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ORIGINAL PAPER

p53 and BCL-2 as Prognostic Markers in Endometrial Carcinoma

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Abstract The objective of this study was to verify the frequency of p53 and BCL-2 immunohistochemical expression in patients with endometrial carcinoma and to correlate it with histological factors (histological type, tumor grade, depth of myometrial invasion, lymph node involvement and surgical staging) and survival. Forty-eight patients with endometrial carcinoma who were submitted to primary surgical treatment were assessed. p53 and BCL-2 immunohistochemical expression was determined using paraffin blocks containing the tumor area. p53 and BCL-2 expression was detected in 39.6% and 58.3% of the tumors, respectively. No significant difference was found regarding the frequency of p53 expression when analyzing histological type (33.3% in endometrioid tumors, 58.3% in non-endometrioid tumors; p=0.176), depth of myometrial invasion (p=0.632) and surgical staging (I—11.1%, II—66.7%, III—57.1%; p=0.061). p53 expression was significantly more frequent in undifferentiated tumors (p=0.007) and in those showing lymph node involvement (p=0.030). Univariate analysis showed a positive association with death (RR, 3.358; CI, 1.386–8.134; p=0.005) and short-term survival. The present study did not reveal any correlation between BCL-2 expression and histopathologic markers or survival. In conclusion, this study showed that p53 expression is directly correlated with undifferentiated tumors, lymph-node involvement and risk of death. On the other hand, BCL-2 expression was not correlated with any known histological factors.

Keywords Endometrial cancer · p53 · BCL-2 · Prognosis

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Introduction

Endometrial carcinoma is one of the most common gynecological malignancies. In Brazil where epidemiological recording systems are inadequate, endometrial carcinoma ranks fifth among all tumors, being surpassed only by breast, cervical, colonic and stomach cancers [1].

Conventional prognostic markers used to determine the clinical course of endometrial carcinomas include histological type, tumor grade, depth of myometrial invasion, lymph-node involvement and stage [2]. This information is obtained through the histological analysis of surgical specimens. Clinical variables, such as age and ethnic group, have also been regarded as important prognostic factors [3].

Most women (75–88%) with endometrial carcinoma have early-stage disease at diagnosis [4, 5]. As a result, these patients have a good prognosis, with a 5-year survival rate of 80–85% [5]. However, in 15–20% of these patients who have an apparently curable disease, the disease will

recur and they will die. There is therefore speculation about the existence of additional factors that may be related to the risk of tumor progression and dissemination.

In the past few years, improvements in molecular biology have led to the suggestion of some biological markers being as important as, or more important than, conventional factors [6]. Several publications have regarded the *TP53* gene (the gene that encodes p53) and the *BCL-2* gene as possible markers of tumor aggressiveness [6-10].

TP53 is considered to be a tumor suppressor gene. This gene expresses a protein called p53 which, due to injuries to the cell genome, halts the progression of the cell cycle and causes apoptosis (programmed cell death). The functional inactivation of p53 protein plays a crucial role in the malignant transformation process. It provides the tumor cell with a higher capacity for division and proliferation [11]. A total of 21–49% of endometrial carcinomas show abnormal p53 expression [12, 13]. The expression of p53, as seen in immunohistochemical analysis, seems to be related to undifferentiated, aggressive tumors, with a poor prognosis [6-8].

The *BCL-2* gene inhibits apoptosis. It encodes a protein of 25–26 kDa (BCL-2), which is located in the mitochondrial membrane, endoplasmic reticulum and nuclear membrane [14]. The levels of BCL-2 expression vary in a cyclic fashion in the normal endometrium. These levels are higher during the proliferative phase and decrease in the secretory phase, thus suggesting hormonal regulation. Moreover, BCL-2 expression, which is relatively high in the normal endometrium, decreases in atypical hyperplasia and adenocarcinoma [15, 16]. Its sustained expression may be important at the early stages of endometrial carcinoma. BCL-2 expression has been related to well-differentiated endometrial tumors, early-stage disease and diseases with a good prognosis [9, 16-19].

