEB1 IHC staining				p-value	EpCAM IHC staining				p-value
			Negative-Low		Moderate-High			Low		High		
	No.	%	No.	%	No.	%		No.	%	No.	%	
Response	(N=32)		(N=8)		(N=24)			(N=3)		(N=29)		
CR	10	31.2	3	37.5	7	29.2	0.012a	3	100	7	24.1	0.043[a]
PR	6	18.8	4	50	2	8.3		0	0	6	20.7	
SD	2	6.2	1	12.50%	1	4.2		0	0	2	6.9	
PD	14	43.8	0	0	14	58.3		0	0	14	48.3	
OAR	16	50	7	87.5	9	37.5	0.037a	3	100	13	44.8	0.226[a]
NR	16	50	1	12.5	15	62.5		0	0	16	55.2	
Relapse	(N=36)		(N=20)		(N=16)			(N=24)		(N=12)		
Absent	17	47.2	17	85	0	0	<0.001a	17	70.8	0	0	<0.001[a]
Present	19	52.8	3	15	16	100		7	29.2	12	100	
Disease Free Survival												
Mean DFS (months)	24.44 months		31.90 months		14.31 months		<0.001b	28.71 months		14.83 months		<0.001[b]
(95%CI)	(20.68 – 28.68)		(29.69 – 34.11)		(10.41 – 18.22)			(25.29 – 32.13)		(9.66 – 20.01)		
12-month DFS	69.4		100		31.3			91.7		25		
18-month DFS	58.3		95		12.5			79.2		16.7		
24-month DFS	52.8		85		12.5			70.8		16.7		
30-month DFS	52.8		85		12.5			70.8		16.7		
Mortality	(N=60)		(N=25)		(N=35)			(N=24)		(N=36)		
Alive	38	63.3	23	92	15	42.9	<0.001a	24	100	14	38.9	<0.001[a]
Died	22	36.7	2	8	20	57.1		0	0	22	61.1	
Overall Survival												
Mean OS (months)	27.46 months		33.96 months		22.27 months		<0.001b	35 months		21.26 months		<0.001[b]
(95%CI)	(24.59 – 30.33)		(31.25 – 36.67)		(18.62 – 25.93)					(17.51 – 25.02)		
12-month OS	81.5		92		79.8			100		69		
18-month OS	65.7		92		46.6			100		41.1		
24-month OS	61.8		92		39.3			100		33.5		
30-month OS	61.8		92		39.3			100		33.5		

Categorical variables were expressed as number (percentage); a, Chi-square test; b, Log-rank test; $p<0.05$ is significant.

participates in occurrence of recurrent SOC possibly via enhancing carboplatin resistance in tumor cells as previously stated (Noh et al., 2012). Our findings confirmed previously reported results that revealed that Nanog overexpression was related to resistance to the therapy protocol, and Nanog messenger RNA was higher in cisplatin- and paclitaxel-resistant SOC cell lines (Siu et al., 2013; Amsterdam et al., 2013). In contrast, Robinson et al. did not succeed to find association between Nanog expression and chemotherapy resistance in contrast to Sox2 (Robinson et al., 2021).

It was supposed that Nanog can adjust p53-related signaling and adversely related to cancer cell pro-apoptosis processes. The NANOG-STAT3-ABCB1 signaling examined in relative to cancer ovary and breast facilitates resistance against many chemotherapeutic treatments (Choi et al., 2012). It was shown that Nanog in ovarian cancer plays role in EMT regulation and therapy- resistance through upregulation of STAT3 pathway (Liu et al., 2016). Previously SOC cell lines have been proven to show an association between EMT and drug resistance. There is likewise approval to detect that EMT occurs in combination with ovarian-cancer progression and metastasis (Zhang et al,. 2021).

EMT is implicated in metastasis, tumor invasion, and drug resistance. Thus, the molecular actors implicated in this cycle are worthy attractive targets in oncology. Among these molecules ZEB1, this molecule inhibits the function of some microRNAs, as miR 183, and miR 200, which act as suppressor of stem like hallmarks and enhancer of epithelial differentiation leading to tumor progression and metastasis (Yang et al., 2014). Previous studies reported that ZEB1 was correlated with a poorer clinical outcome of numerous solid cancers (Zhang et al., 2015).

