Balance of glandular and stromal Bcl2/Bax expression in pre-neoplastic and neoplastic endometrial tissues

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Summary
Apoptosis is regulated by estrogen in normal endometrium and it is essential for the monthly sequential endometrial changes. In this study, epithelial and stromal expression of apoptosis regulated molecules; Bcl-2 and Bax in pathological endometrial lesions, their correlation with progression of endometrial carcinoma, and their association with estrogen receptor (ER) and progesterone receptor (PR) expression were investigated. Tissue sections of 39 endometrial carcinomas and 37 endometrial hyperplastic lesions were evaluated for Bcl-2, Bax, ER, and PR expression by immunohistochemistry. Bcl-2 and Bax expression was detected in most hyperplastic and in 77% and 82% of neoplastic endometrial lesions, respectively. The expression of both molecules was predominantly glandular; however concomitant stromal expression was also demonstrated. Glandular and stromal Bcl-2 and Bax expression was significantly reduced in endometrial carcinoma. There was a strong association between Bcl-2 and Bax expression in hyperplastic endometrium, in neoplastic lesions and in different histological subtypes, different grades and different FIGO stages of endometrial carcinoma at glandular, but not at the stromal level. The glandular expression of both molecules was significantly associated with ER and PR expression in hyperplastic lesions with loss of Bax/ER and Bax/PR association in neoplastic lesions. There was a strong association of stromal Bcl-2 with stromal ER expression in malignant endometrium and a strong association of stromal Bax with stromal ER in hyperplastic, but not in neoplastic endometrial lesions. In conclusion, disruption of hormonal control of Bax in endometrial tissue and subsequent disturbed stromal but not epithelial Bcl-2/Bax association could help early progression of endometrial carcinoma.

Key words: Endometrial carcinoma; Bcl-2, Bax; Glandular and stromal expression; Hormonal control.

Introduction
Programmed cell death (apoptosis) is a crucial process for normal tissue growth and development. Apoptosis has been shown to be an important regulator of endometrial functions [1]. During menstrual cycle, a rapid sequence of proliferation, differentiation, and cell death occurs in normal endometrium. Cell proliferation, as indicated by Ki-67 immune-labeling, is prominent during proliferative endometrial changes while apoptosis progressively increases in glandular epithelium from early to late secretory phase and is most frequent in the menstrual phase of endometrium [2, 3]. The expression of the anti-apoptotic molecule Bcl-2 is high in proliferative endometrium and decreases in secretory phase, being very low or absent during mid- and late secretory and menstrual phases. In contrast, expression of the pro-apoptotic molecule, Bax is moderate in proliferative endometrium and increases dramatically during secretory phase when apoptosis is most prevalent [4, 5]. It has been stated that frequency of apoptosis in endometrial tissue is controlled by the hormonal changes of ovarian cycle under physiological and pathological conditions [1, 6]. Proliferation of endometrial tissue is essentially associated with increased estradiol concentration, high estrogen receptor expression, and high Bcl-2 and Ki-67 expression, while production of progesterone in secretory phase leads to significant reduction of Bcl-2 and Ki-67 expression [2].

Deregulation of apoptosis contributes to neoplastic transformation and progression of several epithelial malignancies [7]. The intracellular ratio of Bcl-2/Bax molecules has been hypothesized to control progression/regression of apoptosis [8]. In postmenopausal women, there is a shift towards high expression of Bcl-2 and Ki-67 proliferation index in non-menstruating endometrial tissue with a tendency towards low expression of the cyto-death molecule caspase-cleaved cytokeratin 18 neopeptide [9]. Uncontrolled estrogen exposure can suppress Bax expression and induces imbalance in Bcl-2/Bax expression level in endometrial cells leading to precancerous lesions and type I endometrial adenocarcinoma [10]. In the same context, Bcl-2/Bax ratio tends to increase in grade 3 compared to grade 1 endometrial carcinoma and the epithelial expression of Bax was shown to be associated with higher clinical stages of this disease [11].

The microenvironment of neoplastic cells plays an essential role for development and subsequent behavior of endometrial carcinoma [12]. It has been shown that ER and...
PR are expressed in endometrial stromal cells [13] and that endometrial stromal cells contribute directly to biosynthesis of active estrogen from androgen precursors [14]. Mutation of endometrial epithelia is associated with activated stroma and subsequent recruitment of fibroblasts and inflammatory cells which support survival and proliferation of carcinoma cells through a paracrine signaling [15]. The role of stromal cells in endometrial carcinogenesis is poorly investigated with no provided data about expression of the apoptotic molecules in endometrial stromal cells. The main goal of this study is to evaluate the association of epithelial and stromal expression of Bcl-2 and Bax in pre-neoplastic and neoplastic endometrial tissues. The association of these apoptosis-related molecules with expression of ER and PR and with different clinic-pathological parameters of endometrial carcinoma was also measured.

Materials and Methods

Samples were collected from archived material at Pathology Department, Sohag University Hospital. Tissue samples of 37 endometrial hyperplastic lesions and 39 endometrial carcinomas were included in the study. The specimens were radical hysterectomy and bilateral salpingo-oophorectomy in 49 cases, simple hysterectomy in seven cases, subtotal hysterectomy in three cases, and dilatation and curettage in 17 cases. The clinical data were retrieved from patients’ files. Hematoxylin and Eosin-stained sections were reviewed and endometrial carcinomas were evaluated for histological type, grade, depth of invasion, and presence of vascular tumor emboli. FIGO staging was specified according to revised FIGO staging for carcinoma of the endometrium or involved less than half of inner myometrium (FIGO IIIA) in six cases, and to vagina (FIGO IIIB) in three cases. Vascular tumor emboli were histologically evaluated in two cases. FIGO staging was specified according to revised FIGO staging for carcinoma of the endometrium or involved less than half of inner myometrium (FIGO IA) and the tumor extended to outer half of myometrium (FIGO IB) in ten cases, to uterine cervix (FIGO II) in three cases, to serosal covering of corpus uteri and/or adnexa (FIGO IIIA) in six cases, and to vagina (FIGO IIIB) in two cases. Vascular tumor emboli were histologically evidenced in 13 out of 35 radically excised malignant endometrial lesions.