The aim of the present study was to check the frequency of p53 and BCL-2 immunohistochemical expression in Brazilian patients who have been surgically treated for endometrial carcinoma in our setting, and to correlate this expression with conventional histological and prognostic factors (histological type, tumor grade, depth of myometrial invasion, lymph node involvement and surgical staging) and survival.

Materials and Methods

Between January 1996 and December 2001, 52 consecutive patients with a histological diagnosis of endometrial carcinoma were submitted to primary surgical treatment at the Division of Genital Oncology of Hospital de Clínicas de Porto Alegre (HCPA), Brazil. This hospital division is considered to be a reference center for the treatment of endometrial carcinoma.

Patients with concomitant history or presence of any other malignancy and/or who had previously been submitted to neoadjuvant therapy were excluded from the study. Therefore, four patients with a previous history of malignancy (two with colon cancer, one with breast cancer and one with cervical cancer) were excluded from the initial group. Altogether, 48 patients participated in the study.

Out of these 48 patients, 11 (23%) were submitted to total hysterectomy with bilateral salpingo-oophorectomy with or without peritoneal cytology, and 37 patients (77%), in addition to total hysterectomy, were submitted to some form of pelvic lymph-node dissection (total dissection or lymph-node sampling) with or without peritoneal cytology. The number of pelvic lymph nodes that were dissected ranged from 2 to 40 (mean, 16.9; standard deviation, 9.71). In addition to pelvic lymphadenectomy, para-aortic lymph-node sampling (2–8 lymph nodes; mean, 3.36) was performed in 11 patients.

It was possible to define surgical staging in 26 patients using the information obtained from the patients' histopathologic records.

Of these 48 patients, 34 were referred for post-surgical adjuvant therapy and, in three cases, no mention of such indication was found in the medical records. Radiotherapy (teletherapy and/or brachytherapy) was the treatment of choice in 31 patients.

A historical cohort was used. Surgical resection was regarded as time zero. The follow-up began after tumor resection, extending up to the patient's death or up to the end of the study period (December 2003). Related death (outcome) was a death that was directly associated with the event (endometrial cancer). Information about the patients' survival was obtained from the medical records and also via telephone calls and regular mail. Outpatients were instructed to see their doctor every 3 months during the first post-operative year, every 4 months in the second year and every 6 months in the third to fifth years, and annually from the sixth year on. The study protocol was approved by the Research and Ethics Committee of the HCPA Graduate Research Group.

Variables Analyzed

Age and description of the histological analysis of the surgical specimens were obtained from the review of medical records. The variables analyzed included age, histological type, tumor grade, depth of myometrial invasion, lymph node involvement and surgical staging.

Histological type and tumor grade were redefined by analyzing the new hematoxylin/eosin-stained slides that were embedded in paraffin blocks containing the tumor area, which were later used for immunohistochemical analysis. The cases in which the classification of the depth

of myometrial invasion was based on outdated criteria (i.e. depth of myometrial invasion divided into thirds) were reclassified. The surgical specimens were obtained from the Division of Pathology of the HCPA. The slides were assessed by two independent pathologists.

The histological classification satisfied the criteria established by the College of American Pathologists [20]. Endometrial lesions were divided into two large groups: endometrioid adenocarcinomas and non-endometrioid adenocarcinomas (the subtypes of which include serous papillary carcinoma, clear-cell carcinoma, mucinous carcinoma, squamous-cell carcinoma, undifferentiated carcinoma and mixed-cell carcinoma). Adenosquamous carcinoma was considered, as is the case in most studies of this type, to be a non-endometrioid lesion [7, 8, 21].

