Original paper

Immunohistochemical evaluation of forkhead box A1 and EphA5 markers in serous ovarian carcinomas, and their impact on the clinical outcome of patients

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Ovarian cancer is the most lethal gynaecological neoplasm in females. In ovarian cancer, forkhead box A1 (FOXA1) aids transcription of YAP-associated protein mediated by the cyclic adenosine monophosphate response element-binding protein. As a result, cellular proliferation and migration increased. The roles of erythropoietin-producing human hepatocellular carcinoma cell (Eph) receptors and ephrin ligands in cell adhesion, migration, cell proliferation regulation in various cancers, and angiogenesis are well characterized.

This study included formalin-fixed, paraffin-embedded tissue specimens from 41 patients with ovarian serous cystadenocarcinoma, including both low- and high-grade tumours. For each case, a paraffin block with tumour tissue was chosen for an immunohistochemical procedure using primary antibodies against EphA5 and FOXA1. By the end of 2017, patients finished their chemotherapy and were followed for the next 3 years.

Positive FOXA1 and EphA5 results were presented in 68.3% and 39% of patients, respectively. A statistically significant correlation was detected between FOXA1 expression and each of CA-125 level, tumour stage, tumour grade, and the presence of lymph node metastasis. In our work, the overall survival was positively correlated with EphA5 expression and inversely correlated to FOXA1 immunoreactivity. The estimated disease-free survival (DFS) and EphA5 immunoreactivity had a significant positive association, whereas DFS and FOXA1 protein expression had a significant inverse link.

FOXA1 and EphA5 expression play a role in ovarian cancer progression and prognosis prediction.

Key words: FOXA1, EphA5, serous ovarian carcinomas.

Introduction

Ovarian serous cancer is considered the most lethal gynaecological neoplasm in females. Its high mortality impact is owing to difficulty in early detection and chemotherapy resistance. The epithelial category of ovarian tumours is the most frequent pathologic subtype, with 90% of them categorized as primary ovarian malignant tumours. Surgical excision combined with platinum- and paclitaxel based chemotherapy is the standard treatment strategies in the management of epithelial ovarian cancer; however, relapse occurs in about two-thirds of patients after initial treatment, associated with resistance to platinum-based chemo-

therapy. Ovarian cancer spreads quickly in the short term and may show resistance to chemotherapy [1].

Several investigations were undertaken in Egypt at various institutions; among them, the studies by Helal *et al*. [2] 2015 and Nassar *et al*. [3] 2016 showed that the rising incidence of serous ovarian cancer among Egyptian females is a significant health problem that requires further investigation. Ovarian cancer accounted for 2.2% of all incident malignancies and 4.4% of all newly diagnosed cancers, according to Ibrahim *et al*. [4]. The inability to diagnose the disease early is the reason for the low patient survival and mortality [5]. Furthermore, nonspecific symptoms that primarily coincide with GIT and urinary symptoms divert the patient's and clinician's attention away from the ovary. Moreover, despite numerous attempts, no efficient screening approach exists [6].

The most frequent variant of epithelial ovarian cancer is serous carcinoma. Based on biological and histological morphologic criteria such as the degree of nuclear atypia and mitotic count, serous carcinomas are currently categorized into 2 distinct subtypes: low-grade serous carcinoma (LGSC) and high-grade serous carcinoma (HGSC). Tumour stage and postoperative residual mass have an impact on the treatment decision, and the emergence of new molecular markers direct physicians considerations towards clinical prognosis via major therapeutic modification [7].

Forkhead box A1 (FOXA1) is a transcription factor with a winged-helix DNA-deoxy binding domain and N-terminal and C-terminal transcriptional domains, which belongs to the forkhead family. Forkhead box A1 is a key player in the cell cycle, facilitating the G1-S and G2-M transitions through Cyclin E2 upregulation [8]. The cyclin family, including CCNA2, CCND1, CCNB1, and CCNE, has important functions in cell cycle regulation [9, 10].

Forkhead box A1 expression is detected in many organs such as breast, liver, pancreas, and prostate. Forkhead box A1 has been described as a "pioneer factor" that binds to chromatin-packaged DNA and allows other transcription factors, including androgen receptor (AR), to bind to the chromatin. In prostate cancer, FOXA1 binds directly to AR and regulates the transcription of prostate-specific genes [11]. According to recent global gene expression analyses of prostate cancer and triple-negative breast cancer, high FOXA1 expression increases tumour proliferation. As a result, FOXA1 expression in prostate cancer and triple-negative breast cancer is thought to be a predictor of poor prognosis [12].

In ovarian cancer, FOXA1 aids transcription of YAP-associated protein mediated by the cyclic adenosine monophosphate response element-binding protein. As a result of the high YAP activation, cellular proliferation, migration, and chemotherapy resistance increases [13].

The erythropoietin-producing human hepatocellular carcinoma cell (Eph) family of receptors and ligands is the most diverse set of tyrosine kinase receptor-ligand systems, with involvement in brain plasticity, axon guidance, cell migration, tissue segmentation, and angiogenesis [14]. Eph receptors and their ephrin ligands are divided into 2 classes, A and B, based on structural homology and binding affinities. Ephrin-A ligands connect to EphA receptors through a glycosylphosphatidylinositol anchor on the cell membrane, whereas ephrin-B ligands bind to EphB receptors via a transmembrane domain [15].

Eph receptors are thought to play a role in influencing developmental events, especially in the nervous system. The involvement of Eph receptors and ephrin ligands in cell adhesion, migration, compartment formation, cell proliferation regulation in various malignancies, and angiogenesis are well characterized [16].

The EphA5 receptor's role as an axon guidance protein throughout nervous system development is well documented. However, nothing is known about EphA5's potential function in human carcinogenesis. Increased methylation of EphA5 is linked to decreased expression in primary breast cancer, according to Fu *et al*. [17]. Pancreatic adenocarcinoma cases with elevated EphA5 expression had considerably higher tumour cell proliferative capability, according to Giaginis *et al*. [18]. There have been no published findings on the role of EphA5 expression in epithelial ovarian cancer up to this point [19].

The present study aimed to evaluate the immunohistochemical expression FOXA1 and EphA5 expression in serous ovarian carcinoma and correlate their expression with patient survival (Figs. 1, 2, 3).

Material and methods

Clinicopathological data and patients

All patients were selected and underwent operative staging surgery, specimens collection for the histopathological diagnosis and postoperative follow up was done in the Obstetrics and Gynaecology Department, Faculty of Medicine, Zagazig University, Egypt. The surgical procedure was debulking surgery including hysterectomy, bilateral oophorectomy, lymphadenectomy, and omentectomy or maximal debulking.

