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To link to this article: https://doi.org/10.1080/01443615.2018.1437717

Published online: 21 Mar 2018.
Expression of p53, Bcl-2 and Bax in endometrial carcinoma, endometrial hyperplasia and normal endometrium: a histopathological study

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ABSTRACT
Our objective was to examine the expression rates of p53, Bcl-2 and Bax in endometrial carcinoma, endometrial hyperplasia and normal endometrium. A total of 94 endometrial frozen sections (carcinoma 48, hyperplasia 21, normal tissue 25) were examined immunohistochemically in terms of the expression rates of p53, Bcl-2 and Bax. All of the specimens in the non-malignant groups were positive for Bax, whereas this rate was 85.4% in the group with malignant specimens ($p = .03$). Conversely, p53 was expressed only in the cancerous group (77.1%, $p < .001$). The Bcl-2 expression rate was 54.2% in the cancer group, 76.2% in the group with hyperplasia and 60% in the group containing normal tissue ($p = .23$). Comparing to the non-malignant specimens, the mean Bcl-2/Bax were significantly higher in the malignant group. In conclusion, Bax under-expression, p53 over-expression and a high Bcl-2 to Bax ratio might be associated with endometrial carcinoma. Bcl-2, however, plays no significant role in this regard.

IMPACT STATEMENT
- What is already known on this subject? The p53, Bcl-2 and Bax are the three major genes that regulate apoptosis. Some studies have suggested that these genes may play a role in the pathogenesis of endometrial carcinoma. The available reports, however, are old and inconclusive.
- What do the results of this study add? Comparing immunohistochemically obtained p53, the Bcl-2 and Bax expression rates between normal endometrial tissue, endometrial specimens with endometrial hyperplasia and specimens with carcinoma showed that Bax under-expression, p53 over-expression and a high Bcl-2 to Bax ratio were associated with malignancy. Using an up-to-date technique to examine the three major regulators of apoptosis at the same time, in a rather large sample size of both normal and abnormal endometrial tissue specimens simultaneously, are the major advantages of the present work.
- What are the implications of these findings for clinical practice and/or further research? According to our findings, the status of p53, Bcl-2 and Bax expression in the endometrial tissue can be used for risk stratification of endometrial carcinoma for both screening and preventive purposes.

Introduction
Endometrial cancer is a common gynaecological malignancy all over the world (de Haydu et al. 2016). By now, various factors have been suggested in connection with this cancer in terms of pathogenesis and prognosis, such as age, ethnicity, subtype and grade (Axtell and Myers 1978; Creasman 1997). The p53 and Bcl-2 genes are important regulators of apoptosis. They have been found to play substantial roles in the pathogenesis of different types of cancers including endometrial carcinoma (Geisler et al. 1996; Geisler et al. 1998; Sakuragi et al. 1998; Geisler et al. 1999). The p53 gene is a tumour suppressor that causes cell cycle cessation and induces apoptosis in cells bearing injured genomes. In addition, p53 gene activity may predict prognosis in patients with cancer (Appel et al. 2008).

In contrast, the Bcl-2 gene acts in the opposite direction by encoding an anti-apoptotic protein (Morsi et al. 2000; Sabeti et al. 2013). While the endometrial expression of Bcl-2 may decrease in atypical hyperplasia and adenocarcinoma, a sustained expression has been found in specimens with early endometrial carcinoma (Coppola et al. 1998; Marone et al. 2000). Bcl-2 expression has been found in association with early-stage, well-differentiated endometrial tumours with a good prognosis (Yamauchi et al. 1996; Saegusa and Okayasu 1997; Taskin et al. 1997).

The Bax gene constitutes the third major component in the axis of apoptosis (Figure 1). Actually, Bax is a member of the Bcl-2 gene and a transcriptional target of p53 (Flaman et al. 1998; Campomenosi et al. 2001). Its proapoptotic protein promotes cell death and neutralises the anti-apoptotic function of Bcl-2. Two previous studies have suggested a
significant role for the Bax protein in the genesis of endometrial carcinomas (Burks et al. 1994; Catasus et al. 1998).

