BCL-2, BAX and P53 Expression Profiles in Endometrial Carcinoma as Studied by Real-time PCR and Immunohistochemistry

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Abstract. *Background: Apoptotic genes regulate apoptosis by the action of their pro- and antiapoptotic products. Among the most important proteins are p53 and Bcl-x family proteins. Patients and Methods: The differential expression of these apoptotic genes were analyzed in relation to clinicopathological criteria in women with endometrial carcinoma. Thirty-three fresh tissues and 191 paraffin-embedded tissues were analyzed by real-time PCR for bcl-2/bax ratio and immunohistochemistry for p53, bcl-2 and bax proteins. Results: Bcl-2/bax ratio tended to increase in grade 3 samples compared to grade 1 tumors. Mutated p53 was frequently observed in serous–papillary endometrial carcinomas (p=0.018). Low (<10%) and moderate (10-50%) expression of mutated p53 was observed in tumors with high expression of bax protein (>0.7). Conclusion: The Bcl-2/bax ratio is increased in grade 3 tumors. Bax protein shows a strong tendency for expression in the third group of clinical staging (stage IIb, III and IV). Poorly differentiated tumors highly expressed mutated p53.*

Apoptosis is a genetically controlled cell suicide pathway which plays an essential role in deleting excess, unwanted or damaged cells during development and tissue homeostasis. Dysfunction of apoptosis contributes to a wide variety of pathological conditions in humans, including carcinogenesis (1).

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The endometrium changes morphologically and functionally due to hormonal response during the menstrual cycle. In 1976, Hopwood and Levison described apoptosis in normal endometrium (2). Later, Tabibzaden *et al.* (3) reported that apoptosis in the endometrium is rare in the proliferative phase, while the number of apoptotic cells increases gradually in the secretory phase. The increase is maximal in the menstrual phase (4).

Bcl-2 is a proto-oncogene which is expressed especially in glandular cells (5) at the end of the proliferative phase and this expression disappears when the secretory phase begins (6, 7). Bax is a pro-apoptotic protein that induces cell death by its homodimerization and heterodimerization with bcl-2 (7) and other members of the bcl-2 protein family.

Wild-type p53 contributes to the suppression of tumors by inhibiting cell proliferation and inducing cell death by apoptosis (8). P53 is a transcriptional factor of *bax* gene and the loss of its normal function may result in diminished expression of bax (7). There is an interaction between p53 and bcl-2 in the regulation of apoptosis in endometrial carcinoma which is different from that in other type of tumors (9).

The aim of this study was to analyze the bcl-2/bax ratio and its correlation with clinicopathological factors in patients with endometrial carcinoma.

Patients and Methods

Study population and specimen collection. Fresh endometrial tissues were obtained from 33 patients with endometrial carcinoma (30 with endometrioid adenocarcinoma, 1 with clear-cell carcinoma and 2 with serous–papillary carcinoma), and 191 paraffin-embedded tissues from patients who were submitted to abdominal hysterectomies in the First Gynecological Department of the Aghios Savvas General Anticancer Hospital of Athens (Greece) from 1986-2007. All patients were Caucasians, with an age range of 32-82 years (mean 63.3 years). The

Table I. *Clinical parameters*.

Variable	No. of patients
Age range (years), 32-82 years (mean 63.3 years)	
Family history (sister and/or mother)	
Endometrial cancer	1
Breast cancer	3
Other neoplasia	3
Personal history (other neoplasia)	
Breast	14
Vulva	2
Thyroid	1
Gastric	1
Parity	
Nulliparous	28
≤2	70
>2	31
Unknown	18
Predisposing factors	
Obesity	6
Tamoxifen users	8
Diabetes	10
Endometrial phase	
Atrophic	38
Proliferative	1
Secretory	1
Tamoxifen users	8

Table II. *Clinicopathological data – complementary therapy and recurrence*.

Variable	No. of cases
Histological cell type	
Endometrioid	144
Serous-papillary	7
Clear cell	2
Adenosquamous	2
FIGO stage	
Ia	23
Ib + Ic + IIa	119
IIb + III + IV	30
FIGO grade	
1	64
2	34
3	61
Lymph-node excision	
No	84
Yes	60
Unknown	2
Lymph-node metastasis	
Negative	53
Positive	7
Peritoneal cytology	
No washings	13
Negative	118
Positive	9
Unknown	8
Follow-up therapy	79
External radiotherapy (RT)	37
External RT + brachytherapy	21
Brachytherapy	4
Chemotherapy	3
External RT + hormonotherapy	1
Hormonotherapy	2
Recurrence	
No	9
Yes	14
Recurrence area	
Vagina	4
Pelvis	3
Abdomen/Intestine	3
Distant	4

Hospital's Ethical Committee provided approval for this study. All participants gave their consent before entrance to the study.