This study used formalin-fixed, paraffin-embedded tissue specimens from 41 patients at the Department of Pathology, Faculty of Medicine, Zagazig University, Egypt, who were diagnosed with ovarian serous cystadenocarcinoma. The cases were selected and received their chemotherapy during the period from 2016 until the end of 2017. Then we followed them for the next 3 years. After surgical excision, cases received platinum-based chemotherapy in the Clinical Oncology Department, Faculty of Medicine, Zagazig University, Egypt.After excluding cases with insufficient evidence

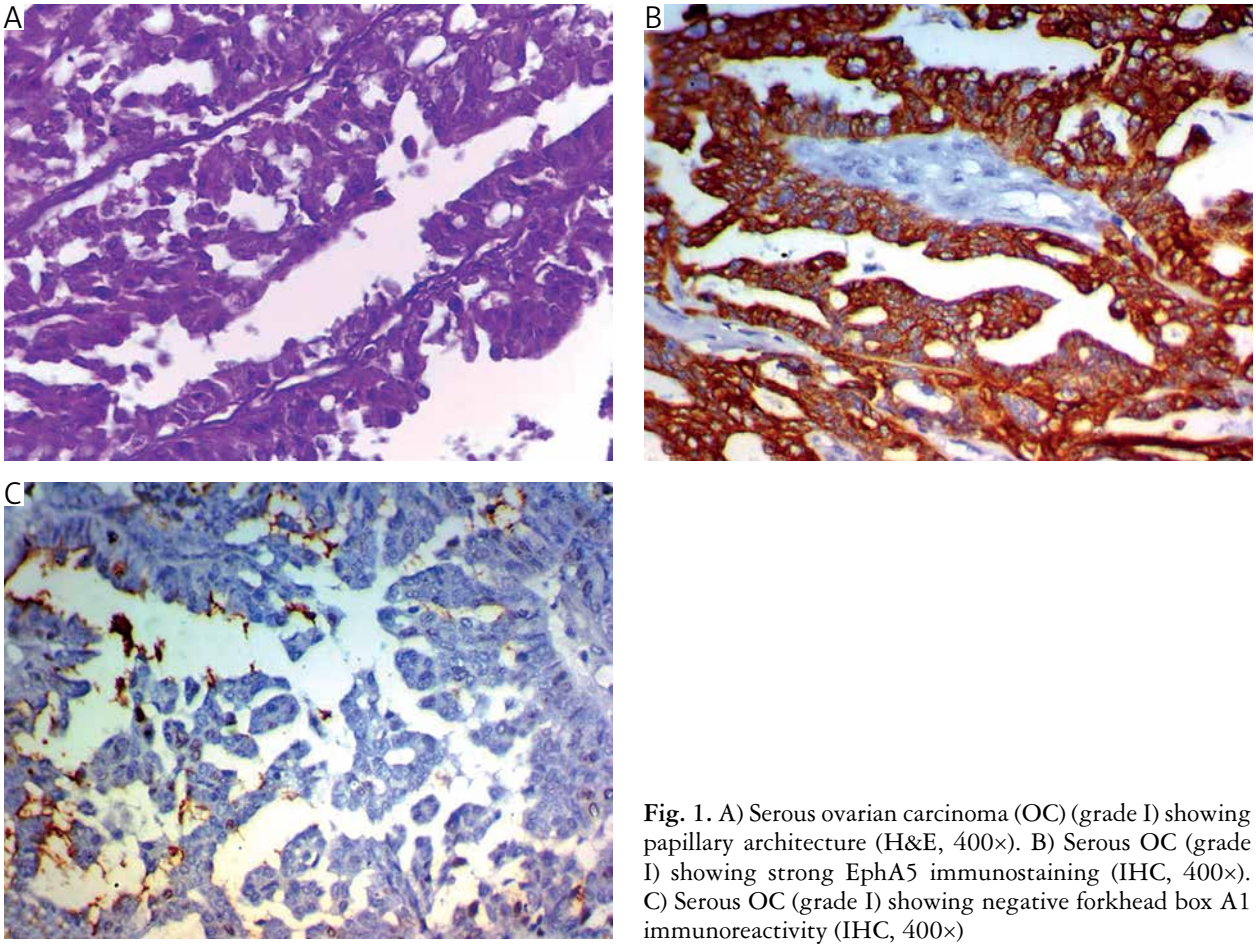

Fig. 1. A) Serous ovarian carcinoma (OC) (grade I) showing papillary architecture (H&E, 400×). B) Serous OC (grade I) showing strong EphA5 immunostaining (IHC, 400×). C) Serous OC (grade I) showing negative forkhead box A1 immunoreactivity (IHC, 400×)

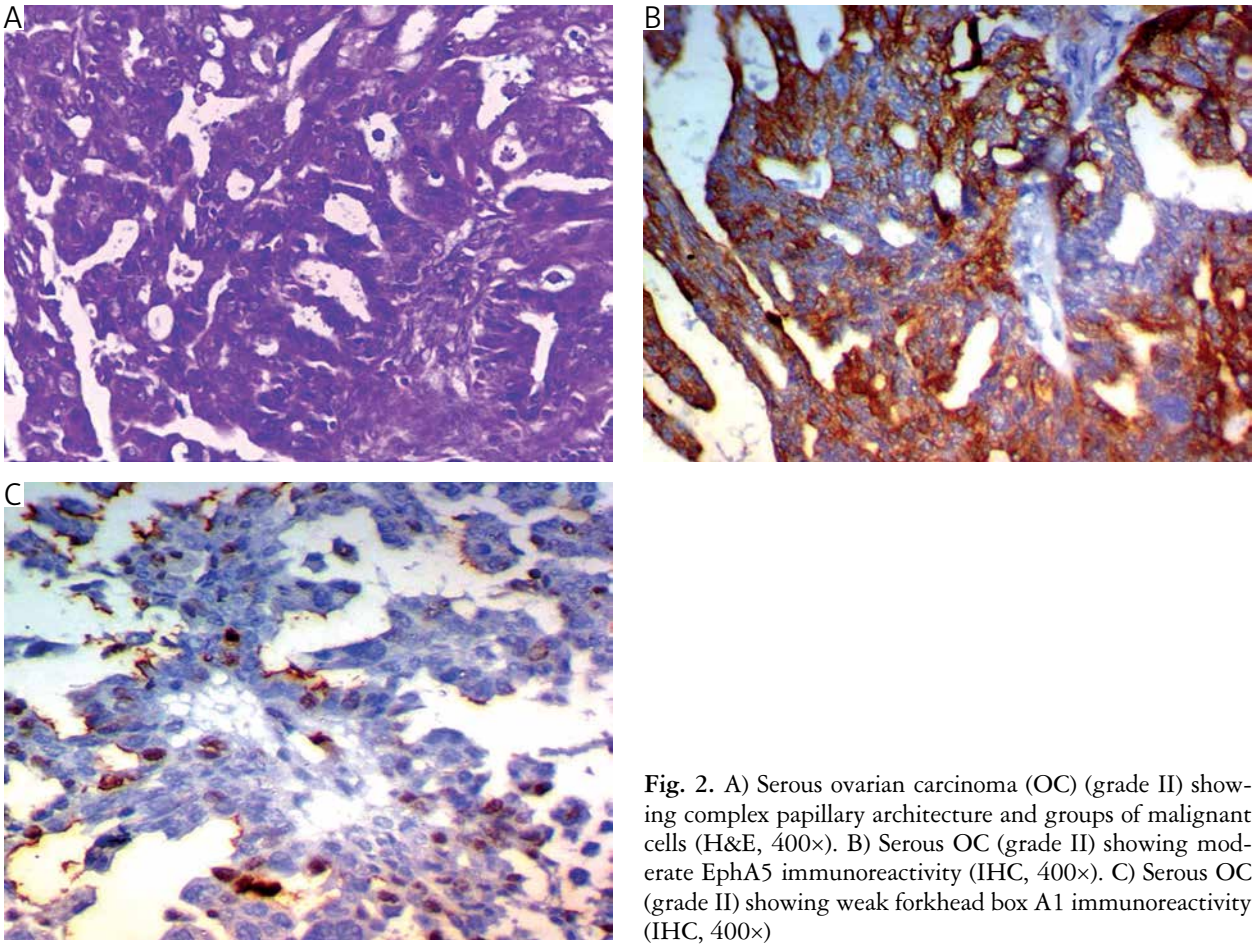

Fig. 2. A) Serous ovarian carcinoma (OC) (grade II) showing complex papillary architecture and groups of malignant cells (H&E, 400×). B) Serous OC (grade II) showing moderate EphA5 immunoreactivity (IHC, 400×). C) Serous OC (grade II) showing weak forkhead box A1 immunoreactivity (IHC, 400×)