According to their histological type, endometrioid adenocarcinomas were classified into the following groups: well-differentiated tumors (G1)—tumors including only glands; moderately differentiated tumors (G2)—combination of glands and solid epithelial masses; and undifferentiated tumors (G3)—predominantly solid proliferations. Undifferentiated, serous-cell and clear-cell carcinomas were considered to be undifferentiated lesions. As no universally accepted criteria exist for the classification of mucinous carcinomas, we used the same classification as was used for endometrioid tumors [20].

The currently used classification for the depth of myometrial invasion is the following: A—no invasion (restricted to the endometrium); B—invasion less than or equal to 50%; and C—invasion greater than 50% [20]. This new classification could not be used in only one case.

The surgical staging of endometrial carcinomas was defined according to the International Federation of Gynecology and Obstetrics (FIGO) staging (1988), as follows: I—disease restricted to the uterine corpus; II—cervical involvement; and III—involvement of uterine adnexa and/or positive retroperitoneal lymph nodes and/or peritoneal cytology.

Immunohistochemical Analysis

The following primary antibodies were used: mouse monoclonal anti-p53 antibody, (clone DO-7, Dako), and monoclonal anti-BCL-2 antibody (Dako).

One section from each paraffin block was submitted to hematoxylin/eosin staining for histological analysis and confirmation of the tumor area. The subsequent sections were submitted to routine immunohistochemical analysis by the Division of Pathology of HCPA, using the described primary antibodies. Only one slide was mounted with each monoclonal antibody, using a single paraffin block. All reactions were performed using positive controls (breast cancer for p53 expression and lymph nodes for BCL-2 expression).

After deparaffinization and rehydration, antigen retrieval, inactivation of endogenous peroxidase and blocking of non-specific reactions, the sections were incubated for 2 h at ambient temperature with a diluted solution of primary antibodies (1:100 for p53 and 1:80 for BCL-2). Primary antibody binding was revealed by streptavidin–biotin peroxidase complex (LSAB, Dako) and diaminobenzidine tetrahydrochloride (DAB, Dako).

Evaluation of Staining for p53 and BCL-2

Tumors were considered positive for p53 expression if 10% or more of the nuclei of tumor cells stained brown, and for BCL-2 expression if 10% or more of the cytoplasm of tumor cells stained brown. The slides were examined by two independent pathologists who were blinded to the histological data. Cases with discrepant scores were later reassessed together until an agreement could be reached.

Statistical Analysis

Continuous variables are described as mean and standard deviation and categorical variables are described as proportions. The correlation between p53 and BCL-2 expression and histopathologic characteristics were analyzed using the Chi-square test. Fisher's exact test was used whenever necessary. The survival analysis was conducted using the Kaplan–Meier method. The impact of each prognostic factor on survival was assessed using the log-rank test. The significance level was established at 5%. The Statistical Package for Social Sciences (SPSS) for Windows version 11.0 was used for statistical analysis.

Results

Forty-eight patients were assessed. Their ages ranged from 34 to 90 years (mean ± SD, 65.6±11.7 years). The mean follow-up period ranged from 20 days to 99.5 months. The mean overall survival was 69.77 months (95% CI, 58.18–81.37). Sixteen patients died during the follow-up period. The major histopathologic characteristics are listed in Table 1.

p53 expression was positive in 19 cases (39.6%), while BCL-2 expression was positive in 28 cases (58.3%). The correlations of p53 and BCL-2 expression with histopathologic prognostic factors are summarized in Table 2. No statistically significant correlation was found between the frequency of p53 expression and histological type, depth of myometrial invasion and surgical staging. p53 expression was more frequent in undifferentiated tumors (p=0.007) and in those with lymph node involvement (p=0.030). No statistically significant correlation was found between the frequency of BCL-2 expression and the histopathologic factors.