The classification of the endometrial phase was carried out according to the criteria of Noyes and Herig (10). Each specimen was reviewed by two expert pathologists and classified according to the FIGO grading system (11). The tumors were also characterized as well differentiated (composed of glands, G1; n=11), moderately differentiated (a combination of glands and masses of solid epithelium, G2; n=7) and poorly differentiated (predominantly solid proliferations, G3; n=4) (12). Our samples were thus categorized according to the clinical staging into three major groups: the first group included stage Ia, the second group stage Ib, Ic and IIa, and the third group IIb, III and IV. Twenty-three out of 186 samples belonged to group 1, 119 to group 2, and 30 to group 3. Patients characteristics are given in Tables I and II.

RNA extraction and cDNA synthesis. As previously described (13), total cellular RNA was obtained from tissue biopsies. Five micrograms of RNA per sample were separated on 1% formaldehyde-agarose gels to assess RNA integrity. RNA concentrations were amended for quality by spectophotometry (E=260 nm). First-strand cDNA was synthesized from 4 μg of total RNA using an Oligo$(dT)_{12\text{-}18}$ primer and Superscript™ II RNase Reverse Transcriptase (Invitrogen, USA). Samples were stored at –20˚C.

Real-time PCR. Thirty-three biopsies were processed for real-time PCR analysis. The quantification of the selected genes by real-time PCR was performed using Rotor Gene 6000 (Corbett Life Science, USA) using the primers shown in Table III. Every reaction consisted of 2 μl cDNA, 1 μl of each primer (400 nM) and 21 μl reaction buffers (Platinum SYBR Green) (total reaction volume 25 μl) (Invitrogen). Real-time PCR cycles consisted of: 2 minutes at 50˚C, 4 minutes at 95˚C for polymerase activation, 45 cycles of 10 seconds at 95˚C (denaturation) 5 seconds at 54˚C, 5 seconds at 72˚C and 15 seconds at 83˚C (annealing and extension). Finally, melting was curried out at 72-95˚C (0.5˚C increments) for 5 seconds for each step. β-Actin of each sample served as intrinsic control. The threshold cycle (CT) of each sample was normalized to β-actin. Relative quantification analysis was carried out with the Rotor Gene 6000 series software version 1.7. The analysis uses the sample's crossing point, the efficiency of the reaction, the number of cycles completed and other values to compare the samples and generate the ratios. The results are expressed as a normalized ratio.

Table III. *Real-time PCR primers used (Invitrogen, USA).*

Gene	Primer
Bcl-2 (F)	5' ATC GCC CTG TGG ATG ACT GAG 3'
Bcl-2 (R)	5'CAG CCA GGA GAA ATC AAA CAG AGG 3'
Bax (F)	5' GGA CGA ACT GGA CAG TAA CAT GG 3'
Bax (R)	5' GCA AAG TAG AAA AGG GCG ACA AC3'
β-Actin (F)	5' TCT GGC ACC ACA CCT TCT ACA ATG 3'
β-Actin(R)	5' AGC ACA GCC TGG ATA GCA ACG 3'

F, Forward; R, reverse.

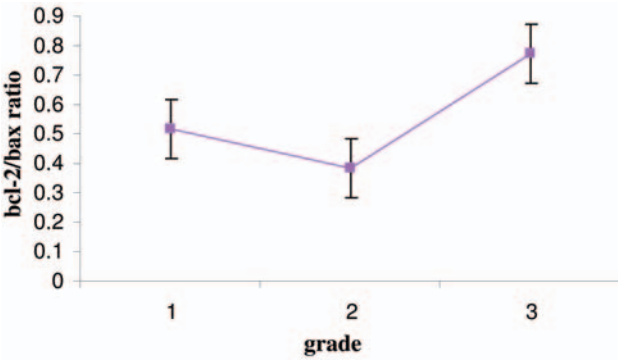

Figure 1. *Real-time PCR analysis for the ratio of bcl-2/bax among different groups of grade.*

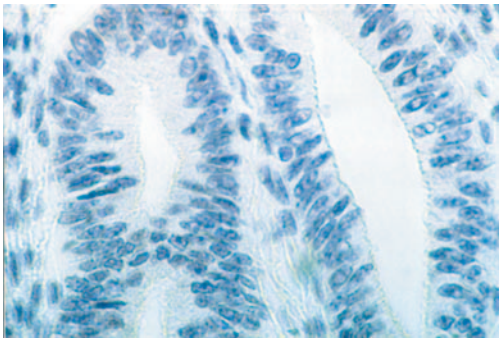

Figure 2. *Negative expression of bax protein in normal proliferative endometrium (×400) (Nikon HFX-IIA).*