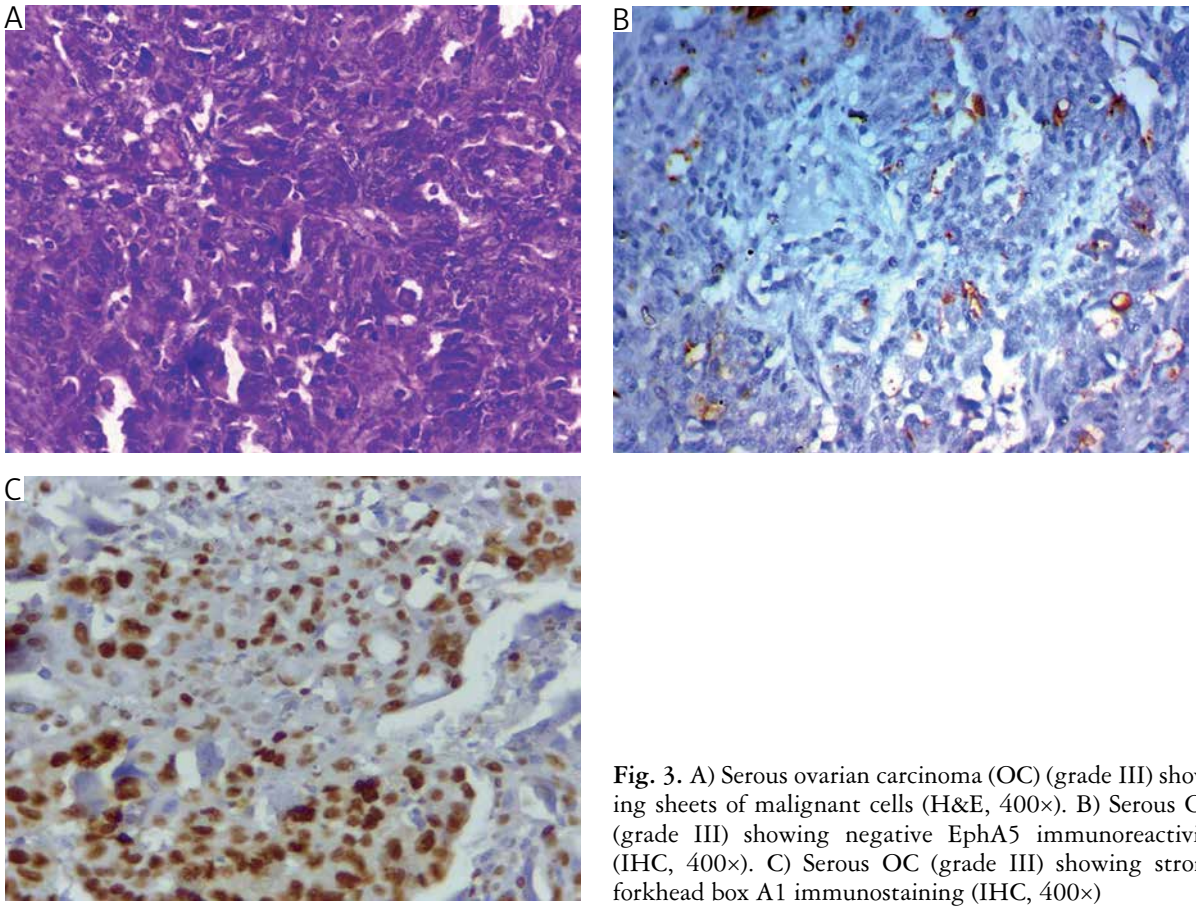

Fig. 3. A) Serous ovarian carcinoma (OC) (grade III) showing sheets of malignant cells (H&E, 400×). B) Serous OC (grade III) showing negative EphA5 immunoreactivity (IHC, 400×). C) Serous OC (grade III) showing strong forkhead box A1 immunostaining (IHC, 400×)

of FIGO stage by accessible slides or tissue blocks and recurrent tumours, the final number of cases was determined (41 cases). All cases were surgical specimens with exclusion of samples from recurring tumours.

Histopathological evaluation of ovarian serous cystadenocarcinoma was made according to the criteria of the World Health Organization. The cases were stratified as LGSC (18 cases) and HGSC (23 cases). Tumour Staging was assessed on the basis of the International Federation of Gynaecology and Obstetrics system (8th edition). This study was undertaken according to the Code of Ethics of the World Medical Association (Declaration of Helsinki) and approved by the research Ethics Committee of Faculty of Medicine, Zagazig University (ZU-IRB #8016).

Immunohistochemical staining

For each case, a representative paraffin-embedded tumour tissue block was chosen for immunohistochemical procedure and serial sections of 3-μm thickness were recut. The staining was done using a typical streptavidin-biotin immunohistochemical procedure. The slides were deparaffinized in xylene and rehydrated in ethanol in a graduated sequence. Antigen retrieval was performed on the deparaffinized sections by boiling for 10 minutes in 0.01 mol/l sodium citrate buffer (pH 6.0) in a microwave oven. We incubated the sections overnight with primary antibodies at 4°C in a humid environment after suppressing endogenous peroxidase activity with 0.3% hydrogen peroxide and 1.5% normal goat serum, respectively. The primary antibody against EphA5 (ab 46CT61.6.4, mouse monoclonal antibody; Thermo Fischer) and FOXA1 (ab JF10-02, rabbit polyclonal antibody; Thermo Fischer) was applied at a dilution 1 : 50 and 1 : 200, respectively, at 4°C in a humid chamber. A biotin-labelled secondary antibody (Universal Link; Agilent Dako, Denmark) was added for 15 min. Then sections were stained for 5 min with 3,3′-diaminobenzidine. Tissues were counterstained with haematoxylin. The primary antibody was replaced with PBS as a negative control.

Evaluation of immunohistochemical data

EphA5 protein expression was evaluated semi-quantitatively according to the intensity of antibody staining in the cytoplasm as follows (0, none; 1, weakly positive; 2, moderately positive; and 3, strongly positive/dark brown). Staining extent was assessed according to the percentage of stained tumour cells and was categorized as follows: 0, 0%; 1, 1% – < 25%; 2, 25–50%; and 3, > 50% positively stained cells. The values of staining extent and staining intensity were added and their final scores were used to define EphA5 protein expression as follows: 0–2, negative (–); and 3–6, positive (+) [20].

