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Expression of apoptosis-related proteins in endometriomas and benign and malignant ovarian tumours

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Abstract Apoptosis is a physiological process by which multicellular organisms eliminate superfluous cells. Alterations in apoptosis play a key role in tumour development. The objective was to evaluate the immunohistochemical expression of p53, p21, bax, bak, fas, bcl-2 and bcl-x proteins in 10 endometriomas, 20 benign ovarian tumours (10 mucinous, 10 serous) and 30 malignant ovarian tumours (9 mucinous, 19 serous; 2 endometrioids). p53 positive cells (mean±SD) in endometriomas, and benign and malignant tumours were 1.9±3.2, 0 and 16.2±33.0, respectively. The difference was significant between benign tumours and endometriomas ($P=0.003$) but not between endometriomas and malignant tumours. P21 expression in endometriomas and benign and malignant tumours was 19.5±27.8, 1.7±6.7 and 4.1±8.6, respectively. Increased p21 expression was found in endometriomas compared with benign ($P=0.001$) and malignant ($P=0.01$) tumours. Bax expression was higher in endometriomas than in benign tumours ($P=0.01$), but no difference was found between endometriomas and malignant tumours. No difference in bak, fas, bcl-2 or bcl-x expression was observed among the groups. In endometriomas, a negative correlation was found between p53 and fas expression ($P=0.04$, $r=0.66$). Although endometriomas have histological features of benign ovarian tumours, endometriomas share with malignant ovarian tumours certain alterations in apoptosis-related proteins.

Keywords Apoptosis · Endometriomas · Ovarian tumours · Ovarian cancer

Introduction

Endometriosis is defined by the presence of ectopic endometrial glands and stroma outside the uterus, and is a cause of pain and infertility. The main etiopathogenetic concepts forwarded to explain the development of endometriosis are the metaplasia and transplantation theories [33, 35]. The metaplasia theory, based on the concept that the peritoneum has the potential to differentiate into Mullerian-type epithelium, postulates that invasive mechanisms assist in the establishment of endometriotic lesions beneath the peritoneal surfaces. The transplantation theory implies that endometrial cells have the ability to detach from the endometrium, reattach after passive transport through the Fallopian tubes into the peritoneum and invade target tissues. The incidence of endometriosis in the general female population ranges from 5% to 15%, but reaches more than 50% in women with dysmenorrhea and/or infertility [34, 38, 40]. Clinically, one of the most frequent locations of endometriosis has been the ovary, with cysts called endometriomas [18]. The malignant transformation of ovarian endometriotic lesions varied from 0.6% to 1% [14].

Apoptosis, also called programmed cell death, was first discovered in 1842 by Vogt [41]. More than a century later, Kerr et al., rekindled apoptosis research [20]. This physiological process is involved in cellular homeostasis, maintaining a balance between cell proliferation and programmed cell death [16]. Apoptosis can be initiated by extracellular survival signals, mainly mediated by the bax/bcl-2 complex and also by intracellular death signals (genome instability) that increase the level of p53, and extracellular death signals involving membrane receptors such as the fas/fas-ligand system. Apoptosis-related protein expression in endometriotic lesions at different sites is controversial [19, 23, 30, 42]. Indeed, in glandular epithelial cells from ovarian endometriotic

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tissue, McLaren et al. [26], contrary to Harada et al. [13], reported positive bcl-2 expression. Previous studies [13, 30, 38] showed lower bcl-2 expression in endometriotic cysts than in peritoneal endometriotic samples. In contrast to bcl-2 expression, few data have been reported on the expression of apoptosis-related proteins such as bax [27] and fas [13, 42], and no data are available on p21 expression in patients with endometriosis. The aims of this study were to evaluate the expression of pro-apoptotic proteins (p53, p21, bax, bak and fas) and anti-apoptotic proteins (bcl-2 and bcl-x) in endometriomas, in comparison with benign and malignant ovarian tumours.