Because of inconsistency among the available reports regarding the importance of p53, Bcl-2 and Bax proteins in patients with endometrial carcinoma and noting that much of the current data come from old series, this study seeks to examine the p53, Bcl-2 and Bax immunohistochemical expression rates in normal endometrial tissue as well as in endometrial specimens with endometrial hyperplasia and carcinoma.

Materials and methods

Between 2011 and 2015, a total of 94 frozen endometrial specimens containing normal tissue (n = 25), atypia-free endometrial hyperplasia (n = 21) and carcinoma (n = 48) were examined in three teaching hospitals. In the first group, all of the specimens were obtained during the proliferative phase. In the third group, specimens from patients with other malignant condition(s) and those with a positive history of neoadjuvant therapy were not included.

Samples were snap-frozen in liquid nitrogen and stored at −80°C. To define histotype in the cancer group, freshly prepared paraffin-embedded hematoxylin-eosin-stained tumour specimens were used. A pathologist with over 10 years of academic experience reported subtypes including endometrioid adenocarcinoma and nonendometrioid adenocarcinomas (serous, clear-cell and undifferentiated carcinomas) according to the established guidelines defined by the College of American Pathologists (http://www.cap.org). Surgical cancer staging was carried out according to the method developed by the International Federation of Gynecology and Obstetrics (FIGO) (Langdon et al. 1997).

Immunohistochemistry

Sections with 5-micron thickness were obtained from the paraffin blocks after a histopathologic confirmation. To detect Bax protein, two rabbit polyclonal antibodies, namely Ab-1 (Calbiochem Novabiochem Corp., Darmstadt, Germany) and P-19 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used. The latter with excess molar ratios of the concomitant blocking peptide was used for the negative control study (Sakuragi et al. 2002).

A mouse monoclonal anti-p53 antibody (clone DO-7, Dako Japan, Co. Ltd, Kyoto, Japan) and a monoclonal anti-Bcl-2 antibody (clone 124, Dako Japan, Co. Ltd, Kyoto, Japan) were used for detecting related proteins. Positive controls were also used (breast cancer for p53 expression and lymph nodes for Bcl-2 expression). The process comprised of the deparaffinisation and rehydration, antigen retrieval, inactivation of endogenous peroxidase and the blocking of non-specific reactions, sequentially. Then, the samples were incubated for two hours at ambient temperature with a diluted solution of primary antibodies according to the guidelines provided by the manufacturers. The indirect streptavidin-biotin-peroxidase complex (LSAB, Dako Japan, Co. Ltd, Kyoto, Japan) method was used as described previously (Sakuragi et al. 1994). The degree of immunohistochemical staining was determined as follows: 0, no staining; 1+, focal (<50% of cells) staining; and 2+, diffuse (>50% of cells) staining (Sakuragi et al. 2002).

Statistics

The statistical analysis was performed using SPSS software Ver. 21 (IBM Corporation, Chicago, IL, USA). A normal distribution of the numerical data was confirmed using the Kolmogorov–Smirnov test. One-way ANOVA, independent samples t-test, Chi-square test and Fisher’s exact test were used for comparisons, where appropriate. A p-value less than .05 was considered statistically significant.

Results

The mean patients’ age was 59.60 ± 16.91 years (33–82) in the control group, 61.14 ± 16.01 years (33–82) in the group with endometrial hyperplasia and 62.42 ± 12.85 years (34–81) in the group with cancer. The endometrial cancer was most commonly of grade I (56.3%) as well as of endometrioid subtype (66.7%) (Table 1).

