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Differential expression of Fas family members and Bcl-2 family members in benign versus malignant epithelial ovarian cancer (EOC) in North Indian population

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Abstract Epithelial ovarian cancer (EOC) represents the most challenging of gynecological malignancies. Defective apoptosis is a major causative factor in the development and progression of cancer. The two important pathways of apoptosis are *extrinsic* death receptor pathway (Fas family) and *intrinsic* mitochondrial pathway (Bcl-2 family). In this study, differential protein expression of the major Fas family members (Fas, FasL, and FAP-1) and Bcl-2 family members (Bax, Bcl-2, and Bcl-X_L) in benign versus malignant surface epithelial ovarian tumors was evaluated at the protein level by immunohistochemistry. The expression of these molecules was compared in 30 benign versus 35 malignant surface epithelial ovarian tumors. The findings of the present study showed that there was no significant difference in the expression of the Fas family members in benign and malignant ovarian tumors. However, benign tumors showed higher levels of anti-apoptotic Bcl-2 protein levels ($p < 0.009$), whereas malignant tumors showed higher levels of pro-apoptotic Bax ($p < 0.001$). In general, there was no significant difference in Bcl-X_L protein levels. The observations made in the present study suggest that alterations in expression of the Fas family and the Bcl-2 family members occur and play a key role in the deregulated growth of epithelial ovarian cancer.

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Introduction

Epithelial ovarian cancer (EOC) is a major cause of morbidity and mortality among all gynecological malignancies [1]. The WHO classification divides each subtype of surface epithelial ovarian tumors into three groups: benign cystadenomas, borderline tumors (intermediate, low malignant potential, LMP), and malignant (invasive) carcinomas, reflecting their clinical behavior [2, 3]. There has been little change in ovarian cancer incidence and mortality over the past 30 years. Although tumor markers are available for monitoring ovarian cancer, their clinical utility remains unclear, and many individual markers are limited in specificity or sensitivity [3]. Over these years, the most important realization in understanding cancer biology is the involvement of apoptosis-regulating genes in the process of oncogenesis.

Cell turnover in normal tissues is regulated by the balance between the rates of cell proliferation and cell death [4]. Consequently, uncontrolled neoplastic growth can be caused not only by increased proliferation but also by a diminished rate of cell death, which can result from the failure of cells to undergo apoptosis. Apoptosis occurs through two main pathways. The first, referred to as the *extrinsic* or death receptor pathway, is triggered through the Fas death receptor. When a death stimulus triggers this pathway, the membrane-bound FasL interacts with the inactive Fas complexes and forms the death-inducing signaling complex (DISC) [5]. The Fas DISC contains the adaptor protein Fas-associated death domain (FADD) protein and caspases-8 and -10, which further activate the

effector caspase-3. Functionally, Fas signaling can be inhibited by a phosphatase termed Fas-associated phosphatase-1 (FAP-1) [6, 7]. Binding of FAP-1 to Fas prevents the binding of FADD and thereby leading to the inhibition of further signaling cascade.

The *intrinsic* or mitochondrial-dependent pathway for apoptosis is governed by Bcl-2 family proteins. Bcl-2 family members act by regulating the efflux of apoptogenic proteins from mitochondria [8, 9]. Members of Bcl-2 family contain from one to four Bcl-2 homology (BH) domains. The number and combination of the BH domains dictate whether the protein is pro-apoptotic or anti-apoptotic. Anti-apoptotic Bcl-2 family members contain all four BH domains and include Bcl-2, Bcl-X_L, Mcl-1, Bcl-w, and Bf1-1/A1. Pro-apoptotic members lack the BH4 domain and are divided into two groups, the "BH3-only" members (Bid, Bik, and Bim) and the multidomain BH1-3 pro-apoptotic members (Bax and Bak) [9]. The ratio of these molecules plays a central role in the regulation of apoptotic machinery. Following a death signal, pro-apoptotic proteins undergo post-translational modifications resulting in their activation and translocation to the mitochondria leading to apoptosis. In response to apoptotic stimuli, the outer mitochondrial membrane becomes permeable; leading to the release of cytochrome *c*. Cytochrome *c* binds and activates Apaf-1 as well as procaspase-9, forming an "apoptosome" [10]. Pro-caspase 9 promotes its self-activation which further leads to the activation of one of the effector caspases. Both *extrinsic* and *intrinsic* pathways of apoptosis converge to a final common pathway involving the activation of a cascade of proteases called caspases that cleave regulatory and structural molecules, culminating in cell death [11].