Materials and methods

Materials

Between 1986 and 2000, tissue samples were obtained from 60 patients undergoing surgery in the Gynaecology Department of Hôtel-Dieu Hospital, Paris; the samples consisted of 10 endometriomas, 20 benign ovarian tumours (10 mucinous, and 10 serous) and 30 malignant ovarian tumours (9 mucinous, 19 serous; 2 endometrioids).

In the endometrioma group, the mean age of the patients was 34 years (range 23–43 years). All the patients were pre-menopausal, and none had received progestin or gonadotrophin releasing hormone (GnRH) analogues for at least 3 months before surgery. They underwent surgery during the proliferative phase of the menstrual cycle in five cases and during the secretory phase in five cases. All underwent cystectomy.

In the benign tumour group, the patients' mean age was 45 years (range 21–75 years). Eight women were post-menopausal (40%), and twelve (60%) were pre-menopausal. No medical treatment was given before surgery, but three post-menopausal women were on hormone replacement therapy (HRT). All the pre-menopausal patients underwent cystectomy during the proliferative phase. One post-menopausal woman underwent bilateral salpingo-oophorectomy.

In the malignant tumour group, the patients' mean age was 55 years (range 26–84 years). Twenty patients (66.6%) were post-menopausal, and ten (33.3%) were pre-menopausal. Five post-menopausal women (25%) were on HRT. The disease was staged according to the FIGO classification (Federation Internationale de Gynecologie et Obstetrique) [17]. Thirteen patients had stage-I, two stage-II, fourteen stage-III, and one stage-IV disease. No neoadjuvant chemotherapy was used before surgery; the latter consisted of hysterectomy with bilateral salpingo-oophorectomy and omentectomy in all cases, and pelvic and para-aortic lymphadenectomy in 7 cases. Surgery was followed by chemotherapy.

The diagnosis was confirmed histologically on each tissue sample by reviewing hematoxylin/eosin-stained slides. None of the endometriomas contained clear nuclear atypia.

Methods

Antibodies

To evaluate pro-apoptotic protein expression, we used an anti-human-p53 mouse monoclonal antibody (Dako, Glostrup, Denmark), an anti-human-p21 mouse monoclonal antibody (Dako), an anti-human-bax mouse monoclonal antibody (Immunotech, Marseille, France), an anti-human-bak rabbit polyclonal antibody (Santa-Cruz Biotechnology, Santa-Cruz, CA, USA), and an anti-human-fas (CD 95) mouse monoclonal antibody (Immunotech). To evaluate anti-apoptotic protein expression, we used an anti-human-bcl-2 rabbit polyclonal antibody (Santa-Cruz Biotechnology), and an anti-human-bcl-x mouse monoclonal antibody (Transduction Laboratories, Lexington, KY, USA).

Immunohistochemical technique

In all cases, 5- μ m-thick, paraffin-embedded sections of formalin-fixed tissues were used. Sections were deparaffinated in xylene and rehydrated through a graded series of ethanol solutions, followed by microwave antigen retrieval for 20 min in 0.01 M sodium citrate buffer. Endogenous peroxidase activity was inhibited with 0.3% hydrogen peroxide for 15 min. After washing in Tris buffer saline-Tween casein (TBS-TC), sections were incubated for 1 h with the primary antibody. The antibodies to p53, p21, bak, fas and bcl-2 were used at a dilution of 1/50. Anti-bax was diluted 1/200, and anti-bcl-x was diluted 1/75. After washing in TBS-TC, the sections were incubated with an anti-mouse or anti-rabbit peroxidase conjugate (Valbiotech, Paris, France) for 40 min. Binding was visualised by incubating sections with DAB (3,3'-diaminobenzidine) (Dako) or AEC (3-amino-9-ethylcarbazol) (Beckman Coulter, Brea, CA, USA) and lightly counterstaining with hematoxylin.

Positive controls for p53, p21, bax, bak, fas, bcl-2 and bcl-x were sections obtained from colon carcinoma, tonsil, normal breast epithelium, normal breast epithelium, rhabdomyosarcoma, lymph node and aortic endothelium, respectively. For negative controls, the primary antibody was replaced by the relevant IgG subtype (monoclonal antibodies) or with non-immune serum (polyclonal antibodies).