The current study revealed moderate-high ZEB1 expression in 58.3% with a significant relation with tumor grading, nodal metastasis, and stage supporting the possible role of ZEBI in advancement of cancer progression and metastasis via promotion of EMT by suppressing genes as E cadherin, and triggering genes needed for transformation to the mesenchymal phenotype as previously confirmed (Peinado et al, 2007; Li et al., 2016) where they approved a significant relation between ZEB1 staining and aggressive phenotypes of EOC. Spoelstra et al showed that ZEB1 was abnormally expressed in advanced undifferentiated endometrioid carcinomas and uterine serous carcinomas (Spoelstra et al., 2006). Chen et al., (2013), reported that silencing

Table 7. Relation between Co-Expression of NANOG/ZEB1/EpCAM IHC Staining and Outcome among 60 Ovarian Carcinoma Patients

	All studied ovarian carcinoma patients		NANOG/ZEB1/EpCAM IHC staining				p-value
			Negative/Negative/Low		Strong/High/High		
	No.	%	No.	%	No.	%	
Response	(N=27)		(N=3)		(N=24)		
CR	10	37	3	100	7	29.2	0.125[a]
PR	2	7.4	0	0	2	8.3	
SD	1	3.7	0	0	1	4.2	
PD	14	51.9	0	0	14	58.3	
OAR	12	44.4	3	100	9	37.5	0.075[a]
NR	15	55.6	0	0	15	62.5	
Relapse	(N=30)		(N=18)		(N=12)		
Absent	15	50	15	83.3	0	0	<0.001[a]
Present	15	50	3	16.7	12	100	
Disease Free Survival							
Mean DFS (months)	25.31 months		31.67 months		14.83 months		<0.001[b]
(95%CI)	(21.20 – 29.42)		(29.24 – 34.09)		(9.66 – 20.01)		
12-month DFS	70		100		25		
18-month DFS	63.3		94.4		16.7		
24-month DFS	56.7		83.3		16.7		
30-month DFS	56.7		83.3		16.7		
Mortality	(N=49)		(N=18)		(N=31)		
Alive	29	59.2	18	100	11	35.5	<0.001[a]
Died	20	40.8	0	0	20	64.5	
Overall Survival							
Mean OS (months)	20.06 months		35 months		20.45 months		<0.001[b]
(95%CI)	(23.01 – 29.11)				(16.71 – 24.18)		
12-month OS	81.4		100		70.5		
18-month OS	62.4		100		39.1		
24-month OS	60.1		100		30.2		
30-month OS	57.5		100		30.2		

Categorical variables were expressed as number (percentage); a, Chi-square test; b, Log-rank test; p<0.05 is significant.

of ZEB1 expression leads to downregulation of colony forming and migration abilities of cells with enhanced miR 200c expression to inhibit EMT in ovarian cancer cells. Previously Yang et al., (2014) found that ZEB1 expression in esophageal squamous cell carcinoma was associated with tumor staging, spread to LN, tumor grading, and depth of invasion. On the other hand, Sakata et al., (2017) stated that ZEB1 expression was not related to any of the clinical or pathologic features as age, histological type, tumor stage, and surgical procedure. These contradictory findings may be related to the varied methodologies, tumor divergency, different stages or sample size comprised in these works.

Based on our analysis, we noted that ZEB1 expression had a significant relation to poor OS and DFS and that were consistent with others (Li et al, 2016); assumed that ZEB1 overexpression may affect adverse prognosis of SOC and its application in clinical practice is possible. Likewise, ZEB1 overexpression was related to unfavorable response to the chemotherapy directing to chemoresistance and SOC progression and even relapse in cases that initially responded to the first-line chemotherapy. These supported the previously detected conclusions (Siebzehnrubl et al., 2013). According to Siebzehnrubl et al., (2013), ZEB1 is a marker of GBM recurrence, and the ability of resisting chemotherapy. Moreover, suppressing ZEB1 could retain the chemosensitivity of lung adenocarcinoma to docetaxel and inhibit their migratory capability via switching the mesenchymal phenotype (Ren et al., 2013).