Table I. Baseline characteristics of the studied patients

Parameters	$n = 41$	%
Age		
< 50 years	13	31.7
≥ 50 years	28	68.3
Family history		
Negative	35	85,4
Positive	6	14.6
Grade		
Low	18	43.9
High	23	56.1
Staging		
I	5	12.2
I	7	17.1
III	20	48.8
IV	9	22.0
Size		
≤ 5	16	39.0
> 5	25	61.0
Lymphovascular invasion		
No	24	58.5
Yes	17	41.5
Ascites		
No	12	29.3
Yes	29	70.7
Lymph node metastasis		
Negative	15	36.6
Positive	26	63.4
Metastasis		
Absent	32	78.0
Present	9	22.0
CA-125		
Normal	15	36.6
High	26	63.4
Recurrence ($n = 32$)		
No	12	37.5
Yes	20	62.5
Death		
No	19	46.3
Yes	22	53.7
Chemo		
Absent	4	9.8
Present	37	90.2
FOXA1		
Low	13	31.7
High	28	68.3
EphA5		
Negative	25	61.0
Positive	16	39.0

Regarding FOXA1 protein expression, nuclear staining was observed in randomly selected high-power fields ($n = 5$) for each specimen. Positive expression extent was graded as follows: negative = 0; 1–50% = 1; 51–74% = 2; and more than 75% = 3. The staining intensity was evaluated as follows: weak = 1; intermediate = 2; and strong = 3. The final score was obtained by multiplying the extent and intensity score as follows: 0 = –; 1–2 = +; 3–4 = ++; 6–9 = +++). We categorized scores (0 and +) as low expression and (++ and +++) as high expression for statistical reasons [21].

Statistical analysis

The statistical tool SPSS, version 15, was used to examine our findings (SPSS Inc., Chicago, Illinois, USA). For quantitative variables, numbers, and percentages, data were reported as mean SD. Fisher's exact test was employed for categorical variables. Pearson's correlation coefficient was used to examine the correlations between EphA5 and FOXA1 expression. Significant was defined as a p-value of less than 0.05.

Results

About 68% of the studied patients were ≥ 50 years old and 61% had tumour size > 5 cm; 56.1% had high grade tumour and 36.6% showed absent lymph node metastasis. Regarding staging, 12.2%, 17.1%, 48.8%, and 22% had stage I, II, III, and IV, respectively. Lymphovascular invasion occurred in 41.5% of the enrolled cases. Positive FOXA1 and EphA5 presented in 68.3% and 39% of patients, respectively. Recurrence and death occurred in 68.3% and 53.7% of patients, respectively.

Forkhead box A1 expression in ovarian serous carcinoma and its correlation with clinicopathological parameters

No relationship was observed between FOXA1 and the age of the studied patients, family history, tumour size, or the presence of metastasis, ascites, or lymphovascular invasion. A statistically significant relationship was detected between nuclear FOXA1 expression and each of the following: CA-125 level ($p < 0.001$), stage ($p = 0.002$), tumour grade ($p = 0.003$), lymph node metastasis ($p < 0.001$), and recurrence ($p = 0.006$). Similarly, we found a statistically significant relationship between high FOXA1 protein expression and death occurrence ($p = 0.001$) (Table I).

EphA5 expression in ovarian serous carcinoma and its correlation with clinicopathological parameters

EphA5 protein was examined in 41 samples of ovarian serous carcinoma. Twenty-five of 41 (61%)

samples showed negative or weak staining with anti-EphA5 antibody; 16/41 (39%) were moderately or strongly stained. Expression of EphA5 was significantly associated with FIGO stage ($p = 0.007$) and tumour grade ($p = 0.01$). No significant association was found between the expression of EphA5 and patients' age ($p = 0.185$), family history ($p = 0.999$), tumour diameter ($p = 0.62$), and distant metastasis ($p = 0.717$) (Table II).

The impact of forkhead box A1 and EphA5 expression on the patients' survival

The 3-year disease-free survival (DFS) rate of our included cases was 31.7% in all cases, 30.8% in FOXA1 high positive expression, and 69.2% among the EphA5-positive patients. We reported shorter 3-year DFS associated with high FOXA1 and negative EphA5 expressions with statistical significance; the mean 3-year DFS survival in low FOXA1 was 32 ±2.01 months vs. 22.38 ±2.26 months in high FOXA1 expression ($p = 0.009$), while the mean DFS in negative EphA5 was 21.6 ±2.3 months vs. 32.5 ±1.6 months in EphA5 positive expression, with statistical significance ($p = 0.004$) (Table III, Fig. 4).

The estimated 3-year overall survival (OS) of our patients was 46.3% in all cases, 42.1% in patients with high FOXA1 nuclear positivity and 63.9% in cytoplasmic EphA5 positive cases. Patients who exhibited high FOXA1 and negative EphA5 expressions showed significantly shorter 3-year OS – we noticed that the mean 3-year OS in low FOXA1 was 33.69 ±1.64 months vs. 29.36 ±1.27 months in high FOXA1 expression ($p = 0.003$). However, we observed that the mean 3-year OS in negative EphA5 was 28.24 ±1.48 months vs. 34.63 ±0.74 months in EphA5 positive expression, with statistical significance ($p = 0.001$) (Table IV, Fig. 4).

In our work, the OS positively correlated with EphA5 expression ($p = 0.001$) and inversely related to nuclear FOXA1 immunoreactivity ($p = 0.001$). The estimated DFS and EphA5 immunoreactivity had a significant positive association ($p = 0.02$), whereas DFS and FOXA1 protein expression had a significant inverse link ($p = 0.006$) (Table II).

There is non-significant negative correlation between FOXA1 and EphA5 levels (Table V).

We detected a significant relation between OS and tumour grade (mean survival in low grade was 33.78 months vs. 28.35 months in high grade, $p = 0.003$) (Table VI, Fig. 5).

Regarding OS time differences in patients with low-grade tumours in relation to expressions of markers, the mean survival in low FOXA1 was 35.1 months vs. 32.13 months in high FOXA1, with no significance ($p = 0.07$). Moreover, the mean survival in negative EphA5 tumours was 33.11 months vs. 34.44 months in EphA5-positive tumours ($p = 0.6$). Whereas, among patients with high-grade tumours a significant relation was detected between OS and EphA5 (mean survival in negative EphA5 was 25.5 months vs. 34.85 months in positive EphA5, $p \leq 0.001$*). No significance was observed between OS and FOXA1 expression among the high-grade group (Tables VII and VIII, Fig. 6).

The relationship between marker expression in different grades and DFS was estimated. We observed a significant inverse correlation between DFS and EphA5 in patients with high-grade tumours (mean survival in negative EphA5 was 15.06 months vs. 28.29 months in positive EphA5) ($p = 0.005$). No such significance was detected between EphA5 expression and DFS in the low-grade group of patients (mean survival in negative EphA5 was 27 months vs. 32 months in positive EphA5, $p > 0.05$). Moreover, we did not detect a significant relationship between FOXA1 expression and the DFS of patients either with low-grade tumours (mean survival in low FOXA1 was 31.6 months vs. 26.88 months in high FOXA1, $p > 0.05$) or high-grade tumours (mean survival in low FOXA1 was 27 months vs. 17.9 months in high FOXA1, $p > 0.05$) (Tables IX and X, Fig. 7).

Discussion

Gynaecological tumours represent a major problem among Egyptian females. Ovarian cancer ranks as the fourth most common cancer in Egypt [4]. Among all the gynaecological cancers, it has the greatest fatality rate [22]. Several factors contribute to the poor prognosis of ovarian cancer: either its late diagnosis in an advanced stage or its vague symptoms or misdiagnosis [23].

Wang *et al*. explored the oncogenic role of FOXA1 protein in ovarian cancer development and pathogenesis. Their results revealed that in FOXA1-silenced ovarian cancer cell lines, cellular proliferation, migration, and invasion were reduced; apoptotic activity was up-regulated with induction of S-phase arrest. Silencing of FOXA1 protein reduced the expression of many factors, including the YAP, CDK1, CCND1, PI3K, E2F1, Bcl-2, and VEGFA proteins [21].