There are only a few and contradictory reports available in the literature regarding the expression of Fas and Fas-related molecules in normal ovaries, benign tumors, and ovarian cancer [12, 13]. Ben-Hur et al. [14] and Zusman et al. [15] have reported decreased Fas expression in malignant ovarian tumors compared to benign and borderline tumors, whereas Munakata et al. [16] found no difference according to the histologic category. In their study, Munakata et al. [16] further reported that tumors expressing FasL showed a less favorable prognosis than those without FasL expression. In another study on FAP-1 molecule, Meinhold–Heerlien et al. [17] have shown that FAP-1 expression correlates significantly with Fas resistance in ovarian cancer cells lines and is commonly expressed in ovarian cancer. Thereafter, no further reports are available till date on FAP-1 expression in ovarian cancer patients. Moreover, reports regarding the differential expression of FAP-1 in benign versus malignant ovarian tumor are lacking. However, altered expression of Bcl-2 family members has also been implicated in ovarian tumorigenesis [18, 19]. Differential expression of Bcl-2 family members has been investigated in epithelial ovarian tumors including serous and mucinous benign, borderline, and malignant tumors [20]. Bcl-2 was found to be strongly expressed in the surface epithelium of normal ovaries and also in benign and borderline ovarian tumors, but only weakly in malignant tumors [21]. In contrast, Zusman et al. [15] reported intermediate Bcl-2 expression in borderline ovarian tumors compared to benign and malignant tumors. Wehrli et al. [20] showed a decreased Bcl-X expression in serous malignant ovarian tumors compared to benign and borderline serous tumors, but no such difference in mucinous ovarian tumors. Previous studies have shown no difference in Bax expression between benign, borderline, and malignant ovarian tumors [16, 20].

Epithelial ovarian cancer is often asymptomatic in its early stages; therefore, most patients are diagnosed late in stage III and IV. The origin of malignant tumors, an intricate process could only be resolved by understanding the molecular mechanisms of tumorigenesis. Therefore, we have evaluated the expression of major Fas family members (Fas, FasL, and FAP-1) and Bcl-2 family members (Bax, Bcl-2, and Bcl-X_L) in benign versus malignant surface epithelial ovarian tumors at the protein level by immunohistochemistry.

Materials and methods

Patients and tissue samples

The case material for this study included patients of ovarian cancer, who were routinely diagnosed and managed by the Department of Obstetrics and Gynaecology, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh. A total of 35 cases of malignant surface epithelial ovarian tumors were included for this study. An informed consent was obtained from every patient enrolled in this study as per Institute's Ethical Committee guidelines before the surgical procedures. After surgery, the ovarian tissue was immersed in 10 % buffered formalin and submitted for routine histopathologic examination in the Department of Cytology and Gynaecological Pathology, PGIMER. After fixation for at least 24 h, the paraffin-embedded blocks from the tissue were prepared. Serial section was cut on slides coated with poly-L-lysine (Sigma Chemical Co., St. Louis, MO, USA) and stored for immunohistochemistry. Following histopathologic confirmation for epithelial ovarian tumors, further molecular analysis was done.

Thirty cases of benign surface epithelial ovarian tumors were retrospectively taken from the archives of the Department of Cytology & Gynaecological Pathology, PGIMER.

