Role of Bcl-2 in Endometrioid Corpus Cancer: An Experimental Study*

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Abstract. *Background: Bcl-2 expression appears to be under hormonal control in normal endometrium and to correlate with hormone receptor status in endometrial cancer. The aim of this study was to assess bcl-2 expression in endometrial cancer. Materials and Methods: Hysterectomy specimens from 125 patients with endometrial cancer were stained for bcl-2. Estrogen receptor (ER) and progesterone receptor (PR) levels were quantified with a dextran-coated charcoal assay. Results: Bcl-2 expression correlated significantly with endometrioid histology and high levels of ER and PR ($p<0.05$). For the entire population, bcl-2 expression was not significantly associated with the presence of extrauterine disease. However, when only patients with grade 1 or 2 endometrioid histology were considered, tumors with bcl-2 expression were significantly ($p<0.05$) more likely to present with extrauterine disease than those not expressing bcl-2. Conclusion: Bcl-2-mediated inhibition of apoptosis may be important in the acquisition of molecular alterations and development of metastases in a subset of hormone-dependent endometrial cancers.*

Endometrial cancer is the most common malignancy of the female reproductive tract in the United States and is exceeded annually in overall frequency only by cancers of the breast, colon and lung. It has been estimated that during the calendar year 2005, 40,880 new cases of endometrial cancer will be diagnosed and 7,310 deaths will occur (1).

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The antiapoptotic protein bcl-2 has a critical regulatory role in normal cell turnover, and its dysregulated expression appears to be involved in the genesis of different neoplasias (2). In breast cancer, bcl-2 expression appears to be modulated by steroid hormones and is reportedly correlated directly with hormone receptors (3). Moreover, it has been suggested that bcl-2 is involved in the inhibition of apoptosis, favoring the acquisition of genetic mutations and the subsequent development of metastases in breast cancer (4).

Bcl-2 expression also appears to be under hormonal control in the normal endometrium (5) and has been shown in endometrial cancer to correlate directly with hormone receptor expression, endometrioid histological subtype and low-grade disease (6). In addition to its role in breast cancer, bcl-2 may have a role in suppressing apoptosis in hormone-dependent endometrial cancers, thereby favoring the acquisition and accumulation of genetic alterations.

With the similarities between hormonally-dependent breast cancer and endometrioid adenocarcinoma of the endometrium, we hypothesized that bcl-2 expression is associated with steroid receptor expression and, ultimately, the clinical behavior of the disease. The aim of this study was to evaluate the expression of bcl-2 in hormone-dependent endometrial carcinogenesis.

Materials and Methods

All patients with surgically treated endometrial cancer from 1984 through 1993 at the Mayo Clinic (Rochester, MN, USA) were eligible for this retrospective study. Approval for the study was obtained from the Institutional Review Board of the Mayo Foundation. Only patients with epithelial endometrial cancer who met the following inclusion criteria were included: i) the disease had been managed surgically with hysterectomy and removal of the remaining adnexal structures, ii) no other malignancy was diagnosed within 5 years before or after the diagnosis of endometrial cancer (except for carcinoma *in situ* or skin cancer other than melanoma), and iii) initial preoperative endometrial sampling was performed at the Mayo Clinic.

Histological characteristics were abstracted from the original pathology reports. The hematoxylin-eosin-stained sections of the hysterectomy specimens from all patients were re-reviewed by two

of the authors (T.J.S., G.L.K.) to confirm the histological diagnosis, grade and subtype.

The histological classification followed the classification of the World Health Organization (7). Architectural grading was based on the degree of glandular differentiation in accordance with the FIGO guidelines (8). The subgroup of endometrioid low-grade corpus cancer were considered to be representative of hormone-dependent endometrial cancer, in accord with the literature (9).

Analysis of molecular parameters. Hematoxylin-eosin-stained sections of tumor-containing paraffin blocks were reviewed by one of the authors (T.J.S.) to identify the most representative block to section. The immunohistochemical staining technique has been described in detail elsewhere (10).

Grading of the slides for the cytoplasmic marker bcl-2 included glandular staining intensity relative to the negative control tissue (*i.e.*, endometrial tissue that did not stain for bcl-2). Normal tonsil tissue was used as a positive control. The reviewer assigned a score of "0" for no staining, "1+" for weak staining, "2+" for moderate and "3+" for strong staining. The reviewer was blinded to the other results of the analysis at the time of quantitation of the tissues. Cases were eliminated from the analysis if the tissue was so poorly preserved that staining was uninterpretable or if no tumor tissue was present on the slide.

Analysis of hormone receptor status. As part of routine clinical care during the study period, estrogen receptor (ER)-ligand and progesterone receptor (PR)-ligand binding levels had been assessed in fresh tissue with the use of a dextran-coated charcoal assay (fmol/mg of protein), as described by Alberts *et al.* (11). The tissue that was immediately adjacent to the area where ER and PR were measured had been submitted for histological examination in paraffin-embedded sections to confirm the presence of cancer. ER and PR levels were abstracted retrospectively from the histology reports.