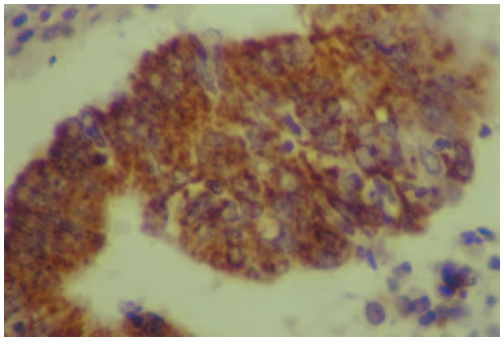

Figure 3. *Immunohistochemical cytoplasmic stain in endometrioid adenocarcinoma for bax protein (grade 3) (×400) (Nikon HFX-IIA).*

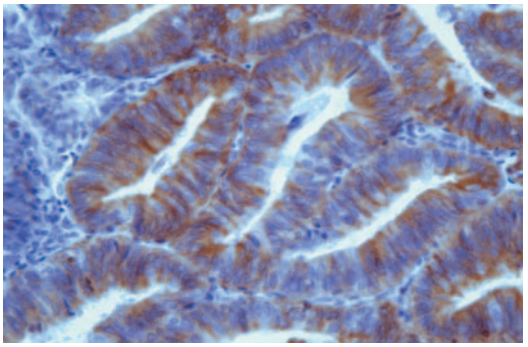

Figure 4. *Immunohistochemical cytoplasmic stain in normal secretory endometrium for bcl-2 (grade 2) (×400) (Nikon HFX-IIA).*

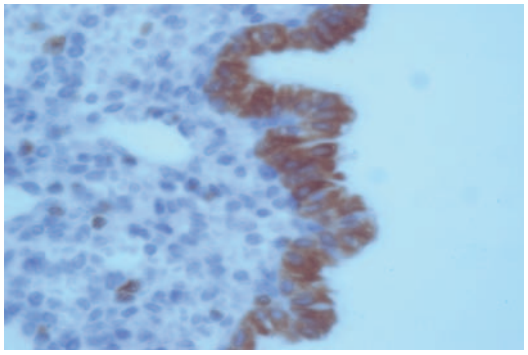

Figure 5. *Immunohistochemical cytoplasmic stain in endometrioid adenocarcinoma for bcl-2 (grade 3) (×400) (Nikon HFX-IIA).*

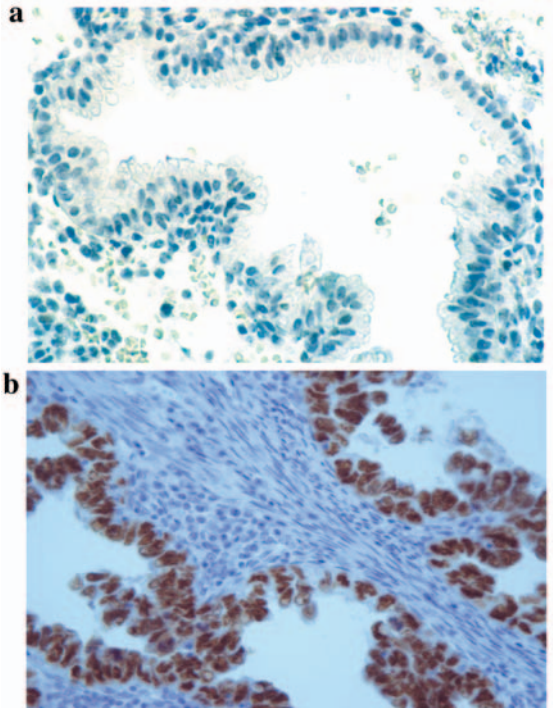

Figure 6. *a, Norman secretory endometrium: negative for mutated p53 M (×200) (Nikon HFX-IIA). b, Endometrioid adenocarcinoma: immunohistochemical expression of mutated p53 (grade 3) (×400) (Nikon HFX-IIA).*

Immunohistochemistry. Formalin-fixed paraffin-embedded tissues were used for this study. Samples were fixed in 4% buffered formalin and then routinely processed for paraffin embedding. Five-micrometer thick sections were obtained by microtome. They were dried in an autoclave at 60˚C for 30 minutes. Trilogy solution was used for deparaffinization, rehydration and unmasking (Cellmarque, USA). The samples were then placed in Perox Free Block solution (Cellmarque) for 10 minutes to inhibit endogenous peroxidases. EnVision and Dual Link System Peroxidase were used for 25 minutes (DakoCytomation, USA). The antibodies that were used for the detection of bcl-2, bax and mutated p53 (mp53) were mouse anti-human monoclonal antibodies Clone 124, Clone SPM336 and Clone DO-7, respectively (DakoCytomation, USA Spring Bioscience, USA). Liquid DAB and the Substrate Chromogen System were used for 6 minutes (DakoCytomation, USA).