Analysis of immunohistochemical results

For qualitative analysis, samples were considered negative when no labelled cell was observed on the tissue section, and positive in all other cases.

For semi-quantitative analysis, positive cells were counted among 500 cells in the most characteristic areas, and the percentage of labelled cells was calculated by two independent observers in five consecutive high-power fields (0.1734 mm² per field) per section. Variations between the two observers were below 5%.

Statistical analysis

We used the Chi-square test for categorical variables, and the Kruskal-Wallis and Mann-Whitney tests for continuous variables. The Spearman correlation test was used to identify correlations between two quantitative variables. *P* values below 0.05 were considered significant.

Results

Qualitative expression of apoptosis-related proteins in endometriomas and benign and malignant ovarian tumours

P53 immunostaining was restricted to the nuclei (Fig. 1a). P53 expression differed among the three groups of tumours ($P=0.01$). The paired differences were significant between endometriomas and benign tumours ($P=0.01$), between benign and malignant tumours ($P<0.001$), but not between endometriomas and malignant ovarian tumours (Table 1).

P21 immunostaining was also restricted to the nuclei (Fig. 1b). P21 expression differed among the three groups of tumours ($P=0.003$). The difference was significant between endometriomas and benign tumours ($P=0.003$) and between endometriomas and malignant ovarian tumours ($P=0.04$).