Table 1 Histopathological characteristics of endometrial tumors resected at the division of genital oncology of the Hospital de Clínicas de Porto Alegre

Characteristics	Number of patients (%)
Histological type (n=48)	
Endometrioid	36 (75)
Non-endometrioid	12 (25)
Serous papillary	4
Adenosquamous	3
Mucinous	2
Undifferentiated	2
Clear-cell	1
Tumor grade[a] (n=48)	
G1	14 (29.2)
G2	20 (41.6)
G3	14 (29.2)
Depth of myometrial invasion[a] (n=48)	
No invasion	2 (4.2)
Less than or equal to 50%	19 (39.6)
Greater than 50%	26 (54.2)
Middle third invasion	1 (2)
Surgical staging[b] (n=26)	
I	9 (34.6)
II	3 (11.5)
III	14 (53.9)
Lymph node involvement (stage IIIC)[b] (n=37)	
Positive	5 (13.5)
Negative	32 (86.5)

[a] Following the College of American Pathologists (ACP) criteria
[b] According to FIGO, 1988

The mean survival for the 19 p53-positive patients was 48.9 months (95% CI, 30.79–67.08); for the 29 p53-negative patients, the mean survival was 83.8 months (95% CI, 71.6–96.1; log rank p=0.0023). In univariate analysis, p53 expression was positively correlated with the occurrence of death (Fig. 1). Of the 16 patients who died, 11 (68.7%) showed p53 expression (RR, 3.358; 95% CI, 1.386–8.134; p=0.005). Conversely, only 8/32 (25%) of living and disease-free patients were p53-positive.

The mean survival for the 28 BCL-2-positive patients was 72.48 months (95% CI, 58.12–86.84); for the 20 BCL-2-negative patients, the mean survival was 65.2 months (95% CI, 47.8–82.6; log rank p=0.53). No correlation was observed between BCL-2 expression and death (Fig. 2). Of the 16 patients who died, nine (56.2%) showed BCL-2 expression (RR, 0.918; 95% CI, 0.411–2.052; p=1.000).

Discussion

Considerable effort has been made to identify the genetic and molecular changes that are observed in carcinomas in general and, in particular, in endometrial carcinoma, and to determine how they relate to the staging and prognosis of the disease.

Several studies carried out in patients with endometrial tumors have indicated that p53 and BCL-2 are important prognostic variables [6–10]. The presence of p53 immunoreactivity, in addition to indicating abnormal protein expression (as a result of structural and/or functional

Table 2 Association between p53 and BCL-2 expression and the histological characteristics of patients with endometrial carcinoma who were submitted for primary surgical treatment

Characteristics	P53-positive	p53-negative	p value	BCL-2-positive	BCL-2-negative	p value
Histological type						
Endometrioid	12 (33.3)	24 (66.7)	0.176	21 (58.3)	15 (41.7)	1.000
Non-endometrioid	7 (58.3)	5 (41.7)		7 (58.3)	5 (41.7)	
Myometrial invasion						
No myometrial invasion	1 (50.0)	1 (50.0)	0.632	1 (50.0)	1 (50.0)	0.855
Less than or equal to 50%	6 (31.6)	13 (66.4)		11 (57.9)	8 (42.1)	
Greater than 50%	12 (46.2)	14 (53.8)		15 (57.7)	11 (42.3)	
Middle third invasion	0 (0)	1 (100)		1 (100)	0 (0)	
Tumor grade						
G1	2 (14.3)	12 (85.7)	0.007	8 (57.1)	6 (42.9)	0.285
G2	7 (35.0)	13 (65.0)		14 (70.0)	6 (30.0)	
G3	10 (71.4)	4 (28.6)		6 (42.9)	8 (57.1)	
Surgical staging						
I	1 (11.1)	8 (88.9)	0.061	4 (44.4)	5 (55.6)	0.608
II	2 (66.7)	1 (33.3)		2 (66.7)	1 (33.3)	
III	8 (57.1)	6 (42.9)		5 (35.7)	9 (64.3)	
Lymph node involvement						
Positive	4 (80.0)	1 (20.0)	0.030	1 (20.0)	4 (80.0)	0.285
Negative	8 (25.0)	24 (75.0)		20 (62.5)	12 (37.5)	

The numbers in parentheses correspond to the percentage values.