Staining evaluation and statistical analysis. The stained slides were microscopically analyzed by two expert pathologists independently. The results of immunostaining for bcl-2 and bax were analyzed semiquantitatively by using an immunohistochemical histological score (HSCORE), which incorporated both the intensity and the distribution of specific staining. The HSCORE was formulated as HS=Σ(Pi x I/100), where Pi denotes the percentage of stained cells and I denotes the intensity of staining which ranges from 1 to 3. The immunostaining for p53 was characterized as 0 for tissues without staining, 1 for 0-10% p53-positive cells, 2 for <50% positive cells and 3 for >50% positive cells.

Data were assessed with ANOVA (analysis of variance) and Chi-square with SPSS software version 13.0 (USA). Statistical significant was accepted at $p \leq 0.05$.

Results

The molecular analysis for *bcl-2* and *bax* gene revealed that there was no statistically significant difference among the different grades. However, the bcl-2/bax ratio appeared to have a tendency to increase in grade 3 samples compared to that in grade 1 tumors. We also find that this ratio was lower in grade 2 tumors. This result may presents due to the small number of the samples (Figure 1).

The analysis of the bcl-2/bax ratio reveals that in 36.5% of samples the ratio was greater than, while in 63.5% of the samples it was below 1. According to the statistical analysis, a ratio greater than 1 was more likely to be found in tumors in group 2 of our clinical staging (p=0.078). However, no significant association was found between the ratio and the histological grading.

The immunohistochemical analysis of the expression of bax protein showed that out of 191 samples, 33 were negative (HSCORE: 0-0.05). In 110 samples, the HSCORE was between 0.051 and 0.69 (low and moderate expression of the protein) and in 48 samples it was >0.7 (high expression) (Figures 2 and 3).

Regarding the expression of bcl-2 protein, 85 out of 180 samples were negative, while 57 and 38 expressed bcl-2 protein with an HSCORE of between 0.051-0.69 and >0.7, respectively (Figures 4 and 5).

The immunohistochemical analysis also revealed that mp53 was not expressed in 37 out of 186 samples. In 119 samples, the protein was present in <10% and in 10-50% of cancer cells, while in 30 samples the protein was expressed in the majority of cancer cells (>50%) (Figure 6a and b).

In the statistical analysis, the expression of all proteins was tested for correlation with histological grading, clinical staging, myometric and lymph-node invasion, recurrence, parity, age, chemotherapy and radiotherapy and other clinicopathological criteria given in Tables I and II.

Tumors which revealed the strongest tendency (p=0.101) for high expression of bax protein (>0.7) belonged in group 3 of our clinical staging, while high expression of bcl-2 (>0.7) seemed to be related (p=0.132) to tumors of the second group.

Mp53 was expressed in 163 samples (64 grade 1, 61 grade 2 and 34 grade 3). Statistical analysis revealed no statistically significant differences among the three groups of clinical staging. We found, however, that mp53 was more frequently observed in serous–papillary endometrial carcinomas (p=0.018). Low (<10%) and moderate (10-50%) mp53 expression was observed in tumors with high expression of bax protein (>0.7). No statistically important results were found for correlation between p53 and bcl-2.

Discussion

In recent years, it has been thought that apoptosis may constitute an important mechanism in carcinogenesis. Apoptotic and antiapoptotic genes produce proteins that regulate apoptosis. The dysfunction of their expression may contribute in carcinogenetic pathways. Bcl-2 as an antiapoptotic protein inhibits apoptotic death and its expression in normal menstrual cycle is an established fact. There are studies which support that overexpression of this protein in endometrial tissue may be the signal which cancer cells need in order to establish their presence in the tissue and grow in a malignant neoplasm (14, 15).

On the other hand, bax protein enhances the apoptotic stimuli, leading cancer cells to apoptosis. Overexpression of this protein may inhibit the malignant progression of a tumor. What happens when in endometrial tissue there are cancer cells in which bcl-2 and bax proteins co-exist? Which of the two proteins will be the dominant signal?