Statistical analysis and design. For statistical analyses, bcl-2 was quantified as a categorical variable and was divided as follows: patients with no (0) staining for bcl-2 ("bcl-") were compared with all the other cases (*i.e.*, 1+, 2+, 3+) ("bcl+"). This was done according to our previous study in which bcl-2 expression had been compared with prognosis (10). The ER and PR levels were analyzed as continuous variables.

Wilcoxon rank sum tests and weighted χ^2 analyses were used for statistical comparisons. Differences between groups were considered statistically significant at $p<0.05$. SAS version 6.12 and S-Plus version 3.4 statistical software packages were used.

From the group of 283 eligible patients, 125 were selected using a case-cohort design, as described previously (10). In brief, the 125 cases included all 49 women who had disease recurrence and 76 randomly chosen progression-free patients. This was not a random sample of the whole cohort, but was designed in accord with the method of Prentice (12).

The data from these 125 cases are reported as nonextrapolated data. Next the assumption was made that the randomly selected subgroup of 76 cases with nonrecurrent disease was representative of the whole population of 234 recurrence-free patients. In this way, every patient who did not have disease recurrence had a higher statistical weight than patients who had disease recurrence. Thus, the results observed in the population of 125 patients were extrapolated to the overall population of 283 patients. We have previously used this statistical design (with recurrence as outcome variable) for predicting recurrence and survival in patients with endometrial cancer on the basis of immunohistochemical results reported on the preoperative endometrial biopsy (10). The current study used the same population as the previous analysis but examined a different outcome. As a consequence of the statistical design, most of the results are reported as estimated percentages for the overall population and not as actual numbers.

Table I. *Correlation of estrogen (ER) and progesterone (PR) receptor levels with bcl-2 expression and presence of extrauterine disease.*

Variable	ER, mean±SD, fmol/mg	*P*	PR, mean±SD, fmol/mg	*P*
	All patients			
Extrauterine disease				
Yes	158±213	0.05	251±378	0.03
No	252±231		425±449	
Lymph node metastases				
Yes	194±288	0.76	303±571	0.20
No	195±207		388±444	
Bcl-2				
0	105±124	0.01	203±344	0.01
1+, 2+, 3+	261±243		414±453	
Grade/histological subtype				
Grade 1-2 and endometrioid	275±228	<0.01	517±507	<0.01
Grade 3 or nonendometrioid	128±199		139±169	
	Grade 1 or 2 endometrioid tumors			
Extrauterine disease				
Yes	242±261	0.33	475±511	0.60
No	289±216		536±517	
Lymph node metastases				
Yes	341±362	0.62	607±802	0.64
No	229±188		523±500	
Bcl-2				
0	169±143	0.15	447±463	0.55
1+, 2+, 3+	306±241		537±527	

Results

All patients had endometrial cancer that was managed with hysterectomy and the removal of both or remaining adnexal structures. During the 10-year interval, 299 patients met the inclusion criteria. Of these 299 patients, 16 did not have the necessary clinicopathological data or histology blocks and were excluded, leaving a cohort of 283 patients. From these 283 patients, immunohistochemical studies were performed on the subgroup of 125 patients, as described in the statistical methods.

Table II. *Assessment of extrauterine disease (EUD) and positive lymph nodes (PLNs) according to histological grade, subtype and bcl-2 expression in patients with endometrial cancer.*

	All patients				Grade 1 or 2 endometrioid tumors			
Variable	% EUD	*P*	% PLNs	*P*	% EUD	*P*	% PLNs	*P*
Grade								
1-2	20	0.009	10	0.18	18	---	10	---
3	35		18		---		---	
Histological subtype								
Endometrioid	20	0.002	9	0.007	18	---	10	---
Nonendometrioid	41		27		---		---	
Bcl-2								
0	17	0.11	7	0.22	6	0.007	0	0.03
1+, 2+, 3+	26		14		23		14	

On the basis of an assessment of traditional pathologic prognostic factors (including intraoperative frozen section analysis), lymphadenectomy was performed in 82 patients, according to the guidelines for surgical staging that were in place during the era of the study. Six cases were eliminated because of technical inadequacies in tissue preparation for staining analysis; also, bcl-2 staining information was not available for 3 other patients. Overall, both bcl-2 staining intensity and ER levels were available for hysterectomy specimens from 74 patients and information about both PR levels and bcl-2 expression for 59.