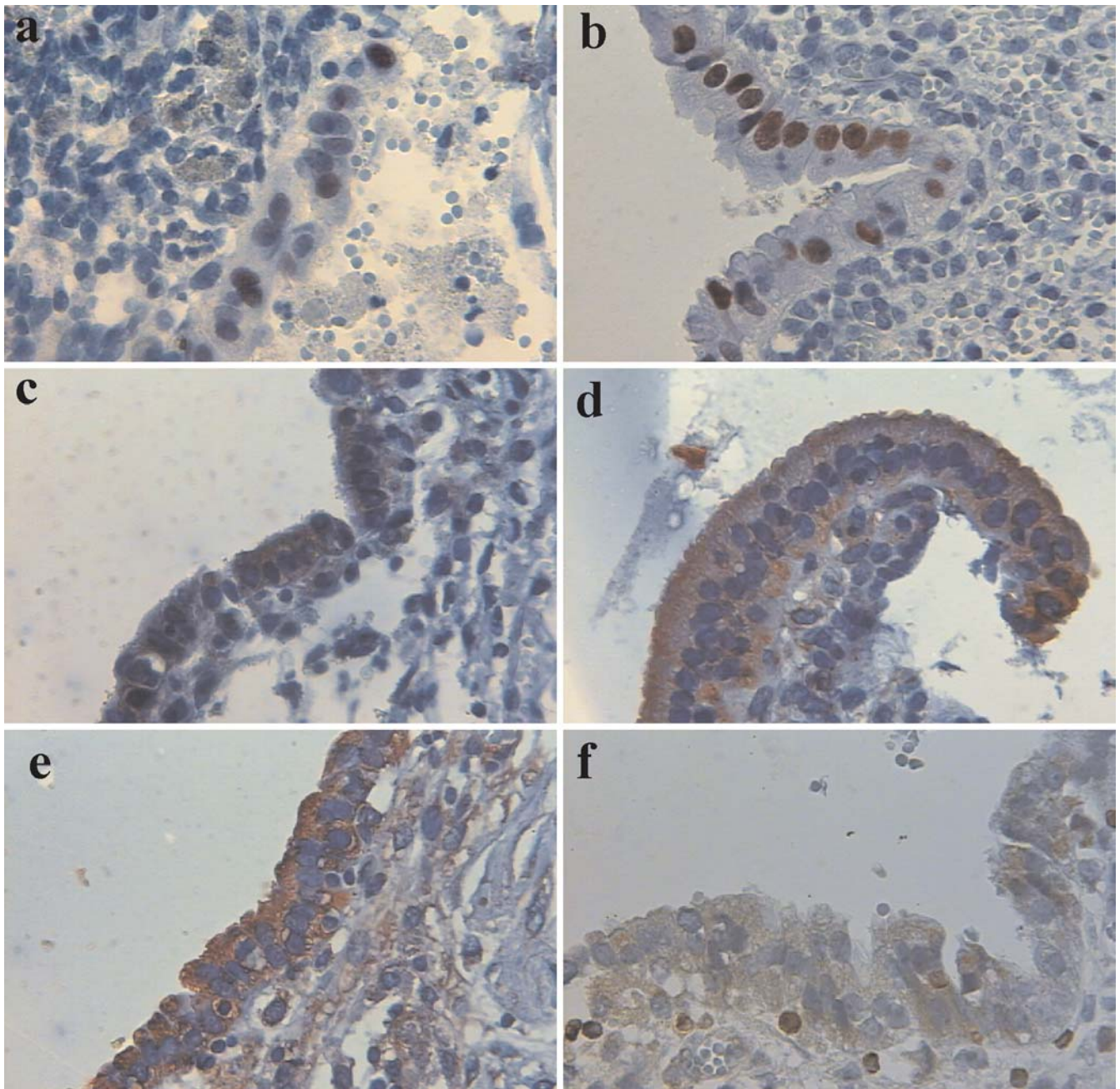


Fig. 1 Immunoperoxidase staining with 3-amino-9-ethylcarbazol (a–e) or diaminobenzidine (f) as chromogen, and nuclear counterstaining with Harris hematoxylin. Representative examples of p53 (a), p21 (b), bax (c), bak (d), fas (e) and bcl-2 (f) staining in

endometriomas. p53 and p21 immunostaining was restricted to the nuclei. Bax, bak and bcl-2 immunostaining was detected in the cytoplasm. Fas labeling was observed in both the outer membrane and the cytoplasm

Table 1 Qualitative expression of apoptosis-related proteins in endometriomas and benign and malignant ovarian tumours. NS not significant

	Endometriomas (n=10)	Benign ovarian tumours (n=20)	Malignant ovarian tumours (n=30)	P value*
p53	4/10	0/20	9/30	0.01
P21	7/10	2/20	9/30	0.003
Bax	7/10	9/20	15/30	NS
Bak	10/10	20/20	30/30	NS
Fas	10/10	18/20	29/30	NS
Bcl-2	7/10	13/20	16/30	NS
Bcl-x	8/10	16/20	27/30	NS

* P value for the three groups using Chi square test

Table 2 Mean±SD percentages of cells positive for p53, p21, bax, bak, fas, bcl-2 and bcl-x in endometriomas and benign and malignant ovarian tumours. NS not significant

	Endometriomas (n=10)	Benign ovarian tumours (n=20)	Malignant ovarian tumours (n=30)	P value*
p53	1.9±3.2	0	16.2±33.0	0.02
p21	19.5±27.8	1.7±6.7	4.1±8.6	0.002
bax	30.5±29.3	6.5±12.7	18.6±26.2	0.04
bak	88.0±14.3	75.3±33.2	90.4±15.7	NS
fas	64.8±32.1	54.7±42.6	68.9±32.2	NS
bcl-2	37.6±40.6	38.0±38.6	35.4±40.0	NS
bcl-x	64.5±36.7	51.0±42.6	68.5±30.7	NS

* P value for the three groups using Kruskal Wallis test

Bax immunostaining (Fig. 1c) and bak immunostaining (Fig. 1d) was restricted to the cytoplasmic compartment. Fas immunostaining was observed on the outer cell membrane and in the cytoplasmic compartment (Fig. 1e). Bcl-2 and bcl-x immunostaining was restricted to the cytoplasmic compartment (Fig. 1f). No difference in bax, bak, fas, bcl-2 or bcl-x expression was observed among the three groups of tumours.

In endometriomas, no qualitative difference in immunostaining was found for p53, p21, bax, bak, fas, bcl-2 or bcl-x according to the phase of the menstrual cycle. In benign and malignant tumours, no difference in qualitative immunostaining was observed according to menopausal status.