Forkhead box A1 over-expression is closely related to lung cancer, prostate cancer, and oesophageal cancer pathogenesis. Forkhead box A1 has a distinct role in the prognosis of androgen receptor-dependent prostate cancer as well as oestrogen receptor-positive breast cancer. As regards bladder cancer, muscle-invasive pathological subtypes are associated with reduced FOXA1 expression. Recently, the enhancer elements at epithelial signature genes that are repressed by SNAIL1 in colorectal cancer were found to be significantly associated with FOXA transcription factors. SNAIL1 repression activity enhances the epithelial-mesenchymal transition (EMT) of the tumour

Table II. Relationship between forkhead box A1, EphA5, and both baseline characteristics and outcome of the studied patients

Parameters	Total $N = 41$	FOXA1		*p*	EphA5		*p*
		Low $n = 13$ (%)	High $n = 28$ (%)		Negative $n = 25$ (%)	Positive $n = 16$ (%)	
Age							
< 50 years	13	4 (30.8)	9 (69.2)	> 0.999‡	6 (46.2)	7 (53.8)	0.185‡
≥ 50 years	28	9 (32.1)	19 (67.9)		19 (67.9)	9 (32.1)	
Family history							
Negative	35	11 (31.4)	24 (68.6)	> 0.999‡	21 (60.0)	14 (40.0)	> 0.999‡
Positive	6	2 (33.3)	4 (66.7)		4 (66.7)	2 (33.3)	
Grade							
Low	18	10 (55.6)	8 (44.4)	0.003‡*	7 (38.9)	11 (60.1)	0.01*§
High	23	3 (13.0)	20 (87.0)		18 (78.3)	5 (21.7)	
Staging							
I	5	4 (80.0)	1 (20.0)	0.002*§	1 (20.0)	4 (80.0)	0.007*§
II	7	5 (71.4)	2 (57.1)		3 (42.9)	4 (57.1)	
III	20	2 (25.0)	18 (75.0)		13 (65.0)	7 (35.0)	
IV	9	2 (22.2)	7 (77.8)		8 (88.9)	1 (11.1)	
Size							
≤ 5	16	7 (43.8)	9 (56.2)	0.185‡	9 (56.2)	7 (43.8)	0.62‡
> 5	25	6 (24.0)	19 (76.0)		16 (64.0)	9 (36.0)	
Lymphovascular invasion							
No	24	9 (37.5)	15 (62.5)	0.344‡	14 (58.3)	10 (41.7)	< 0.68‡
Yes	17	4 (23.5)	13 (76.5)		11 (44.0)	14 (56.0)	
Ascites							
No	12	6 (50)	6 (50)	0.105‡	6 (50)	6 (50)	0.354‡
Yes	29	7 (24.1)	22 (75.9)		19 (65.5)	10 (34.5)	
Lymph node metastasis							
Negative	15	10 (66.7)	5 (33.3)	< 0.001*‡	5 (33.3)	10 (66.7)	0.006*‡
Positive	26	3 (11.5)	23 (88.5)		20 (76.9)	6 (23.1)	
Metastasis							
Absent	32	11 (73.3)	21 (26.7)	0.692‡	20 (62.5)	12 (37.5)	0.717‡
Present	9	2 (7.7)	18 (77.8)		5 (55.6)	4 (44.4)	
CA-125							
Normal	15	11 (73.3)	4 (26.7)	< 0.001*‡	5 (33.3)	10 (66.7)	0.006‡*
High	26	2 (7.7)	24 (92.3)		20 (76.9)	6 (23.1)	
Chemo							
Absent	4	2 (50)	2 (50)	0.579	2 (50.0)	2 (50.0)	0.637‡
Present	37	11 (29.7)	26 (70.3)		23 (62.2)	14 (37.8)	
Recurrence ($n = 32$)							
No	12	8 (66.7)	4 (33.3)	0.006*‡	4 (33.3)	8 (66.7)	0.021*‡
Yes	20	3 (15.0)	17 (85.0)		16 (80.0)	4 (20.0)	
Death							
No	19	11 (57.9)	8 (42.1)	0.001*‡	6 (36.1)	13 (63.9)	0.001*‡
Yes	22	2 (9.1)	20 (90.9)		19 (86.4)	3 (13.6)	

FOXA1 – forkhead box A
*‡χ^2, * $p < 0.005$ is statistically significant, §χ^2 for trend test*

Table III. Kaplan-Meier survival curves illustrating disease-free survival time differences in patients regarding marker expressions

Parameters		Total *N*	*N* of events	Censored		Survival time, months		*p*
				N	%	Mean		
						Estimate ±SD	95% CI	
FOXA1	Low	11	3	8	72.7	32.0 ±2.01	28.06–35.94	0.009*
	High	21	17	4	19.0	22.38 ±2.26	17.95–26.81	
EphA5	Negative	20	16	4	20.0	21.6 ±2.31	17.08–26.13	0.004*
	Positive	12	4	8	66.7	32.5 ±1.61	29.34–35.66	
Overall		32	28	12	37.5	25.69 ±1.82	22.12–29.26	