According to the statistical design described above, we estimated that, in the overall population of 283 patients, 26% had histological grade 3 disease, 19% had nonendometrioid histological subtype and 73% were bcl+. The mean ER and PR levels were 254±346 fmol/mg (range, 0-883) and 434±678 fmol/mg (range, 0-2,260), respectively. Of the patients who had grade 1 or 2 endometrioid endometrial cancer (an estimated 69% of the overall population), 75% were bcl+. In this subgroup, the mean ER and PR levels were 275±228 fmol/mg (range, 0-813) and 517±507 fmol/mg (range, 0-2,260), respectively, and the mean levels of hormone receptors were significantly higher than those of patients with grade 3 tumor or nonendometrioid histological subtype ($p<0.01$) (Table I).

When considering the entire extrapolated population, bcl+ was significantly associated with endometrioid tumors but not with histological grade. In fact, 75% of tumors of endometrioid histological subtype were bcl+, compared with 60% of nonendometrioid subtypes ($p=0.04$). Bcl-2 expression did not correlate with grade (72% of grade 1 or 2 tumors *vs.* 75% of grade 3 tumors [$p=0.63$]). Overall, bcl+ tumors had significantly higher ER and PR levels than bcl- tumors ($p=0.01$) (Table I). However, when only patients with grade 1 or 2 endometrioid endometrial cancer were considered, the ER and PR levels were not significantly associated with bcl+ tumors (Table I).

Extrauterine disease was observed in 23% of the population, and 12% of patients who had lymphadenectomy had positive lymph nodes. For the subset of patients who had grade 1 or 2 endometrioid endometrial cancer, 18% had extrauterine disease and 10% had positive lymph nodes.

It was estimated that in the overall population of 283 patients, histological grade 3, nonendometrioid histological subtype and low levels of ER and PR were significantly associated with extrauterine disease ($p\leq0.05$) (Tables I and II). Furthermore, the nonendometrioid histological subtype was significantly associated with positive lymph nodes ($p<0.01$) (Table II). However, when only patients with grade 1 or 2 endometrioid endometrial cancer were considered, neither the ER nor PR levels were significantly associated with extrauterine disease (Table I).

It was estimated that in the overall population of 283 patients, bcl-2 expression was not significantly associated with extrauterine disease. However, of patients with grade 1 or 2 endometrioid histological subtype (disease generally considered hormone-dependent), 23% of those who were bcl+ had extrauterine disease and 14% had positive lymph nodes, while of those who were bcl–, 6% had extrauterine disease ($p<0.01$) and 0% had positive lymph nodes ($p=0.03$) (Table II).

To test the validity of our extrapolated calculations based on the study design, nonextrapolated data from the original population of 125 patients were separately analyzed and reported . Overall, 8 out of 31 patients (26%) with negative staining for bcl-2 had extrauterine disease compared with 30 out of 85 (35%) of patients with positive staining for bcl-2 ($p=0.34$) (bcl-2 information was missing for 9 patients [see Materials and Methods]). Moreover, considering only the 82 patients who had lymph node dissection, 3 out of 19 (16%) with negative bcl-2 staining had positive lymph nodes, compared with 12 out of 58 (21%) with positive bcl-2 staining ($p=0.64$) (bcl-2 information was missing for 5 patients). Most importantly, however, when considering only the 85

patients with an endometrioid grade 1 out or 2 tumor, 1 out of 19 (5%) with a bcl-2-negative tumor had extrauterine disease compared with 18 out of 60 (30%) with a bcl-2-positive tumor ($p=0.03$) (bcl-2 information was missing for 6 patients). This difference was even more striking when selecting only the 58 patients with an endometrioid grade 1 or 2 tumor who had lymph node dissection; none of the 12 (0%) patients with a bcl-2-negative tumor had positive lymph nodes compared with 9 of the 42 (21%) with a bcl-2-positive tumor ($p=0.08$) (bcl-2 information was missing for 4 patients).

Discussion

In normal endometrium, estrogens promote epithelial cell proliferation and progesterone induces epithelial cell differentiation. Hormonal changes in the endometrium have been shown to be modulated by apoptosis (13). In the endometrium, bcl-2 expression changes during the different phases of the ovulatory cycle, being more prominent during the proliferative phase than the secretory phase (5). It has been reported that PRs and ERs increase during the proliferative phase of the cycle in parallel with bcl-2, which is consistent with a correlation between hormonal activity and bcl-2 expression (5). These findings suggest that bcl-2 expression may be under hormonal control (*i.e.*, stimulated by estrogen and down-regulated by progesterone) and may regulate the apoptotic involution of the endometrium. More than 50% of endometrial carcinomas show bcl-2 persistence (14), suggesting a possible role for bcl-2 in preventing apoptosis and favoring endometrial carcinogenesis. In corpus cancer, bcl-2 expression has been demonstrated to be correlated with favorable prognosis, endometrioid histological subtype, low histological grade, superficial myometrial invasion and early stage disease. Moreover, a positive association was found between bcl-2 expression and ER and PR positivity in endometrial cancer (14).