Analysis of protein expression by immunohistochemistry (Fas, FasL, FAP-1, Bcl-2, Bcl-X_L, and Bax)

For the comparison of expression of these molecules, 30 benign and 35 malignant surface epithelial ovarian tumors were included. Immunohistochemistry was done by the Biotin–Streptavidin–Peroxidase Complex method. In this method, an unconjugated primary antibody binds to the antigen in the specimen. This attachment was detected by the binding of a biotinylated secondary antibody followed by the use of horseradish-peroxidase-conjugated streptavidin and an appropriate substrate such as 3-3′ diaminobenzidine tetrahydrochloride (DAB). From the paraffin-embedded blocks of formalin-fixed tissues, 5-μm sections were made and mounted on slides coated with poly-L-lysine (Sigma Chemical Co., St. Louis, MO, USA). The sections were then fixed at 56 °C for 30 min and stored for subsequent staining. Before staining, the sections were deparaffinized by heating them at 60 °C, followed by serial passages through few changes of xylene and graded alcohol (100 %, 95 % and 70 %). The endogenous peroxidase activity was blocked by incubating the sections with the blocking solution (0.5 % H_2O_2 in methanol) for 20 min. For Bcl-2, Bcl-X_L, and Bax the antigenic sites were unmasked by means of three cycles of 5-min microwave irradiation in 10 mM citrate buffer (pH 6.0). This treatment for unmasking the antigenic sites was not done for Fas, FasL, and FAP-1. This was followed by blocking with normal horse serum (Novastain Universal Detection Kit, Novacastra Laboratories Ltd., Newcastle, UK) for 1 h. The primary antibodies against the respective proteins were applied onto the sections which were then incubated overnight at 4 °C. All the primary antibodies were obtained from Santa Cruz Biotechnology Inc. CA, USA. The specifications of the primary antibodies and the concentrations used are given in Table 1.

Primary antibody binding was revealed using the Novastain Universal Detection Kit (Ready to use, Novacastra Laboratories Ltd., Newcastle, UK). The sections were then incubated with the biotinylated secondary antibody for 45 min at room temperature, washed three times with PBS followed by application of ready to use streptavidin-peroxidase complex reagent for 45 min at room temperature. After PBS washings, the sections were incubated in peroxidase substrate solution, i.e., with 3-3′ Diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co., St. Louis, USA) for 5–10 min. The sections were then rinsed in tap water, counter stained with hematoxylin, and mounted with DPX. The brown product obtained was visualized and scored by light microscopy. In all batches, a section of tonsil or reactive lymph node served as a positive control. The negative control was served by the omission of the primary antibody.

Scoring of immunostaining

Scoring of immunohistochemistry was done by the scoring system used previously by Miyamoto et al. [22]. The immunohistochemistry results were scored by taking the percentage positivity and staining intensity into account. An intensity score of 0 (no staining), 1 (weak positivity), 2 (moderate positivity), and 3 (strong positivity) was given. The immunohistochemistry (IHC) score was recorded by multiplying the percentage positivity with intensity score obtained.

$$\text{IHC score} = \%\text{age positivity} \times \text{intensity score}$$

Therefore, the final IHC score can range from 0 to 300.

Statistical analysis

The mean and median levels of the expression of each molecule investigated in this study were calculated. For comparison of the expression in different groups, Student's *t* test (comparison of mean levels) and Mann–Whitney *U* test (comparison of median levels) were done. For the analysis, a two-tailed *p* value of less than 0.05 was considered a significant difference.

Results

In the present study, 35 cases of malignant surface epithelial ovarian cancer were included prospectively. Thirty cases of benign surface epithelial ovarian tumors were retrospectively taken from the archives for comparison.

Table 1 List of antibodies used in the study

Antigen	Antibody clone	Antigen retrieval	Primary antibody incubation	Concentration used
Fas	Polyab Fas Clone N-20	None	Overnight at 4 °C	2 μg/ml (1:100)
FasL	Polyab FasL Clone C-20	None	Overnight at 4 °C	4 μg/ml (1:50)
FAP-1	Polyab FAP-1 Clone H-300	None	Overnight at 4 °C	4 μg/ml (1:50)
Bcl-2	Mab Bcl-2 Clone 100	Microwave	2 h at RT	4 μg/ml (1:50)
Bcl-X_L	Mab Bcl-X_L Clone H-5	Microwave	2 h at RT	4 μg/ml (1:50)
Bax	Mab Bax Clone B-9	Microwave	2 h at RT	4 μg/ml (1:50)

The differential protein expression of major Fas family members (Fas, FasL, and FAP-1) and major Bcl-2 family members (Bax, Bcl-2, and Bcl-X_L) in benign versus malignant ovarian tumors was investigated. The result of their expression in malignant surface epithelial tumors versus their benign counterparts is presented.