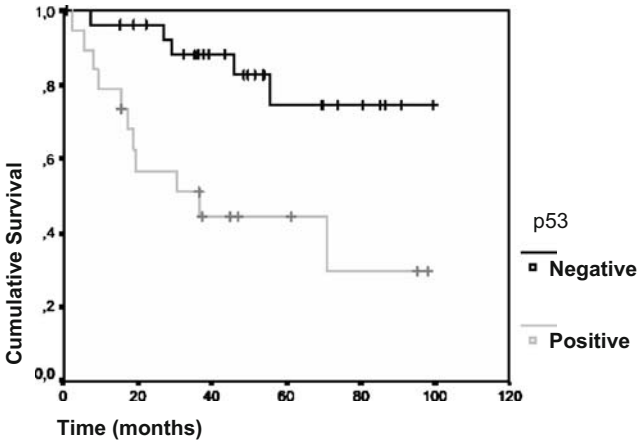

Fig. 1 Survival curves according to p53 expression

changes), seems to be correlated with adverse factors. Undifferentiated lesions, deep myometrial invasion, lymph node involvement and advanced stage of the disease have been associated with p53 positivity [6, 8]. Conversely, BCL-2 expression has been directly correlated with a favorable clinical outcome and good histopathologic prognostic factors (i.e. well-differentiated endometrioid tumors with superficial myometrial invasion and early staging; [9, 16-19]). The theoretical basis for these clinical observations shows that p53 immunohistochemical expression and/or the loss or absence of BCL-2 expression are associated with tumors that are characterized by a high level of apoptosis and cellular proliferation. These characteristics are related to the possibility of tumor progression and dissemination [22, 23].

A recent model of endometrial carcinogenesis revealed that there are two different types of tumor: endometrioid adenocarcinoma (with a good prognosis) and non-endometrioid adenocarcinoma (with a dismal prognosis). Endometrioid tumors develop slowly and progressively as a result of endometrial hyperplasia in unopposed estrogen activity. These tumors are characterized by sustained BCL-2 expression and late mutations to the *TP53* gene, as confirmed by the absence of p53 in atypical hyperplasia and in low-grade lesions (stage I and II; [6, 12, 24]). On the other hand, non-endometrioid adenocarcinomas (in particular, serous-cell and clear-cell carcinomas) develop in an atrophic epithelium without estrogen stimulation, are aggressive and occur in a distinct biological environment. Gene mutation and p53 protein accumulation are often found in intraepithelial endometrial carcinoma, which is a histopathologic entity that is identified as a precursor lesion for serous papillary carcinoma, and is observed in approximately 90% of cases [24-29]. A study conducted by Kounelis et al. [12] showed the extension of p53 immunoreactivity to atypical and atrophic endometrial glands adjacent to the area of the invasive papillary tumor. This information proves that, in these cases, mutations to the *TP53* gene occur early on in carcinogenesis.

Our study analyzed p53 and BCL-2 expression and how they relate to conventional prognostic factors. Immunohistochemistry (IHC) was used to determine this expression. This method, due to its easy application and low cost, has been widely used in studies of this type. Furthermore, it allows the use of stored biological material that has been preserved for long periods of time in paraffin blocks [13, 30, 31].

Unlike the techniques that use polymerase chain reaction (PCR), immunohistochemical analysis does not allow the identification of gene abnormalities. Positive results are usually associated with structural changes in the proteins that have been encoded by the gene. Simply put, we may say that abnormal proteins result from abnormal and mutant genes. However, immunoreactivity may occur only as a result of the binding of these proteins to other proteins, which could provide them with increased stability and allow them to accumulate in neoplastic cells. Conversely, the presence of specific types of mutations (e.g. frame-shift mutations) produces truncated or unstable proteins which go undetected by IHC [32].