To our knowledge, our study is the first research approving that ZEB1 expression was strongly associated with a bad outcome in SOC. These findings were relayed on the metastasis or chemo-resistant promoting effects of ZEB1, but further investigation is needed to explain the molecular mechanisms of ZEB1. The current results suggest that ZEB1 immunoexpression might be an essential predictor of patients with poor prognosis which helps to choose the appropriate therapy strategies.

EpCAM is mitogenic signal transducer via initiation of cell proliferation by controlling the cell cycle, enhancement of cell cycle regulating genes and inducing signal transduction into the nucleus through the

Wnt-signaling pathway (Imrich et al., 2012). Several studies have supported the idea that EpCAM increases tumor progression and metastasis, and EpCAM positive tumor cells show a higher affinity for proliferation in comparison to negative cells. Ep-CAM expression is associated with tumor grading, stage, and metastasis (Ni et al., 2013). The current study reported that the grade of SOC and FIGO stage was significantly related to EpCAM expression which was in line to Zheng et al., (2017), who revealed a significant association between EpCAM expression and grade and stage (p-value=0.03), but in disagreement with Woopen et al., (2014), who approved no correlation between EpCAM with tumor grade.

As previously reported Tayama et al., (2017), we retrieved that SOC with high EpCAM achieved chemoresistance to chemotherapy suggesting that EpCAM could be a predictive marker of response to chemotherapy. In agreement with our notes, former in vitro assays revealed that the EpCAM positive cells has a higher viability as compared to EpCAM-negative cells in response to cisplatin through inhibition of chemotherapy induced apoptosis, that's controlled by EpCAM-Bcl-2 axis (Ni et al., 2013). Moreover, previous in vivo mouse model, the platinum agents contrarily eliminated EpCAM-negative cells compared to EpCAM-positive cells, indicating that the remaining EpCAM-positive cells enhance tumor recurrence after chemotherapy which is verified in our study. So high EpCAM is related to bad prognosis in SOC (Wang et al., 2015). Our findings offer a justification for EpCAM-targeted therapy to enhance chemoresistance. Targeting the EpCAM could be a hopeful line to efficiently eradicate the CSCs as the assumed root of ovarian cancer.

In conclusions, based on the data of our study, the risk of recurrence in SOC could be related to Nanog, ZEB1, and EpCAM overexpression contributing to an aggressive serous ovarian carcinoma with a high incidence of recurrence and unfavorable response to the chemotherapy. Further investigations on larger number of cases are advised to verify these findings and prove the potential use of the present markers as outstanding targets in SOC.

Author Contribution Statement

Conception: Aziza E. Abdelrahman, Hanna M. Ibrahim; Interpretation or analysis of data: Eman Elesbai; Preparation of the manuscript: Aziza E. Abdelrahman, Mohamed S.H. Ramadan; Revision for important intellectual content: Shimaa Gharieb, Moamna M. Fahmy; Supervision: Mohamed A. Wasfy

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Ethical Approval

The present protocol has been approved by Faculty of Medicine- Zagazig University - Egypt.

Availability of data

The data that support the results of our study will be available from the corresponding author upon reasonable request.

Informed consent

Informed consent was obtained from all participants enrolled in the study.

Compliance with Ethical Standards

All the procedures performed involving human participants were in agreement with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict of Interest

Authors declare that's no conflict of interest.

References

Amsterdam A, Raanan C, Schreiber L, et al (2013). Localization of the stem cell markers LGR5 and Nanog in the normal and the cancerous human ovary and their inter-relationship. *Acta Histochem*, **115**, 330Y338.

Bast RC, Hennessy B, Mills GB (2009). The biology of ovarian cancer: new opportunities for translation. *Nat Rev Cancer*, **9**, 415–28.