Differential protein expression of major Fas family and Bcl-2 family members in benign versus malignant surface epithelial ovarian tumors

Immunohistochemistry was performed to evaluate the difference in protein expression of major Fas family members (Fas, FasL, and FAP-1) and the Bcl-2 family members (Bax, Bcl-2, and Bcl-X_L) in benign versus malignant surface epithelial ovarian tumors. Immunohistochemistry was performed using the antibodies mentioned in "Materials and methods" section. The staining for Fas, FasL, and FAP-1 [Fig. 1] and for Bax, Bcl-2, and Bcl-X_L [Fig. 2] was mainly identified in the cytoplasm of the cells as diffuse or sometimes focal positivity. In addition, the staining for Fas was also seen at the cell membrane. Scoring of the immunohistochemistry (IHC) was performed as described previously.

Fas family members (Fas, FasL, and FAP-1)

Comparison of the IHC scores in benign versus malignant tumors was performed by the Student's *t* test and the Mann–Whitney *U* test, and the results are shown in Table 2 and in the histogram shown in Fig. 3.

In general, both benign as well as malignant tumors showed moderate levels of Fas and FAP-1 with no statistically significant differences in their expression in the two groups. On the other hand, FasL was expressed at very low levels in both groups; the difference was also not statistically significant.

Bcl-2 family members (Bax, Bcl-2, and Bcl-X_L)

The immunoreactivity for the Bcl-2 family proteins was cytoplasmic (Fig. 2). The difference in mean and median immunohistochemical scores for the Bcl-2 family members in the benign versus the malignant tumors is shown in Table 3 and in the histogram shown in Fig. 3.

Bax, the prototype pro-apoptotic molecule, showed a significant difference in the benign versus the malignant ovarian tumors ($p = 0.001$). It was observed that most of the benign tumors showed low levels or were negative for Bax expression as compared to malignant tumors, where most of the tumors were strongly positive. On the other hand, the anti-apoptotic Bcl-2 levels were significantly lower in malignant tumors as compared to benign ovarian tumors ($p = 0.009$, Student's *t* test). No difference was observed for Bcl-X_L protein levels in the two groups.

The observations made in the present study suggest that alterations in expression of the Bcl-2 family members could play a key role in the deregulated growth of epithelial ovarian cancer. Malignant surface epithelial tumors show high levels of the pro-apoptotic Bax protein and lower levels of the anti-apoptotic Bcl-2 protein tilting the balance in favor of higher apoptotic rates in malignant tumors.

Discussion

In the present study, we investigated the protein expression of Fas family (Fas, FasL, and FAP-1) and Bcl-2 (Bax, Bcl-2, and Bcl-X_L) family members in benign versus malignant surface epithelial ovarian tumors by immunohistochemistry.

There are a few reports [13, 16, 23] where the expression of Fas and FasL is compared in benign versus malignant surface epithelial ovarian cancer; this is the first study to compare the differential protein expression of FAP-1 in benign and malignant surface epithelial ovarian cancer. The protein levels were analyzed by immunohistochemical scores to make it more objective. There was no difference in the expression of Fas, FasL, and FAP-1 in benign versus malignant tumors. Munakata et al. [16] in their study reported that there was no difference in Fas expression in benign versus malignant epithelial ovarian tumors and this study confirms their observation. On the other hand, while FasL expression was higher in malignant ovarian tumors compared to the benign tumors in previous reports [13, 16, 23], no such difference was observed in the present study. This discrepancy could be because of the differences in the scoring methods. Although the benign tumors showed higher levels of FAP-1 as compared to malignant tumors, the difference was not statistically significant. There are only a few studies on the expression of Fas and Fas-related molecules in normal ovaries and ovarian cancer. van Haften-Day et al. [13] have studied Fas and FasL expression in benign, borderline, and malignant ovarian epithelial tumors and reported a significantly increased Fas expression in borderline tumors, whereas FasL expression was increased in malignant tumors.