In endometrial carcinomas, p53 expression by IHC may not be associated with gene mutation, especially in endometrioid tumors. In these cases, the binding of p53 to other nuclear proteins, forming highly stable protein complexes, apparently explains its immunoreactivity. On the other hand, in non-endometrioid lesions, especially in serous tumors (which are more aggressive), gene mutations are more common [29, 33]. With regard to BCL-2, its expression in solid tumors has not been associated with abnormal genes [34].

In our study, we detected nuclear p53 expression in 39.6% of cases. This overall frequency is consistent with

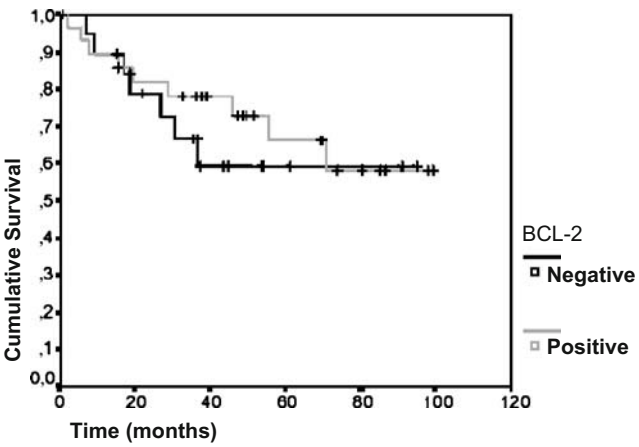

Fig. 2 Survival curves according to BCL-2 expression

Table 3 Association between p53 expression and extensive pelvic lymphadenectomy (n=48)

Extensive pelvic lymphadenectomy	p53-positive	p53-negative	Total	p value
Yes	7 (36.8)	7 (24.1)	14	0.517
No	12 (63.2)	22 (75.9)	34	
Total	19	29	48	

The numbers in parentheses correspond to the percentage values.

those reported in the literature, which describes rates of 21–49% [12, 13].

As far as clinical variables are concerned, we did not find any association of p53 expression with patient age. This was also described by Geisler et al. [17]. We could not assess the correlation of p53 expression with the ethnic group, as race was not properly specified in the medical records. Kohler et al. [13] observed an increased p53 expression in endometrial tumors in black patients. The influence of ethnic differences and high rates of miscegenation of the Brazilian population on biological markers and on other prognostic factors has not yet been clarified.

As to histological type, we found that 58.3% of non-endometrioid tumors and 33.3% of endometrioid tumors expressed p53. Although this difference shows a tendency towards higher p53 expression in non-endometrioid tumors, it was not statistically significant (p=0.176). In previous publications, the difference in p53 expression between these two types of tumors has proved to be more distinct. Geisler et al. [7] for example, observed that 57.3% of endometrioid tumors and 94.1% of non-endometrioid tumors expressed p53 (p<0.001). In Brazil, Bonfitto et al. [35] studied 51 cases and observed p53 expression in 16% of endometrioid tumors and in 71% of non-endometrioid tumors (p<0.05) in uterine curettage specimens. By analyzing only serous-cell and clear-cell tumors, Kounelis et al. [12] found positivity rates in 76–100% of cases. In our study, the separate assessment of these subtypes also showed a high p53 expression (80%), although the number of cases was small (five cases).

We noted an increased p53 expression in poorly differentiated tumors. Similar results were obtained in several other studies [7, 13, 36, 37]. Contrary to what has been reported [6, 8], we did not find any significant difference in p53 expression in relation to the depth of myometrial invasion. Geisler et al. [7] studying 46 patients with endometrial carcinoma, also could not find a statistically significant difference in levels of myometrial invasion and p53 expression.

p53 expression was not found to be statistically different when surgical staging was analyzed. However, when only lymph-node involvement, which characterizes stage IIIC, was analyzed, we observed that p53 immunoreactivity was more frequent. Lymph node involvement is currently regarded as being the most important prognostic variable [6]. The literature abounds with studies that show a direct association of advanced surgical staging and lymph-node involvement with p53 expression [7, 13, 36, 37]. Extensive lymph node dissection or only using lymph node sampling, and the inability to determine surgical staging in 22 cases, are possible limitations of this study. Patients who were submitted to pelvic lymph node sampling, a surgical approach that was commonly used in the first cases of this study, may have been understaged and may have interfered with the results.