<table>
<thead>
<tr>
<th>Variable</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIGO stage</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>27 (56.3)</td>
</tr>
<tr>
<td>II</td>
<td>7 (14.6)</td>
</tr>
<tr>
<td>III</td>
<td>7 (14.6)</td>
</tr>
<tr>
<td>IV</td>
<td>7 (14.6)</td>
</tr>
<tr>
<td>Histotype</td>
<td></td>
</tr>
<tr>
<td>Endometrioid</td>
<td>32 (66.7)</td>
</tr>
<tr>
<td>Serous</td>
<td>9 (18.8)</td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>4 (8.3)</td>
</tr>
<tr>
<td>Clear-cell</td>
<td>3 (6.3)</td>
</tr>
</tbody>
</table>

The expression rates of the three immunohistochemistry markers are summarised in Table 2. Accordingly, Bax was expressed less frequently in the malignancy (85.4%) as compared to the normal (100%) and hyperplastic (100%) groups (Chi-square test, p = .03). In contrast, p53 was expressed only in the cancer group (77.1%) (Chi-square test, p < .001). The mean Bcl-2 to Bax ratio was significantly higher in the cancer
group, in comparison with that in the normal and hyperplastic groups (One-way ANOVA, \( p < .001 \) and .02, respectively). A significant difference was also present in this regard between the latter non-malignant groups (\( p = .03 \)).

The expression rates of the three immunohistochemistry markers in the malignant group are stratified by the grade and type of cancer in Table 3. There was no significant difference between the four stages of malignancy in terms of the expression rate of Bax (Chi-square test, \( p = .71 \)), Bcl-2 (Chi-square test, \( p = .31 \)) and p53 (Chi-square test, \( p = .48 \)). The mean Bcl-2/Bax, however, was significantly higher in stages III–IV than in stages I–II (0.82 ± 0.67 vs. 0.40 ± 0.62; independent samples t-test, \( p = .04 \)) (Figure 3). The endometroid and non-endometrioid subtypes of malignancy were also comparable as the expression rate of Bax (Fisher’s exact test, \( p = .40 \)), \( Bcl-2 \) (Chi-square test, \( p = .41 \)) and p53 (Fisher’s exact test, \( p = .07 \)) (Figure 2). The mean Bcl-2/Bax, however, was significantly higher in the non-endometrioid group as compared to that in the endometrioid group (0.81 ± 0.73 vs. 0.38 ± 0.58; independent samples t-test, \( p = .03 \)) (Figure 3).

**Table 2. Immunohistochemistry findings in three study groups.**

<table>
<thead>
<tr>
<th>Positive marker</th>
<th>Normal ( n ) (%)</th>
<th>Hyperplasia ( n ) (%)</th>
<th>Carcinoma ( n ) (%)</th>
<th>( p ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax</td>
<td>25 (100)</td>
<td>21 (100)</td>
<td>41 (85.4)</td>
<td>0.03*</td>
</tr>
<tr>
<td>+</td>
<td>18 (72)</td>
<td>13 (61.9)</td>
<td>25 (52.1)</td>
<td>0.09</td>
</tr>
<tr>
<td>++</td>
<td>7 (28)</td>
<td>8 (38.1)</td>
<td>16 (33.3)</td>
<td></td>
</tr>
<tr>
<td>Bcl-2</td>
<td>15 (60)</td>
<td>16 (76.2)</td>
<td>26 (54.2)</td>
<td>0.23</td>
</tr>
<tr>
<td>+</td>
<td>9 (36)</td>
<td>6 (28.6)</td>
<td>13 (27.1)</td>
<td>0.31</td>
</tr>
<tr>
<td>++</td>
<td>6 (24)</td>
<td>10 (47.6)</td>
<td>13 (27.1)</td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>37 (77.1)</td>
<td>&lt;.001*</td>
</tr>
<tr>
<td>+</td>
<td>–</td>
<td>–</td>
<td>16 (33.3)</td>
<td>–</td>
</tr>
<tr>
<td>++</td>
<td>–</td>
<td>–</td>
<td>21 (43.8)</td>
<td></td>
</tr>
<tr>
<td>Bcl-2/Bax</td>
<td>0.16 ± 0.22</td>
<td>0.33 ± 0.34</td>
<td>0.52 ± 0.66</td>
<td>&lt;.001*</td>
</tr>
</tbody>
</table>

A \( p \)-value <.05 is considered statistically significant (\*). Table 3. Expression rates of Bax, Bcl-2 and p53 stratified by grade and subtype of endometrial carcinoma.