Semi-quantitative expression of apoptosis-related proteins in endometriomas and benign and malignant ovarian tumours

A difference in p53 immunostaining was observed among the three groups of tumours ($P=0.02$). The difference was significant between endometriomas and benign tumours ($P=0.003$), and between benign and malignant tumours ($P=0.008$), but not between endometriomas and malignant tumours (Table 2).

A difference in p21 immunostaining was also found among the three groups of tumours ($P=0.002$). P21 expression was higher in endometriomas than in benign ($P=0.001$) or malignant ovarian tumours ($P=0.01$), but was not different between benign and malignant tumours.

A difference in Bax immunostaining was observed among the three groups of tumours ($P=0.04$). Bax expression was higher in endometriomas than in benign tumours ($P=0.01$), but no difference was noted between endometriomas and malignant ovarian tumours.

No difference in bak, fas, bcl-2 or bcl-x immunostaining was found among the three groups of ovarian tumours.

In endometriomas, no difference in semi-quantitative apoptosis-related protein expression was found according to the phase of the menstrual cycle. In benign and malignant tumours, no difference was observed according to menopausal status.

Correlations between the expression of individual apoptosis-related proteins in endometriomas

A negative correlation was found between p53 and fas expression ($P=0.04$, $r=0.66$). A trend towards a positive correlation was observed between p53 and p21 expression ($P=0.06$, $r=0.62$). No correlation was found between the other apoptosis-related proteins.

Discussion

Qualitative and semi-quantitative alterations in apoptosis-related protein expressions were observed in endometriomas relative to benign and/or malignant ovarian tumours. Although none of the endometriotic ovarian cysts presented features of nuclear atypia, four of the ten tissue samples showed positive nuclear staining for p53. These results contrast with those from previous reports showing no p53 expression in endometriomas without atypia, with atypia, or with reactive atypia, and positive staining in endometrioid carcinomas [3, 36]. Nevertheless, our results are in line with those from Nakayama et al. [29] showing focal p53 expression in endometriotic epithelial cells without nuclear atypia. Using fragment length polymorphism scanning and polymerase chain reaction DNA sequencing, these authors [29] found no mutations in p53 exons 5–8. No loss of heterozygosity or microsatellite instability was detected either, suggesting that p53 expression by endometriotic cells could be due to overexpression of wild-type p53 protein [29]. Recently, Chang et al. [7] have reported that endometriosis is associated with p53 polymorphism and that heterozygotes and proline homozygotes have a higher risk for endometriosis.

P53 expression was increased in endometriomas compared with benign tumours, but no difference was observed between endometriomas and malignant ovarian lesions. Few data have been reported on p53 expression in endometriomas compared with that of benign or malignant ovarian tumours. In contrast to Nehzat et al. [31], in our experience, a positive p53 staining was found in 40% benign cystic endometriotic lesions. Moreover, these authors have shown that p53 expression was only found in benign endometriotic lesions next to endometrioid or clear cell carcinoma, suggesting a potential continuum from endometriosis to atypical endometriosis to carcinoma. The discrepancy of our results with those reported by

Nezhat et al. [31] could be in relation to the absence of a cut-off value taken in our study. However, this p53 overexpression found in our experience is reminiscent of previously reported data on cadherins, CD44 protein and ICAM-1, demonstrating that endometriomas share biological features with borderline and/or malignant ovarian tumours [8, 9].

We also observed p21 overexpression in endometriomas compared with benign and malignant ovarian tumours. Furthermore, despite the small number of endometriotic cysts included in this study, a trend towards a positive correlation was found between p53 and p21 expression. The p21 overexpression in endometriotic cysts partly contrasts with the lack of any association found by Hsieh et al. [15] between the p21 gene codon 31 arginine/serine polymorphism and endometriosis.

From the pathological point of view, overexpression of both p21 and p53 in endometriomas is in keeping with previous data [11], underlining the interaction of these apoptotic proteins. Indeed, the p53-inducible gene *p21* encodes an inhibitor of cyclin-dependent kinases involved in G1 arrest [2, 5, 10]. Moreover, these latter studies also showed that p21 was not necessarily required for p53-mediated apoptosis. Recently, in univariate analysis of a series of borderline ovarian tumours, Werness et al. [43] showed the prognostic relevance of p53 expression in the absence of p21 expression.