Previous studies report that bcl-2 forms heterodimer complexes with bax *in vivo* and the bcl-2/bax ratio is determinant of the induction as well as the inhibition of apoptosis in human tissues (7). When this ratio is greater than 1, the antiapoptotic signal is dominant among the cells, when the ratio is <1, then apoptotic mechanism should prevail.

To indentify the role of this ratio in endometrial carcinogenesis, we analyzed the expression of these two proteins with real-time PCR. The results were correlated with clinicopathological data. To our knowledge, there are no

previous studies for real-time PCR analysis for bcl-2/bax ratio in endometrial carcinoma. We found that the bcl-2/bax ratio was raised in poorly differentiated tumors (grade 3). The results of the immunohistochemical analysis were similar. This is in contrast with other molecular analysis studies (15-17) in endometrial and other carcinomas. This is justified since bcl-2 inhibits apoptosis. In malignant cells, bcl-2 expression should be high, since impairment of apoptosis seems to have an important role in carcinogenesis. Grade 3 tumors, which are more aggressive, overexpressed the anti-apoptotic signal in the cell, as we can note from the bcl-2/bax ratio. However, because of the small number of fresh tissue samples we had available for real-time PCR, we are not able to derive statistical results about the correlation of the bcl-2/bax ratio with the grade of differentiation of the tumor. This might also be the explanation for the observation that in our study, the bcl-2/bax ratio appeared to decrease in grade 2 as compared to grade 1 tumors.

Another interesting observation is that tumors which were classified as Ic in clinical staging appeared to have a greater possibility of having a bcl-2/bax ratio >1. If our results are confirmed in a greater number of samples, then the ratio might be used in the future as an important diagnostic or even prognostic indicator in endometrial cancer.

Bax protein showed a strong tendency of expression in the third group of clinical staging as in previous studies (18). This result was not expected considering that tumors in this group have the worst progression, while the expression of bax protein should protect the tissue from malignant transformation. However, there are other studies that disagree regarding the correlation of the protein with clinicopathological prognostic criteria such as clinical staging and histological subtype of the tumors (7). Previous studies (16) have shown a correlation between bax protein and grade of differentiation, while in our study this was not observed.

We also found no any statistically important differences in correlation with the grade of differentiation as previous studies have shown (19, 20); some studies have shown that bcl-2 expression was low in grade 3 tumors (14, 18, 19, 21). It is well known that poorly differentiated tumors are often metastatic. The overexpression of bcl-2 may be a negative factor for the progression of the neoplasm. Thus, the inhibition of the apoptotic mechanism may be considered as a prognostic factor for the progression of the disease. The correlation of bcl-2 expression with clinical staging has already been reported in some studies (22), while others do not support this finding (23, 24).

Mp53 was found to be highly expressed in poorly differentiated tumors (19) a finding that was also observed in our samples. The mutated form of p53 protein was observed in 17-52% of endometroid adenocarcinoma, while in serous carcinomas the expression of this form was 50-86% (25-27). In our study, this mutated form was expressed in a very high percentage of our samples.

In our study, increased expression of bax protein (>0.7) is more likely to arise from cells with high expression of p53 (more than 10% of the tumor cells or in 10-50% of them). This finding is possibly related to the fact that p53 protein is a transcriptional factor of bax. Tumors with lower expression of mp53 seemed to intensely express the pro-apoptotic protein bax. This observation suggests that loss of expression of normal p53 is associated with a lower expression of bax protein. Poorly differentiated carcinomas (grade 3) usually overexpress mp53, which means that the absence of the pre-apoptotic signal is related to the evolution and the characteristics of the neoplasm. A number of studies report that since p53 is a transcriptional factor of bax, p53 mutations inhibit bax transcription (28). Decreased bax expression may also be the result of various mutations of the *bax* gene (28). However, other papers do not correlate the alteration of bax expression with p53 or even bcl-2.

Considering p53 and bcl-2 expression, our study did not infer any statistically significant difference. However, some studies do support the theory of inversely related expression of p53 and bcl-2 (possibly bcl-2 functions as a transregulator of p53 (19)). Specifically, the poorer the tumor differentiation is, the more bcl-2 expression is reduced and the more p53 expression is increased.

The findings of this study suggest that the difference between cell proliferation and apoptosis may be an important factor in the development of malignant neoplasias in endometrium. It is already known that carcinogenesis cannot be simply explained as the enhancement of cell growth, but may be caused by the loss of growth inhibition and changes in apoptotic cell death. The results from many studies of the molecular and genetic changes in carcinogenesis of the endometrium are inconsistent, which reflects the complexity and diversity of this process. Further investigation of the expression of p53, bcl-2 and bax proteins may be reveal their use as new diagnostic or/and prognostic tools for clinical practice.

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