Chen D, Wang J, Zhang Y, et al (2013). Effect of down-regulated transcriptional repressor ZEB1 on the epithelial-mesenchymal transition of ovarian cancer cells. *Int J Gynecol Cancer*, **23**, 1357-66.

Cho IJ, Lui P, Obajdin J, et al (2019), Mechanisms, Hallmarks, and Implications of Stem Cell Quiescence. *Stem Cell Reports*, **1**, 1190-1200.

Choi SC, Choi JH, Park CY, et al (2012). Nanog regulates molecules involved in stemness and cell cycle-signaling pathway for maintenance of pluripotency of P19 embryonal carcinoma stem cells. *J Cell Physiol*, **227**, 3678-92.

Clark AT, Rodriguez RT, Bodnar MS, et al (2004). Human STELLAR, NANOG, and GDF3 genes are expressed in pluripotent cells and map to chromosome 12p13, a hotspot for teratocarcinoma. *Stem Cells*, **22**, 169–79.

Davidson B, Holth A, Hellesylt E, et al (2015). The clinical role of epithelial-mesenchymal transition and stem cell markers in advanced-stage ovarian serous carcinoma effusions. *Hum Pathol*, **46**, 1-8.

Farrag M, Emarah Z, Elrefaie W, et al (2021). EGFR and HER2 expression in primary ovarian high-grade serous carcinoma and their prognostic value. *Res Oncol*, **17**, 9-16.

Feng G, Wang X, Cao X, et al (2014). ZEB1 expression in endometrial biopsy predicts lymph node metastases in patient with endometrial cancer. *Dis Markers*, 680361.

Imrich S, Hachmeister M and GIRES O (2012). EpCAM and its potential role in tumor-initiating cells. *Cell Adh-Migr*, **6**, 30-8.

Kenda Suster N, Smrkolj S, Virant-Klun I (2017). Putative stem cells and epithelial-mesenchymal transition revealed in sections of ovarian tumor in patients with serous ovarian carcinoma using immunohistochemistry for vimentin and pluripotency-related markers. *J Ovarian Res*, **10**, 11.

Lee M, Nam EJ, Kim SW, et al (2012). Prognostic impact of the cancer stem cell related marker NANOG in ovarian serous carcinoma. *Int J Gynecol Cancer*, **22**, 1489-96.

Li X, Huang R, Li RH, Trope CG, et al (2016). Expression of zinc finger E-box-binding homeobox factor 1 in epithelial ovarian cancer: A clinicopathological analysis of 238 patients. *Mol Clin Oncol*, **4**, 18-22.

Liu S, Sun J, Cai B, et al (2016). NANOG regulates epithelial-mesenchymal transition and chemoresistance through activation of the STAT3 pathway in epithelial ovarian cancer.