We observed increased bax protein expression in endometriomas compared with benign ovarian tumours. However, no difference in bax expression was observed between endometriomas and malignant ovarian tumours, probably owing to marked overlap of the relevant values. Few data have been reported on bax expression in endometriotic lesions. Goumenou et al. [12] reported similar bax expression in adenomyotic and endometriotic samples, and no variation according to the phase of the menstrual cycle. Furthermore, previous studies have shown no difference in endometrial bax expression between women with and without endometriosis [27] or between eutopic and ectopic endometrium [26], whereas bax expression was higher in endometrioid carcinomas than in normal and hyperplastic endometrium [22]. We found no correlation between p53 and bax expression. However, previous studies have shown that *bax* induction by p53 is necessary to inhibit tumour growth [44] and that the contribution of bax to p53-mediated apoptosis is cell-type dependent [21, 25].

The fas/fas-ligand system is a major pathway for the induction of apoptosis in cells and tissues [28]. It has been suggested that cellular fas ligand expression protects the host tissue from attack by T cells, which are killed through fas ligation. In the present study, despite higher fas expression in endometriomas than in benign tumours, the difference did not reach significance. However, a negative correlation was found between p53 and fas expression. This is in keeping with data showing that transcription of the death receptor CD95/fas/Apo1 is dependent on p53, through a p53-response element located within the first intron of the CD95 gene [32].

The B-cell lymphoma/leukaemia-2 gene (*bcl-2*) is a proto-oncogene that prevents apoptosis [39]. Using reverse-transcriptase polymerase chain reaction and Western blotting [24] or immunohistochemistry [6], a higher *bcl-2* expression has been reported in normal compared with neoplastic ovarian tissues. We found no difference in *bcl-2* expression among the three groups of tumours. This contrasts with a study from Ben-Hur et al. [4], Chan et al. [6] and Zusman et al. [45], showing a decrease in *bcl-2* expression from benign to borderline and malignant ovarian tumours. However, *bcl-2* expression is cell- and tissue dependent, and both p53 and *bcl-2* expression are reduced in serous ovarian carcinomas [1]. Indeed, recently, Nezhat and Kalir [30] have demonstrated a qualitative underexpression of *bcl-2* in cystic compared with non-cystic endometriotic lesions suggesting a differential expression pattern of *bcl-2* according to endometriotic lesions. Moreover, Nezhat et al. [31] reported a less frequent *bcl-2* expression in benign endometriotic cysts than endometrioid, clear cell and serous papillary carcinomas. These results suggest that *bcl-2* pattern expression differs according to histological type of epithelial ovarian tumours.

We found no variation in apoptosis-related protein expression according to the phase of the menstrual cycle. This is in line with previous reports on *bcl-2* [12, 13, 42], *fas* [42] and *bax* expression [12]. Watanabe et al. [42] suggested that the absence of cyclic changes in apoptosis-related proteins in ectopic endometrium points to a difference in the mechanisms of proliferation or differentiation between eutopic and ectopic endometrium. Furthermore, we found no difference in apoptosis-related proteins according to menopausal status in women with benign and malignant ovarian tumours.

The main limitation of this study is the small number of endometriotic cyst samples studied. This was partly due to the inclusion of tissue samples from women without previous medical treatment, at least for 3 months before surgery, and without cytologic atypia. Despite this limitation, in accordance with previous studies [11, 12, 27, 29], our data show that endometriomas share a similar pattern of apoptosis-related protein expression with malignant ovarian lesions.

In conclusion, our results point to a lack of cyclical regulation of apoptosis-related protein expression in endometriomas. Moreover, although endometriomas have histological features of benign ovarian tumours, their pattern of apoptotic protein expression was closer to that of malignant than benign ovarian lesions. From the pathological point of view, these data could suggest a role of defective apoptosis in endometriomas.

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