Immunohistochemical results for the Bcl-2 family members showed that malignant tumors showed a significantly higher expression of Bax and lower levels of Bcl-2 as compared to benign tumors with no difference in Bcl-X_L expression suggesting that Bcl-2 is a stronger than Bcl-X_L in inhibiting apoptosis in ovarian tissue. Differential expression of Bcl-2 family members has been investigated previously in epithelial ovarian tumors including serous and mucinous benign, borderline, and malignant tumors. In

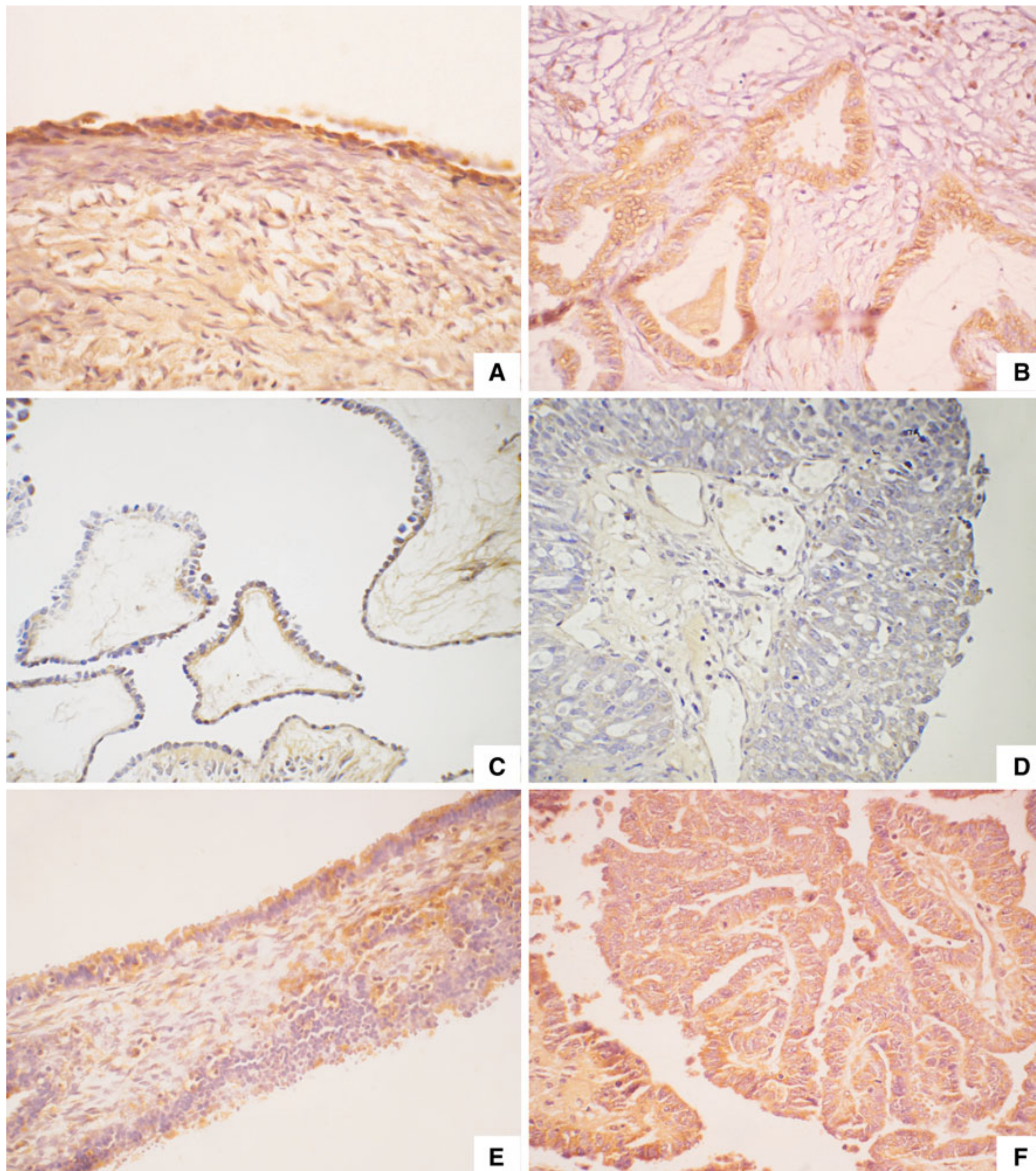

Fig. 1 Immunohistochemical expression of Fas family members in Benign (**a**, **c**, **e**) versus Malignant (**b**, **d**, **f**) surface epithelial ovarian tumors. **a**, **b**: Fas; **c**, **d**: FasL; **e**, **f**: FAP-1 (Biotin–Streptavidin–Peroxidase immunostaining; OM. ×400)

general, Bcl-2 expression is higher in normal epithelium and benign tumors as compared to their malignant counterparts [14, 18–21]. The converse is true of Bax, which is present at higher levels in malignant tumors [18, 19]. Bcl-X_L has been examined in an occasional study and no significant difference was observed [24]. Wehrli et al. [20] have also reported that malignant tumors showed a significantly less immunoreactivity for Bcl-2 and Bcl-X proteins as compared to their benign counterparts. No difference was seen in immunostaining for Bax or Mcl-1. In another study, differential expression of Bcl-2 family members has been investigated in epithelial ovarian tumors including serous and mucinous benign, borderline, and malignant tumors; it was reported that Bcl-2 was higher in normal tissue, whereas Bax and Bcl-X_L were higher in carcinoma [19]. Thus, our study is consistent with the previous reports on the differential expression of Bcl-2 family members in benign versus malignant surface epithelial ovarian tumors. The differential expression of Bax and Bcl-2 in benign versus malignant surface epithelial