FOXA1 – forkhead box A
**p < 0.05 is statistically significant*

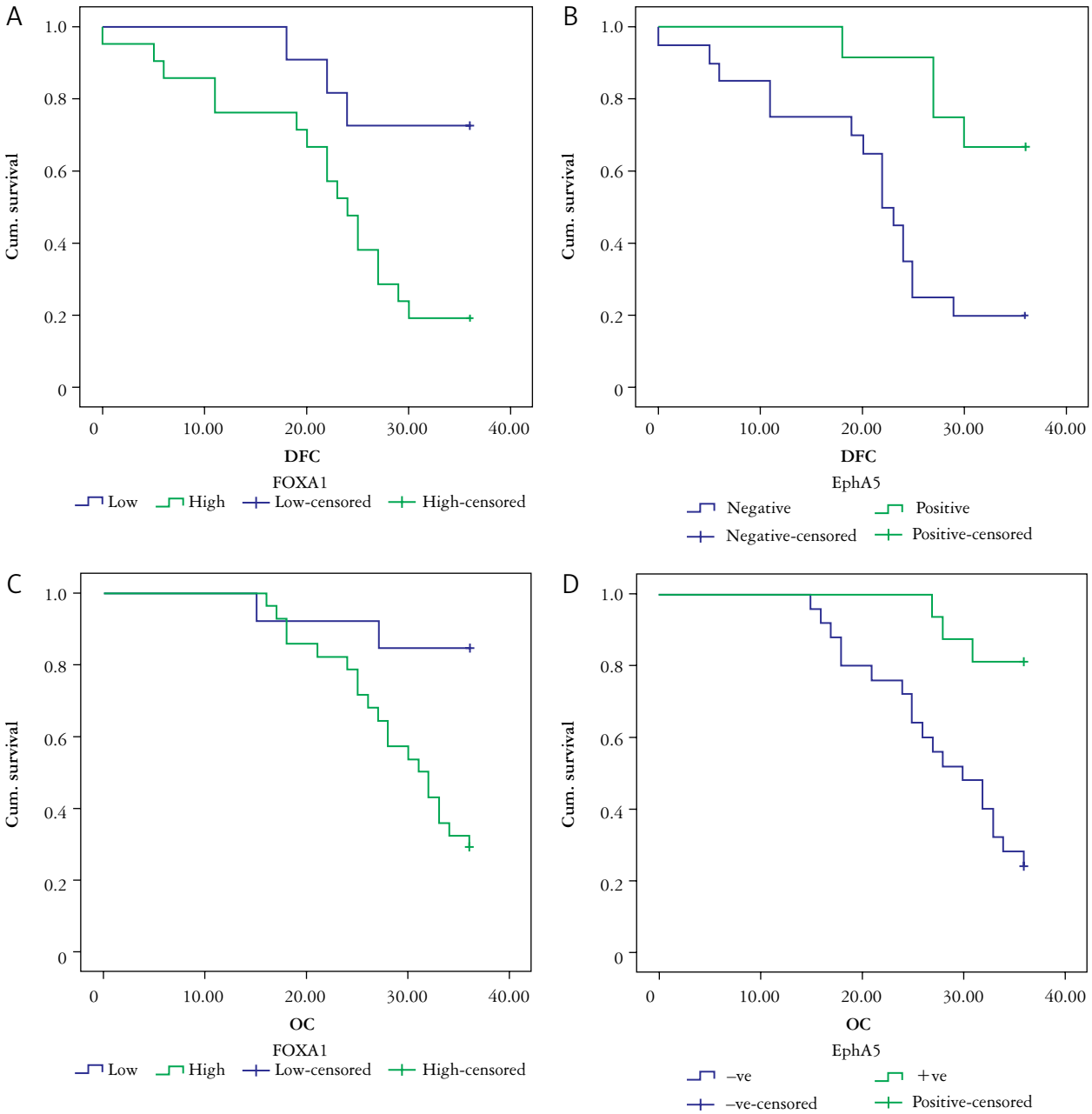

Fig. 4. Kaplan-Meier plot showing the relationship between the following: A) disease-free survival (DFS) of all patients and forkhead box A1 (FOXA1) expression. B) DFS of all patients and EphA5 expression. C) overall survival (OS) of all patients and FOXA1 expression. D) OS and EphA5 expression

DFS – disease-free survival, FOXA1 – forkhead box A1

Table IV. Kaplan-Meier survival curves illustrating overall survival time differences in patients regarding marker expressions

Parameters		Total *N*	*N* of events	Censored		Survival time, months		*p*
				N	%	Mean		
						Estimate ±SD	95% CI	
FOXA1	Low	13	2	11	84.6	33.69 ±1.64	30.48–36.9	0.003*
	High	28	20	8	28.6	29.36 ±1.27	26.86–31.85	
EphA5	Negative	25	19	6	24.0	28.24 ±1.48	25.35–31.13	0.001*
	Positive	16	3	13	81.2	34.63 ±0.74	33.18–36.07	
Overall		41	22	19	46.3	30.73 ±1.07	28.64–32.82	

FOXA1 – forkhead box A
**p < 0.05 is statistically significant*

Table V. Correlation between forkhead box A1 and EphA5 among the studied patients

Parameters	FOXA1		EphA5	
	Phi	*p*	Phi	*p*
FOXA1			–0.207	0.185
EphA5	–0.207	0.185		

FOXA1 – forkhead box A

cells, which confirms the essential role of FOXA factors in maintaining the physiological expression of the epithelial gene network [24].

The mean age of our patients was 58.7 ±6.2 years. This is close to the mean age reported in previous research: 56.44 ±10.08 years [25] and 58.9 years [26].

Among our patients, classic presentation was advanced; stages III and IV were detected in 70.8% of the enrolled cases. Malik [27] reported that stage III or IV accounted for 78% of the cases. Paes *et al*. [28] reported that 56.2% of their cases were stages III and IV. However, Abdel Aziz *et al*. [29] found a higher percentage of stages III and IV (84.3%) among their patients. This could be explained by the low socioeconomic standard in developing countries resulting in tumour progression and late presentation.

In our study, low and high nuclear FOXA1 immunoreactivity was detected in 13/41 (31.7%) and 28/41 (68.2%) of the cases, respectively. This is slightly lower than the results of Wang *et al*. [30]. We found no relationship between FOXA1 protein expression and patients' age, tumour size, or family history of ovarian cancer. Similar results were found in a previous study [30].

The percentage of FOXA1-positive expressing cells increased with an increasing tumour grade: 87% of high-grade and 44% of low-grade tumours exhibited high expression of FOXA1, with statistical significance ($p = 0.003$) (Table II). Our results were in agreement with Wang *et al*. [30].

Among our cases, we observed a significant relationship between FOXA1 expression and tumour stage ($p = 0.002$) as about two thirds of stage IV showed high FOXA1 expression compared to 20% of stage I. Similarly, a highly significant relationship was detected between the serum level of CA125 and nuclear FOXA1expression ($p < 0.001$).

Our cases had a 46.3% OS rate after 3 years of follow-up. The percentage fell within a previously reported range of 40.3-68% [31, 32]. The overall survival was shown to be inversely associated with FOXA1 immunopositivity in our study ($p = 0.001$). This finding is consistent with another study [30], which concluded that FOXA1 is an independent prognostic factor associated with a poor prognosis.

In the current study, we observed an association between high FOXA1 protein expression and unfavourable clinicopathological characteristics: DFS as well as OS. These findings were compatible with those reported in colorectal [33], prostatic [34], and cervical cancer [35]. In contrast, favourable associations had been detected in breast carcinoma [36], hepatocellular [37], cholangiocarcinoma [38], and en-

Table VI. Kaplan-Meier survival curves illustrating overall survival time differences in patients regarding tumour grade

Parameters		Total *N*	*N* of events	Censored		Survival time, months		*p*
				N	%	Mean		
						Estimate ±SD	95% CI	
Grade	Low	18	5	13	72.2	33.78 ±1.16	31.51–36.05	0.003*
	High	23	17	6	26.1	28.35 ±1.48	25.44–31.26	
Overall		41	22	19	46.3	30.73 ±1.07	28.64–32.82	

dometrial carcinoma [39], supporting the hypothesis that the FOXA1 gene may act as an oncogene or tumour-suppressor gene.

The epigenetic alterations of DNA methylation at the promoter region regulate gene transcription. The EphA5 gene has been shown to be suppressed by methylation in prostate cancer, breast cancer, and colorectal carcinoma, showing that EphA5 hypermethylation is crucial during carcinogenesis and tumour progression [40].

EphA5 protein expression was detected in 39% of patients, which is slightly higher than the result obtained by Chen *et al*. [19] (31% of cases). The fact that our study covered both low and high grades could explain this disparity. No significant association was found between the expression of EphA5 and the patients' age ($p = 0.185$), tumour size ($p = 0.62$), and metastasis ($p = 0.717$). This was in concordance with the results of Chen *et al*. [19].

Notably, we found a significant association between EphA5 protein expression and lower tumour grades ($p = 0.0$), early staging ($p = 0.007$), negative lymph nodes ($p = 0.006$), and normal CA-125 levels ($p = 0.006$). The aforementioned association with favourable clinicopathological findings was in agreement with Zhang *et al*. [41], who stated that the levels of Snail and N-cadherin were upregulated in the EphA5 knockdown cells whereas the level of E-cadherin protein was downregulated compared with the enrolled negative controls. This proves the role of EphA5 inhibition in tumour migration and invasion by EMT promotion. Moreover, a recent