Similar to endometrial cancer, bcl-2 expression in breast cancer has been found to be correlated directly with hormone receptors (3). It has been suggested that, during the first steps of malignant transformation of breast cancer, epithelial cells undergo numerous mitoses under the influence of estrogens, with a subsequent progressive accumulation of genetic alterations, such as *HER* amplification and *p53* deletion (15). The anti-apoptotic role of bcl-2 may potentially favor the occurrence of new mutations in a subset of hormone-dependent breast cancers (4).

It is generally accepted that endometrial cancer can be divided into type I and type II. Type I is the endometrioid histological subtype; it usually is low grade, diagnosed at an early stage, associated with hyperplasia and responsive to hormone therapy. Type I endometrial cancer arises during excessive epithelial proliferation stimulated by estrogens. In comparison, type II endometrial cancer is more aggressive, of nonendometrioid histological subtype, high grade and not responsive to hormonal therapy (9). Whereas type I endometrial tumors are associated with the expression of bcl-2 and uncommonly p53 (except in poorly-differientiated lesions or advanced stage), type II endometrial cancer is characterized by *HER-2/neu* overexpression, the presence of p53 and the absence of bcl-2 staining (9, 16). We hypothesized that the role of bcl-2 in preventing apoptosis may be important in less aggressive hormone-dependent endometrial cancer (type I) in which the extended life span of cells induced by bcl-2 may favor the acquisition and accumulation of genetic alterations. In contrast, the growth of more aggressive endometrial cancer (type II) is presumably aided by other factors that impede programmed cell death (9), and the role of bcl-2 in the process of carcinogenesis may become less important. In the present study, the subgroup of endometrioid low-grade corpus cancer were considered to be representative of type I hormone-dependent endometrial cancer. Support for the hypothesis of the hormone-dependency of this subgroup of tumors is its significant association with high levels of both ERs and PRs (Table I).

In our analysis, previously reported population of patients with endometrial cancer were used (10, 16, 17). The main advantage of using the case-cohort design was that it was possible to estimate the results in a consecutive series of patients, thus eliminating the selection bias due to the inclusion in the study group of all patients who had recurrence of disease and only a portion of patients who were free of recurrence. Moreover, the estimation of results in the overall population of 283 patients permitted the statistical results to be based on an extrapolated higher number of patients. However, according to the statistical design, it was necessary to assume that the limited group of 76 patients who did not have recurrence and who were included in the present analysis was representative of all patients without recurrence in the overall population. Therefore, all the results were based on an estimation and reported as percentages rather than actual numbers. To verify that our main conclusions (Table II) were not only a consequence of the statistical design, the association between bcl-2 and extrauterine disease was also testted using the actual population of 125 patients. This association was still found to be significant.

In our analysis, the levels of ER and PR were measured with a dextran-coated charcoal assay. Because of the retrospective nature of the analysis, the results had been previously described in the pathology reports, using quantitative ligand-binding assays. This technique has been replaced at our institution by immunohistochemical assays. Immunohistochemistry provides a more direct histological control of receptor determination than the quantitative

techniques, because nonmalignant tissue components may contribute to the total amount of receptors identified in a biopsy specimen from an endometrial tumor (18). However, in our series, the tissue immediately adjacent to the biopsy specimen submitted for ER and PR quantification was generally used for histological examination of paraffin-embedded sections to confirm the presence of cancer. This reduced the probability of measuring nonmalignant tissue. Moreover, the correlation between ligand-binding assays and immunohistochemical assays has been shown to be excellent, with ligand-binding assays having a higher sensitivity than immunohistochemical assays for detecting both ER and PR (18).

In our study, considering the whole population of endometrial cancers, it was observed that nonendometrioid histological subtype, high grade, and low levels of ER and PR were significantly associated with extrauterine disease (Tables I and II), confirming earlier reports (19). Patients with tumors expressing bcl-2 reportedly have a lower risk of presenting with extrauterine disease or positive lymph nodes (17, 19). However, not all authors agree with this (10). These divergent reports may reflect differences in patient populations and inherent frequencies of the histological subtypes and p53 overexpression (17). For this reason, we focused on the possible role of bcl-2 in type I cancers, although we recognized the aggressive behavior of bcl-2-negative type II endometrial cancers. When considering the whole population of endometrial cancers, bcl-2 expression was not significantly associated with extrauterine disease. However, when only endometrioid grade 1 or 2 endometrial cancers were analyzed, bcl-2 expression was significantly associated with extrauterine disease and lymph node invasion (Table II). These findings suggest that bcl-2-mediated inhibition of apoptosis may be an important step in the acquisition of additional molecular alterations and the subsequent development of metastases in low-grade endometrioid hormone-dependent endometrial cancers.

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