Tumour Biol, **37**, 9671-80.
Mohtar MA, Syafruddin SE, Nasir SN, et al (2020). Revisiting the Roles of Pro-Metastatic EpCAM in Cancer. *Biomolecules*, **10**, 255.
Ni J, Cozzi P, Hao J, et al (2013). Epithelial cell adhesion molecule (EpCAM) is associated with prostate cancer metastasis and chemo/radioresistance via the PI3K/Akt/mTOR signaling pathway. *Int J Biochem Cell Biol*, **45**, 2736–48.
Noh KH, Kim BW, Song KH, et al (2012). Nanog signaling in cancer promotes stem-like phenotype and immune evasion. *J Clin Invest*, **122**, 4077-93.
Noh KH, Lee YH, Jeon JH, et al (2012). Cancer vaccination drives Nanog-dependent evolution of tumor cells toward an immune-resistant and stem-like phenotype. *Cancer Res*, **72**, 1717-27.
Pan Y, Jiao J, Zhou C, et al (2010). Nanog is highly expressed in ovarian serous cystadenocarcinoma and correlated with clinical stage and pathological grade. *Pathobiology*, **77**, 283-8.
Peinado H, Olmeda D, Cano A (2007). Snail, Zeb and bHLH factors in tumour progression: An alliance against the epithelial phenotype?. *Nat Rev Cancer*, **7**, 415 28.
Rampes S, Choy S (2022). Early diagnosis of symptomatic ovarian cancer in primary care in the UK: Opportunities and challenges. *Prim Health Care Res Dev*, **23**, E52.
Ren J, Chen Y, Song H, et al (2013). Inhibition of ZEB1 reverses EMT and chemoresistance in docetaxel resistant human lung adenocarcinoma cell line. *J Cell Biochem*, **114**, 1395 1403.
Robinson M, Gilbert SF, Waters JA, et al (2021). Characterization of SOX2, OCT4 and NANOG in ovarian cancer tumor-initiating cells. *Cancers Basel*, **13**, 262.
Sakata J, Kajiyama H, Shiro S, et al (2017). Impact of positive ZEB1 expression in patients with epithelial ovarian carcinoma as an oncologic outcome predicting indicator. *Oncol Lett*, **14**, 4287-93.
Siebzehnrubl FA, Silver DJ, Tugertimur B, et al (2013). The ZEB1 pathway links glioblastoma initiation, invasion and chemoresistance. *EMBO Mol Med*, **5**, 1196-1212.
Siu MK, Wong ES, Kong DS, et al (2013). Stem cell transcription factor NANOG controls cell migration and invasion via dysregulation of E-cadherin and FoxJ1 and contributes to adverse clinical outcomes in ovarian cancers. *Oncogene*, **32**, 3500Y3509.
Spoelstra NS, Manning NG, Higashi Y, et al (2006). The transcription factor ZEB1 is aberrantly expressed in aggressive uterine cancers. *Cancer Res*, **66**, 3893-902.
Šuster KN, Grazio FS, Virant-Klun I, Verdenik I, Smrkolj Š (2017). Cancer Stem Cell-Related Marker NANOG Expression in Ovarian Serous Tumors: A Clinicopathological Study of 159 Cases. *Int J Gynecol Cancer*, **27**, 2006-13.
Tayama S, Motohart, Narantuy D, et al (2017). The impact of EpCAM expression on response to chemotherapy and clinical outcomes in patients with epithelial ovarian cancer. *Oncotarget*, **8**, 44312-25.
Torre LA, Trabert B, DeSantis CE, et al (2018). Ovarian cancer statistics, 2018. *CA Cancer J Clin*, **68**, 284-96.
Wang H, Wang H, Makki MS, et al (2014). Overexpression of β-catenin and cyclinD1 predicts a poor prognosis in ovarian serous carcinomas. *Int J Clin Exp Pathol*, **7**, 264–71.
Wang ML, Chiou SH, Wu CW (2013). Targeting cancer stem cells: emerging role of Nanog transcription factor. *Onco Targets Ther*, **6**, 1207-20.
Wang X, Li X, Fu X, et al (2015). Eliminating ovarian cancer stem cells: a potential therapeutic target for ovarian cancer chemoresistance. *Curr Protein Pept Sci*, **16**, 270–8.
Woopen H, Pietzner K, Richter R, et al (2014). Overexpression of the epithelial cell adhesion molecule is associated with a more favorable prognosis and response to platinum-based chemotherapy in ovarian cancer. *J Gynecol Oncol*, **25**, 221–8.
Yang X, Wang Q, Dai W, et al (2014). Overexpression of zinc finger E box binding homeobox factor 1 promotes tumor invasiveness and confers unfavorable prognosis in esophageal squamous cell carcinoma. *Tumour Biol*, **35**, 11977-84.
Zhang J, Guan W, Xu X, et al (2021). A novel homeostatic loop of sorcin drives paclitaxel-resistance and malignant progression via Smad4/ZEB1/miR-142-5p in human ovarian cancer. *Oncogene*, **40**, 4906–18.
Zhang P, Sun Y and Ma L (2015). ZEB1: At the crossroads of epithelial mesenchymal transition, metastasis, and therapy resistance. *Cell Cycle*, **14**, 481-7.
Zheng J, Zhao L, Wang Y, et al (2017). Clinicopathology of EpCAM and EGFR in human epithe-lial ovarian carcinoma. *Open Med*, **12**, 39-44.