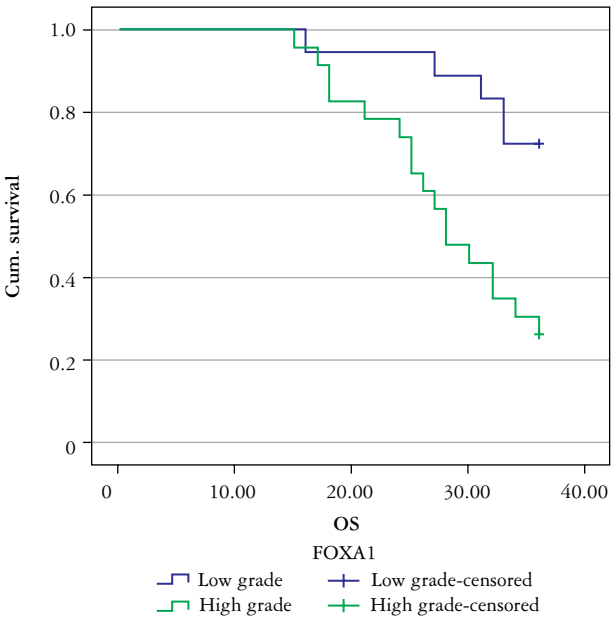

Fig. 5. Kaplan-Meier plot showing relation between overall survival and grade (mean survival in low-grade was 33.78 months vs. 28.35 months in high-grade) ($p < 0.05$)
OS – overall survival

Table VII. Kaplan-Meier survival curves illustrating overall survival time differences in patients regarding marker expressions in low-grade tumours

Parameters		Total *N*	*N* of events	Censored		Survival time, months		*p*
				N	%	Mean		
						Estimate ±SD	95% CI	
FOXA1	Low	10	2	9	90.0	35.1 ±0.85	33.43–36.77	0.079
	High	8	20	4	50.0	32.13 ±2.25	27.72–36.53	
EphA5	Negative	9	3	6	66.7	33.11 ±2.06	25.35–31.13	0.674
	Positive	9	2	7	77.8	34.44 ±1.02	33.18–36.07	
Overall		18	5	13	72.2	33.78 ±1.16	31.51–36.05	

FOXA1 – forkhead box A

Table VIII. Kaplan-Meier survival curves illustrating overall survival time differences in patients regarding marker expressions in high-grade tumours

Parameters		Total *N*	*N* of events	Censored		Survival time, months		*p*
				N	%	Mean		
						Estimate ±SD	95% CI	
FOXA1	Low	3	1	2	66.7%	29.0 ±5.72	17.8–40.2	0.274
	High	20	16	4	20.0	28.25 ±1.46	25.39–31.11	
EphA5	Negative	16	16	0	0.0	25.5 ±1.6	22.37–28.64	< 0.001*
	Positive	7	1	6	85.7	34.86 ±1.06	32.78–36.93	
Overall		23	17	6	26.1	28.35 ±1.48	25.44–31.26	

FOXA1 – forkhead box A

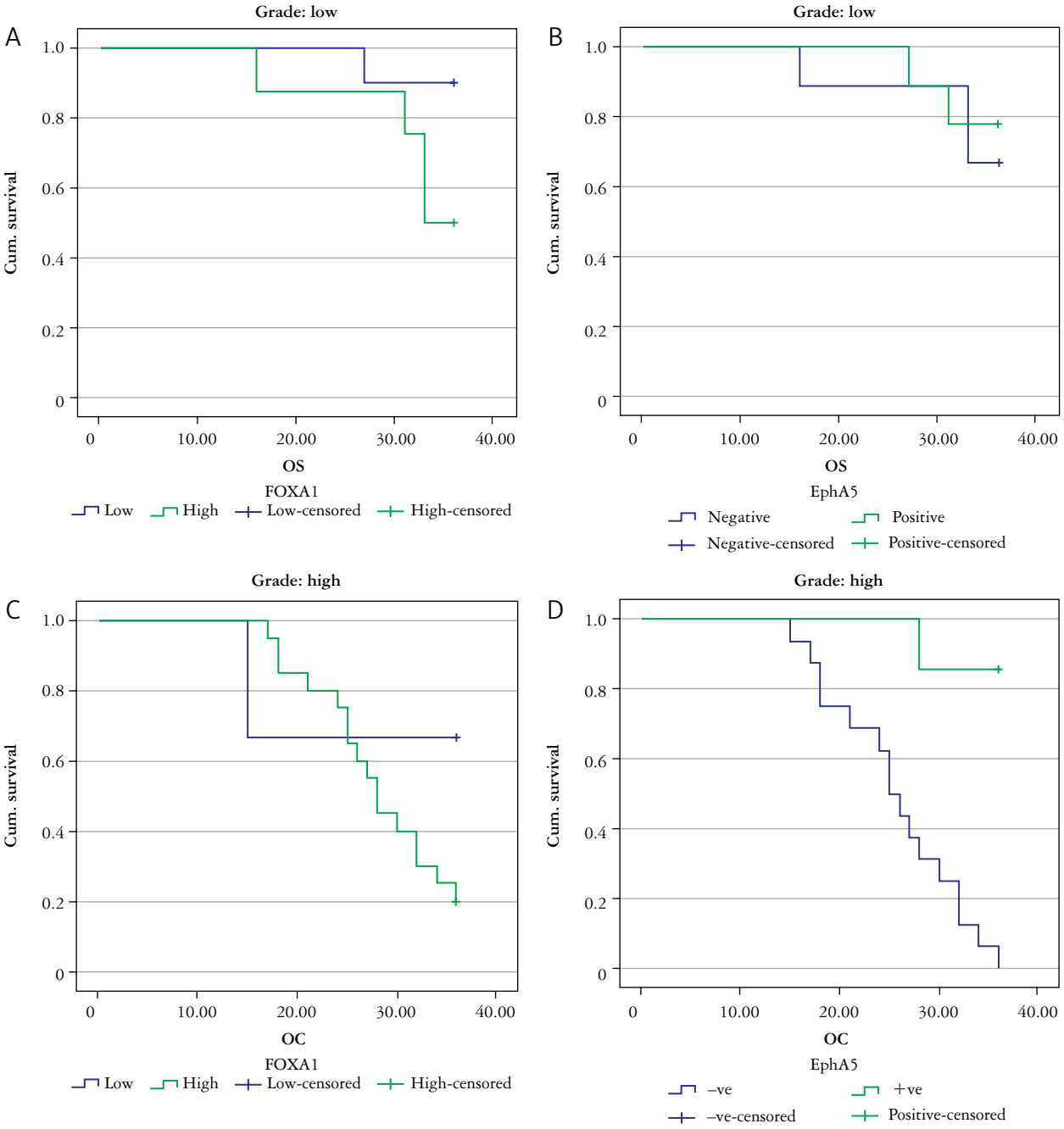

Fig. 6. Kaplan-Meier plot showing the relationship between the following: A) overall survival (OS) in low-grade tumours and forkhead box A1 (FOXA1) expression. B) OS in low-grade tumours and EphA5 expression. C) OS in high-grade tumours and FOXA1 expression. D) OS in high-grade tumours and EphA5 expression

FOXA1 – forkhead box A1, OS – overall survival

study conducted by Li *et al*. [42] showed that loss of EphA5 was associated with higher expression of cancer stem cell (CSC) markers in HER2-positive breast cancer.

Consistent with our research, several authors reported that low EphA5 expression was correlated with lymph node metastasis of colorectal cancer [43], breast cancer [44], gastric cancer [20], and ovarian cancer [19]. In contrast, Staquicini *et al*. [45] discovered that increased EphA5 expression in lung cancer was linked to a higher recurrence rate and a shorter overall patient survival. However, no link was found between EphA5 immunopositivity and lymph node metastases or vascular invasion. EphA5 overexpression has also been observed in high-grade hepatocellular carcinoma [46, 47].