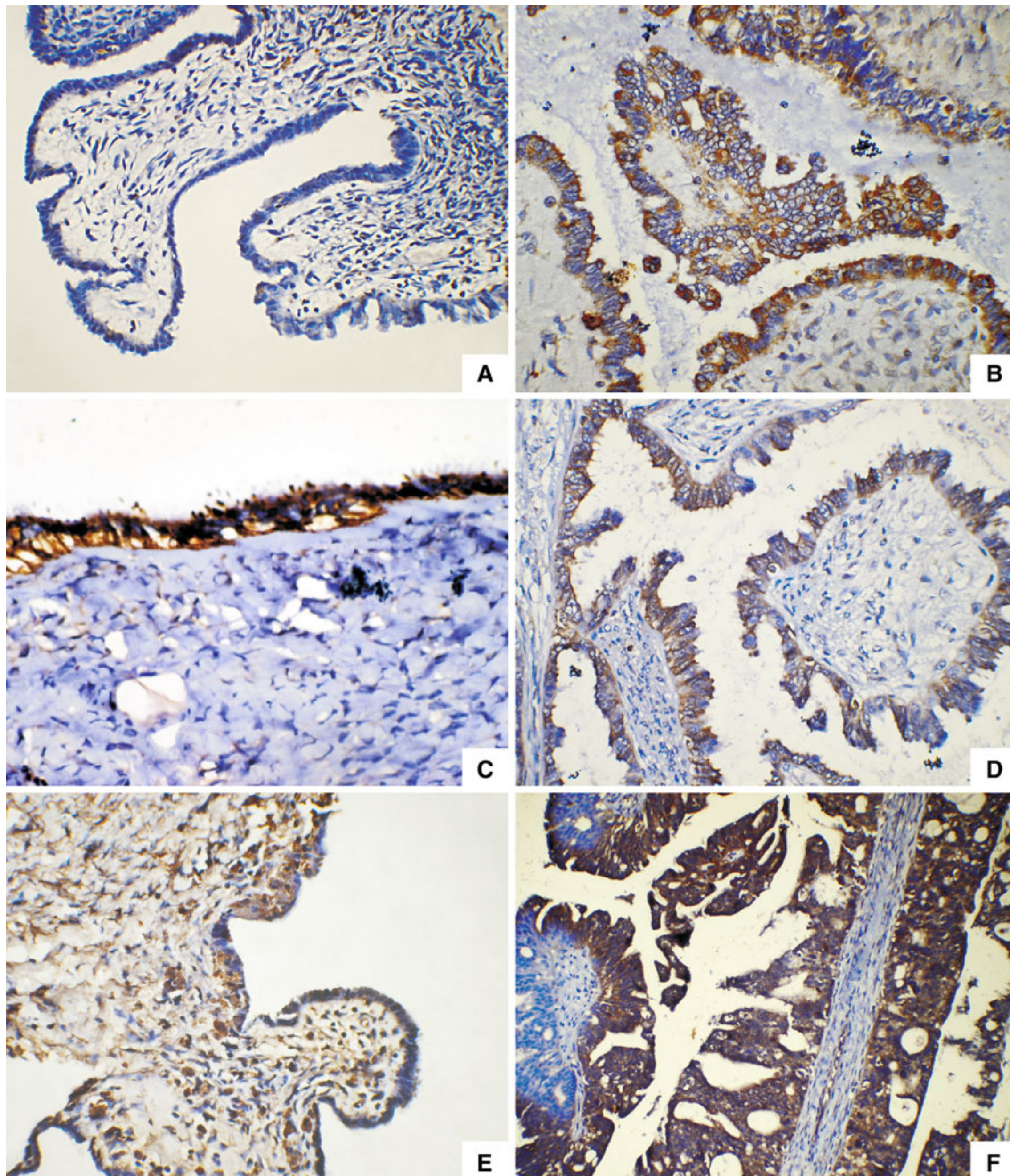

Fig. 2 Immunohistochemical expression of Bcl-2 family members in Benign (**a**, **c**, **e**) versus Malignant (**b**, **d**, **f**) surface epithelial ovarian tumors. **a**, **b**: Bax; **c**, **d**: Bcl-2; **e**, **f**: Bcl-X_L (Biotin–Streptavidin–Peroxidase immunostaining; OM. ×400)

Table 2 Statistical analysis of the differences in the protein levels of Fas family members in benign versus malignant surface epithelial ovarian tumors

	Benign tumors		Malignant tumors		Student's *t* test (*p* value)	Mann–Whitney *U* test (*p* value)
	Mean ± SD	Median	Mean ± SD	Median		
Fas	110 ± 94	100	121 ± 88	100	0.637	0.678
FasL	72 ± 84	0	41 ± 66	0	0.106	0.144
FAP-1	110 ± 105	100	113 ± 93	100	0.901	0.810