Studies have correlated p53 expression with a decrease in overall survival in patients with endometrial tumors. Ohkouchi et al. [6] noted that the 5-year survival for stage III/IV patients varied according to p53 expression. Its presence and absence was related to 40.4% and 75.7% of survival, respectively. Oreskovic et al. [32] also observed that p53 positivity was associated with poor survival in G1 and G2 tumors. Ito et al. [38] studied 221 patients with early-stage disease and found that p53 expression was present in 50% of the patients who died and in only 8% of living and disease-free patients. In our study, according to the univariate analysis, p53 positivity was associated with risk of death. This result was not influenced by the extension of the surgical procedure (lymph-node sampling or extensive pelvic lymphadenectomy) or by adjuvant therapy (factors potentially related to recurrence and/or survival), as we did not find a statistically significant difference when these variables and p53 expression were analyzed (Tables 3 and 4).

The multivariate analysis did not allow us to characterize p53 expression as an independent prognostic factor compared with histological type and tumor grade, myometrial invasion and staging. This finding is not consistent with the data that is described in the literature [6, 8, 31, 38]. Nevertheless, our study is not suitable for this type of analysis because of the small number of cases and outcomes.

Cytoplasmic BCL-2 expression was observed in 58.3% of the carcinomas that we analyzed. The literature describes rates of 34–85% [12], depending on the selection of patients.

Table 4 Association between p53 expression and adjuvant therapy (n=45)

Adjuvant therapy	p53-positive	p53-negative	Total	p value
Yes	14 (77.8)	20 (74.1)	34	1.000
No	4 (22.2)	7 (25.9)	11	
Total	18	27	45	

The numbers in parentheses correspond to the percentage values.

Our study did not demonstrate any correlation between BCL-2 expression and age, histological type and tumor grade, depth of myometrial invasion and survival.

Controversy exists in the literature over the correlation between BCL-2 expression and prognostic factors. Some prostate tumors and neuroblastomas with positive BCL-2 immunoreactivity have highly aggressive biological behavior. This can be explained by the resistance to hormone and/or cytotoxic therapy, due to the persistence of BCL-2 expression and its capacity to inhibit apoptosis [39]. With regard to endometrial carcinoma, however, several studies show that increased BCL-2 expression is associated with some favorable prognostic factors—endometrioid-like, well-differentiated lesions in initial stages [9, 16-19]. Far from being a clearly defined issue, some other studies do not show similar results [12, 21]. Kounelis et al. [12] studied 61 patients and did not find any significant difference in BCL-2 expression when histological type, tumor grade and staging were analyzed. Giatromanolaki et al. [21] assessed 133 patients and did not find any relationship of this marker with tumor grade, depth of myometrial invasion and lymphovascular invasion. Sakuragi et al. [10] did not observe any significant differences in BCL-2 expression when lymph node involvement and tumor grade were analyzed, but this expression was inversely proportional to myometrial invasion ($p<0.025$).

In conclusion, our study showed that p53 expression was directly associated with death and with a dismal prognosis (tumor grade, serous-cell and clear-cell lesions and lymph node involvement). This finding relates p53 expression to increased tumor aggressiveness. On the other hand, BCL-2 expression was not associated with known prognostic factors. This does not invalidate the importance that has been attributed by the literature to markers of apoptosis, as apoptosis seems to result from a highly complex system, with the participation of several genes. With regard to the endometrium, possible influences on BCL-2 expression include the activity of hormone receptors (especially progesterone receptors), and the activity of BCL-2 homologous proteins (namely, BAX, BCL-x, and BCL-xs) which were not included in our study.

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