To explain the abovementioned contradictory data, Zhang *et al*. [41] used EphA5 overexpressed plasmids to transfect EphA5 knockout KYSE150 cells. With EphA5 knockdown, they discovered that

Table IX. Kaplan-Meier survival curves illustrating disease-free survival time differences in patients regarding marker expressions in low-grade tumours

Parameters		Total *N*	*n* of events	Censored		Survival time, months		*p*
				n	%	Mean		
						Estimate ±SD	95% CI	
FOXA1	Low	10	3	7	70.0	31.6 ±2.17	27.35–35.85	0.249
	High	8	5	3	37.5	26.88 ±3.01	20.98–32.77	
EphA5	Negative	9	5	4	44.4	27.0 ±2.93	21.26–32.74	0.228
	Positive	9	3	6	66.7	32.0 ±2.06	27.97–36.03	
Overall		18	8	10	55.6	29.5 ±1.88	25.81–33.19	

FOXA1 – forkhead box A

Table X. Kaplan-Meier survival curves illustrating disease-free survival time differences in patients regarding marker expressions in high-grade tumours

Parameters		Total *N*	*n* of events	Censored		Survival time, months		*p*
				n	%	Mean		
						Estimate ±SD	95% CI	
FOXA1	Low	3	1	2	66.7%	27.0 ±7.35	12.6–41.4	0.079
	High	20	19	1	5.0	17.9 ±2.16	13.66–22.14	
EphA5	Negative	16	16	0	0.0	15.06 ±2.34	10.48–19.65	0.005*
	Positive	7	4	3	42.9	28.29 ±2.21	22.61–33.97	
Overall		23	20	3	13.0	19.09 ±2.21	14.76–23.41	

FOXA1 – forkhead box A

EphA5 overexpression could reverse the cancer-associated characteristics in the KYSE150 cells. The latter finding was concordant with a study by Li *et al*. [40], which proved that EphA5 overexpression suppressed the ability of prostatic cancer cells to migrate and invade adjacent as well as distant sites. EphA5 may play various functions in different cancers, which could be the reason for this.

We studied the correlation between the marker expression and survival among patients with different tumour grades. We observed a significant inverse correlation between OS, DFS, and EphA5 in patients with high-grade tumours. No significant association was found between EphA5 expression and survival in patients with low-grade tumours. Similarly, no relation was established between FOXA1 expression and survival in patients either with low- or high-grade tumours. We reviewed the published literature and did not find any data concerning the relation between FOXA1, EphA5 expression, and survival among patients with different tumour grades. To our knowledge, none of the researchers divided their studied ovarian serous carcinomas into low-grade and high-grade groups and evaluated the survival analysis for each group separately. As a result, this point needs further research and studies because it is a worthy subject as both low- and high-grade SC have different prognoses.

Conclusions

Forkhead box A1 is considered an oncogene that plays a key role in ovarian cancer progression through the up-regulation of variable proteins. Consequently, recent methods for diagnosing and treating, and future target and immune therapies with more exploration and a focus on the molecular mechanisms involved in ovarian cancer are warranted, as well as EphA5 expression, which plays an important role in prognosis prediction.

Recommendation

- Studies enrolling more patients with ovarian serous carcinoma are required to evaluate the prognostic role of forkhead box A1 and EphA5, especially in the presence of such contradictions.
- Evaluation of the survival analysis in relation to the marker expression should be conducted separately for low-grade and high-grade tumours.

The authors declare no conflict of interest.

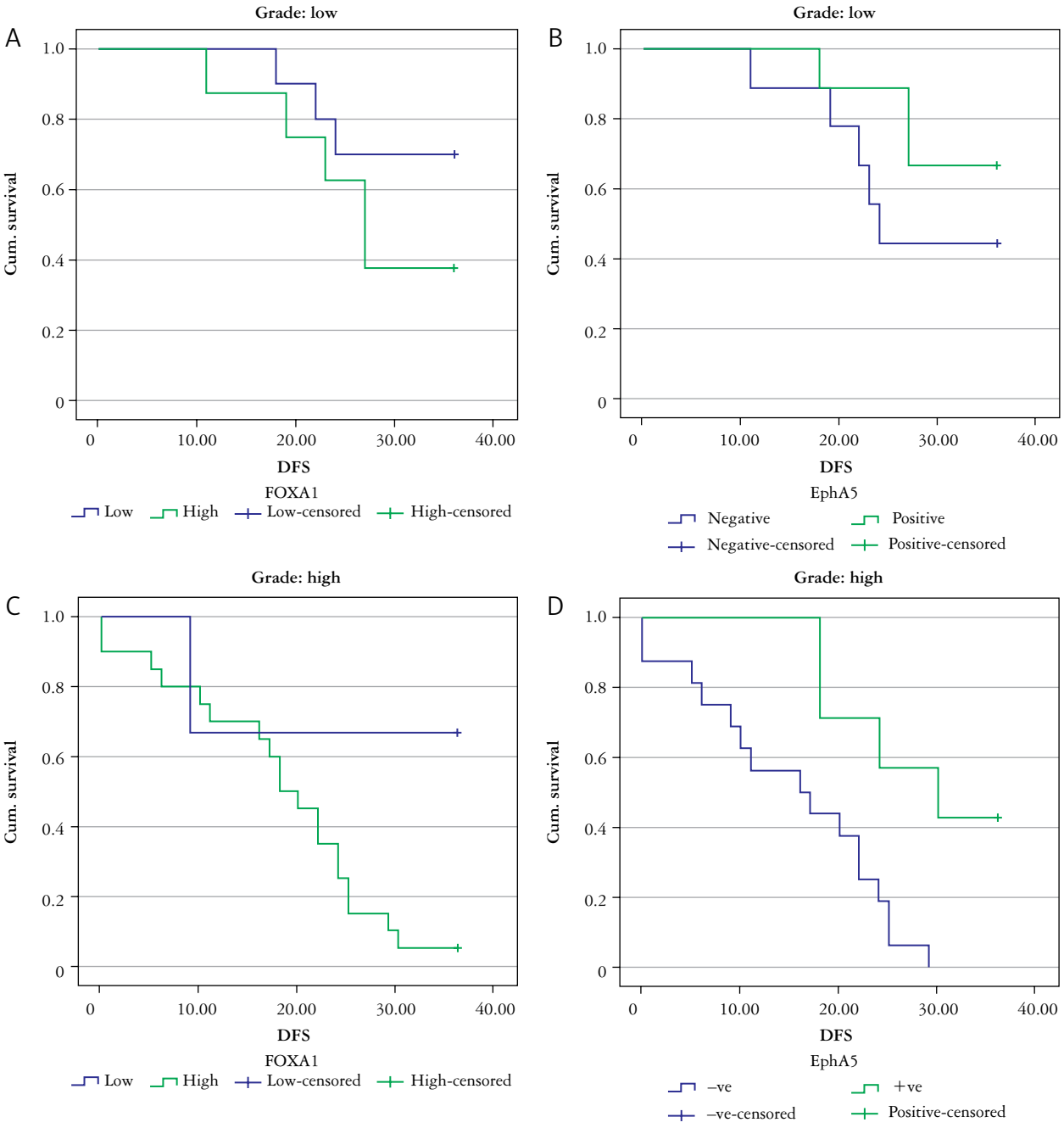

Fig. 7. Kaplan-Meier plot showing the relationship between the following. A) Disease-free survival (DFS) in the low-grade tumours and forkhead box A1 (FOXA1) expression. B) DFS in the low-grade tumours and EphA5 expression. C) DFS in high-grade tumours and FOXA1 expression. D) DFS in high-grade tumours and EphA5 expression

DFS – disease-free survival, FOXA1 – forkhead box A1

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