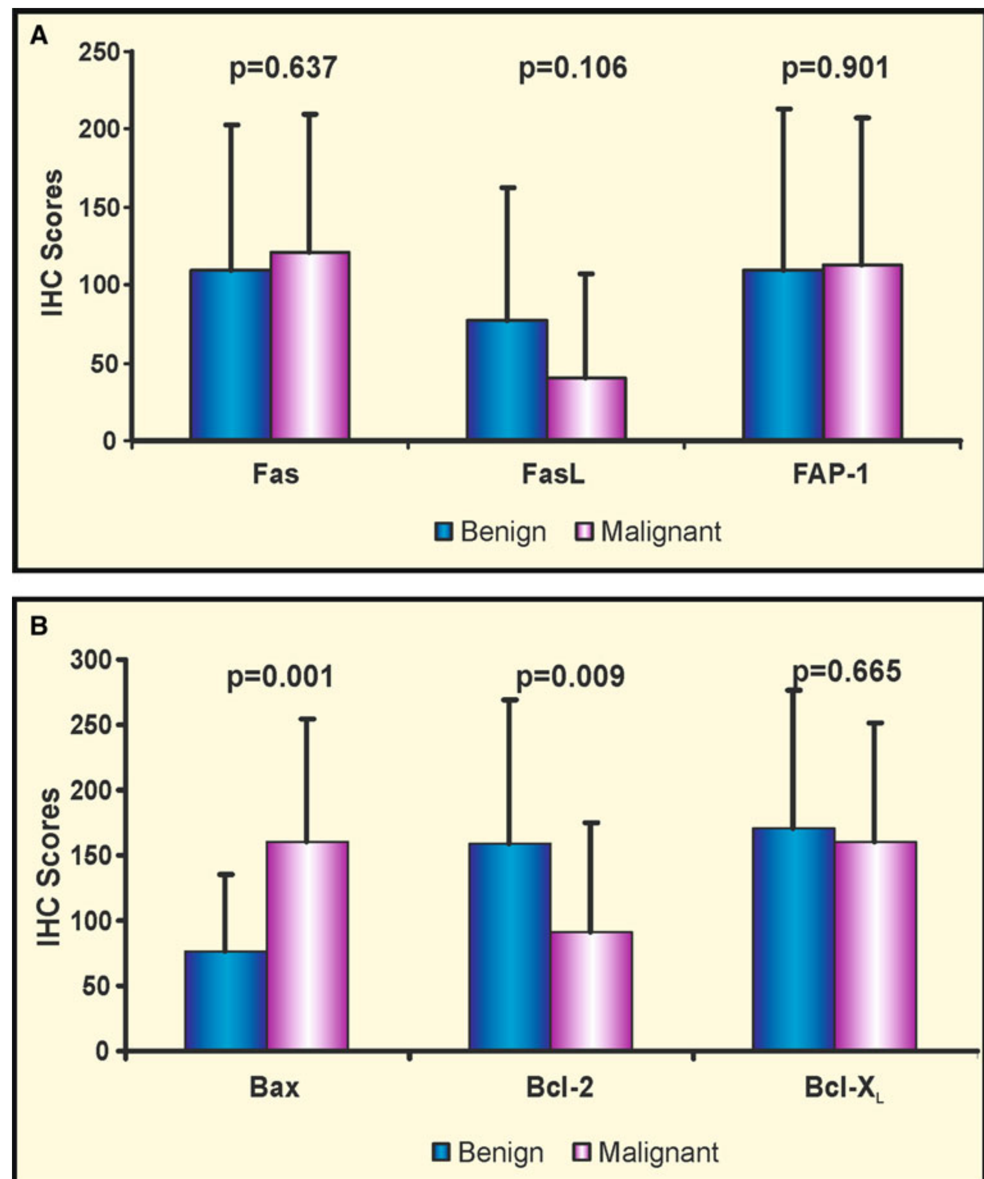

Fig. 3 Histograms showing differential expression of the protein levels of (**a**) Fas family members and (**b**) Bcl-2 family members in *Benign* versus *Malignant* surface epithelial ovarian tumors. Note a significant difference in the protein levels of Bax and Bcl-2

Table 3 Statistical analysis of the differences in the protein levels of Bcl-2 family members in benign versus malignant surface epithelial ovarian tumors

	Benign tumors		Malignant tumors		Student's *t* test (*p* value)	Mann–Whitney *U* test (*p* value)
	Mean ± SD	Median	Mean ± SD	Median		
Bax	76 ± 60	80	160 ± 95	125	0.001	0.001
Bcl-2	159 ± 110	150	91 ± 84	90	0.009	0.009
Bcl-X$_L$	171 ± 105	200	160 ± 92	200	0.665	0.595

ovarian tumors thus plays an important role in the development or maintenance of malignant phenotype.

In conclusion, deregulation in the expression of Bcl-2 family members might play a functional role in the biology of epithelial ovarian tumors. These results suggest that not only the expression but also a balance between anti-apoptotic Bcl-2 and pro-apoptotic Bax is crucial for the apoptosis induction in epithelial ovarian tumors. Further studies

are required to determine whether the expression pattern of these apoptosis-related proteins in epithelial ovarian tumors is related to clinical or histologic prognostic factors.

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Conflict of interest There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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