**Discussion**

The usefulness of various tumour markers in managing the patients with endometrial cancer has been studied extensively. Owing to the advancing technology and the development of new techniques in molecular biology, however, the topic is still intriguing for researchers (Morsi et al. 2000; Abdalla Ribeiro et al. 2014; Chand et al. 2014; Feng et al. 2014; Shan et al. 2015; Wang et al. 2015). In our study, a higher expression of p53 (77.1 vs. 0%) and a lower expression of Bax (85.4 vs. 100%) were significantly associated with malignancy. In contrast, Bcl-2 played no significant role in this regard (54.2% in malignant specimens, 60% in normal specimens, 76.2% in hyperplastic specimens).

In an immunohistochemical study by Appel et al. (2008) on specimens with endometrial carcinoma, p53 and Bcl-2 were expressed in 39.6% and 58.3% of the cases, respectively. Erkanli et al. (2004) examined nine samples with proliferative endometrium, five samples with endometrial hyperplasia and 35 samples with endometrial carcinoma. Bcl-2 was expressed in 100% of non-malignant specimens and in 60% of malignant specimens. In this study, the expression rate of p53 was 20% in malignant specimens. In another series, Sakuragi et al. (2002) examined 56 endometrial frozen specimens with normal tissue (\( n = 14 \)), hyperplasia (\( n = 13 \)) and carcinoma (\( n = 29 \)). In line with our findings, Bax was expressed in all the normal and hyperplastic specimens and in 73.9% of the malignant samples. In other studies on the specimens with endometrial carcinoma, the expression rate of p53 varied between 17% and 86% (Kohler et al. 1996; Kounelis et al. 2000; Erkanli et al. 2004; Lax 2004; Sari et al. 2004; Lomo et al. 2008) and the expression rate of Bcl-2 was between 34% and 85% (Kounelis et al. 2000). In conformity with our findings, Ohkouchi et al. (2002), Kounelis et al. (2000) and Sakuragi et al. (2001) demonstrated a significant association...
between the p53 expression rate and the endometrial malignant changes. Similarly, Kokawa et al. (2001) found no significant relationship between Bcl-2 expression and endometrial cancer. In contrast, Feng et al. (2014) and Porichi et al. (2009) proposed that Bax over-expression may represent endometrial malignant changes and aggressiveness, and Ohkouchi et al. (2002) found both p53 and Bcl-2 genes in association with a tumour aggressiveness. Finally, Bonnefoy-Berard et al. (2004) suggested that a low or absent Bcl-2 expression could predict a tumour progression and dissemination in cases with endometrial carcinoma.

This heterogeneity in reports may stem from racial/ethnic variations of study populations. For example, Kohler et al. (1996) showed a higher rate of p53 expression among African-Americans with endometrial cancer in comparison with Caucasians. Inconsistency in histopathologic classification, tissue preparation, selection of antibodies, staining protocols and definition of "positivity" may also play a role in this regard (Sherman 2000).

In conformity with previous studies (Kounelis et al. 2000; Marone et al. 2000; Halperin et al. 2001; Sakuragi et al. 2002; Erdem et al. 2003; Appel et al. 2008), we found no association between the expression rates of the examined biomarkers and the stage/subtype of endometrial cancer.

Fluctuations in Bcl-2 expression rates in the normal endometrium (highest in the proliferative phase, lowest in the secretory phase) posit a possible hormonal regulation. Marone et al. (2000) showed that the Bcl-2 expression rate is higher in the normal endometrium as compared to that in the hyperplastic/cancerous tissue. A sustained over-expression, however, may suggest a malignancy (Appel et al. 2008). Apoptosis is a normal process in the endometrium. (Hopwood and Levison 1976; Goumenou et al. 2004). A characteristic feature of the normal endometrium is cyclic morphological and functional changes associated with the ovulatory cycle. In a nutshell, oestrogen induces Bcl-2 expression and an elevation in Bcl-2 to Bax ratio prevents apoptosis and the cell survives (proliferative phase). Apoptotic stimuli, on the other hand, activate p53 expression, resulting in a Bax up-regulation and Bcl-2 suppression. So, the Bcl-2 to Bax ratio plummets, apoptosis ensues and the endometrium sloughs off (secretory phase) (Miyashita et al. 1994; Perillo et al. 2000; Vaskivuo et al. 2000; Choi et al. 2001; Sakuragi et al. 2002) (Figure 1). The malfunction of p53, as a transcriptional factor of Bax gene, causes under-expression of this gene. Interactions between p53, Bcl-2 and Bax as apoptotic (p53 and Bax) and antiapoptotic (Bcl-2) factors is unique in the endometrium and necessary for its normal function (Campomenosi et al. 2001; Cinel et al. 2002; Mertens et al. 2002; Sakuragi et al. 2002; Maia et al. 2004). Any abnormality of the p53-Bcl-2-Bax signalling pathway may cause a malignancy (Peiro et al. 2001; Sakuragi et al. 2002; Mitselou et al. 2003). Since the Bax protein promotes the apoptosis of cancer cells, its over-expression is thought to be an anti-malignancy mechanism in the endometrium (Porichi et al. 2009). This explains a lower expression rate of Bax in our malignant specimens as compared to that in the non-malignant counterparts. Therefore, any unopposed oestrogen stimulation and/or the mutation of p53 gene could increase cancer risk (Sakuragi et al. 2002). Like in our study, Sakuragi et al. (2002) found that in some patients with endometrial cancer, both p53 and Bax genes are over-expressed. Sturm et al. (2000) suggested that a p53 gene mutation is not the sole contributor to the negative Bax expression in an endometrial cancer.
carcinoma. An example is a DNA repair factor, Ku70, which has been recently found as a potent regulator of a Bax expression (Hada et al. 2016). So, incidental abnormalities in the Bax expression may be encountered in some patients with an endometrial malignancy.

Because of a possible connection between p53 and Bax genes, it has been suggested that the Bcl-2 to Bax ratio could be a better parameter in the assessment of a malignancy risk, and an increased Bcl-2 to Bax ratio has been found in association with an elevated cancer risk (Costa et al. 1998; Zeren et al. 2014). We also showed a similar connection between the increased Bcl-2 to Bax ratio and the malignancy in the present study. Likewise, we showed a significant association between the increased Bcl-2 to Bax ratio and the severity of endometrial carcinoma, which is in conformity with a previous report (Porichí et al. 2009).

The simultaneous examination of all three major components that play a role in the axis of apoptosis in the endometrium, as well as the employment of normal and hyperplastic specimens as controls are the main advantages of our study. We used immunohistochemistry to assess the expression rate of study variables in the current study because this method has previously proved to be easy to perform and inexpensive, as it allows working on preserved tissues (Soong et al. 1996). Using more sophisticated techniques such as polymerase chain reaction (PCR) to detect possible accompanying gene abnormalities, however, is imperative and should be considered in future studies.

In conclusion, we showed that in comparison with non-malignant conditions, p53 is over-expressed, Bax is under-expressed and the Bcl2 to Bax ratio is higher in endometrial cancer. Bcl-2 expression, however, was not associated with malignant changes.

Disclosure statement
The authors report no declarations of interest.

References