Expression of Bcl-2 and Bax was demonstrated in all cases of non-atypical endometrial hyperplasia. Bcl-2 was expressed by all cases of atypical endometrial hyperplasia and by 30/39 of malignant endometrial lesions, while Bax expression was detected in 21/22 of atypical hyperplastic endometrial lesions and in 32/39 of malignant endometrial lesions. The expression of both molecules was generally cytoplasmic and predominantly glandular (Figures 1 A-D).
Figure 1. — Expression of Bcl2, Bax, ER, and PR in endometrial lesions. Cytoplasmic immunostaining of Bcl-2 (A and B) and Bax (C and D) and nuclear immunostaining of ER (E and F) and PR (G and H) in hyperplastic and neoplastic endometrial lesions. Magnification is ×400.
Concomitant glandular and stromal expression of Bcl-2 was demonstrated in 26 cases and concomitant glandular and stromal expression of Bax was demonstrated in 17 cases. None of the cases showed pure stromal expression of either Bcl-2 or Bax molecules.

Glandular Bcl-2 histoscore ranged between 0 and 1000 with a mean (± SD) of 488 ± 310. About two-thirds of the investigated cases (63.2%) had Bcl-2 histoscores higher than 400 and six cases showed diffuse strong Bcl-2 expression (histoscore of 1000). The glandular expression of Bax also ranged between 0 and 1000 with a mean (± SD) of 446 ± 315. Diffuse strong Bax expression (histoscore of 1000) was recorded in seven cases and more than half of the cases (53.9%) had Bax histoscores higher than 400. There were strong positive association between Bcl-2 and Bax expression in both hyperplastic (Pearson’s correlation, R=0.528, p<0.001) and neoplastic (Pearson’s correlation, R=0.497, p<0.001) endometrial lesions.

There was no significant difference between epithelial expression of Bcl-2 or Bax molecules among non-atypical and atypical endometrial hyperplasia (Mann-Whitney test, p=0.48 and p=0.93). Malignant endometrial tissue showed significant reduction of glandular Bcl-2 and Bax expression compared to hyperplastic lesions (Mann-Whitney, p=0.006, and p=0.014, respectively). None of these parameters: tumor size, histological type, FIGO Stage or presence of lymphovascular invasion was associated with either glandular Bcl-2 or Bax expression (Table 1). The glandular expression of both Bcl-2 and Bax showed a steady decline as the tumor progressed from low to high grade (p=0.102 and p=0.029, respectively). The strong association of glandular Bcl-2 expression with glandular Bax expression was

Table 1. — Association of glandular and stromal Bcl2 and Bax expression with pathological parameters of endometrial lesions.

| Variable                              | n     | Glandular expression (histoscore) | Stromal expression (histoscore) |
|---------------------------------------|-------|-----------------------------------|---------------------------------|
|                                       |       | (Mean±SD)                         | (Mean±SD)                       |
|                                       |       | Bcl-2                             | Bax                             |
|                                       |       | Pearson’s correlation              | Pearson’s correlation            |
| Menopausal status                     |       |                                   |                                 |
| Pre-menopausal                        | 5     | 300±195                           | 400±268                         |
|                                       |       | p=0.819                           | 90±175                          |
| Peri-menopausal                       | 16    | 580±276                           | 522±319                         |
|                                       |       | p=0.027                           | 159±226                         |
| Post-menopausal                       | 55    | 478±322                           | 428±320                         |
|                                       |       | p<0.0001                          | 46±107                          |
| Kruskal-Wallis                        |       | p=0.134                           | p<0.575                         |
|                                       |       |                                   |                                 |
| Hyperplastic lesions                  |       |                                   |                                 |
| Non atypical                          | 15    | 560±260                           | 530±330                         |
|                                       |       | p=0.003                           | 107±142                         |
| Atypical                              | 22    | 617±269                           | 549±362                         |
|                                       |       | p=0.048                           | 139±181                         |
| Mann-Whitney                          |       | p=0.476                           | p=0.051                         |
| Hyperplasia/carcinoma                 |       |                                   |                                 |
| Endometrial hyperplasia               | 37    | 594±264                           | 541±345                         |
|                                       |       | p=0.001                           | 126±165                         |
| Endometrial carcinoma                 | 39    | 388±320                           | 355±258                         |
|                                       |       | p=0.001                           | 35±115                          |
| Mann-Whitney                          |       | p=0.006                           | p<0.001                         |
| Tumor size (millimetre)               | 34    | 35±137                            | 339±269                         |
|                                        |       | p=0.003                           | 35±115                          |
| Mann-Whitney                          |       | p=0.476                           | p=0.051                         |
| Tumor histological type               |       |                                   |                                 |
| Endometrioid carcinoma                | 33    | 422±306                           | 375±263                         |
|                                       |       | p=0.008                           | 41±125                          |
| Serous carcinoma                      | 6     | 200±357                           | 248±211                         |
|                                       |       | p=0.158                           | 0±0                             |
| Mann-Whitney                          |       | p=0.078                           | p=0.315                         |
| Tumor grade                           |       |                                   |                                 |
| Grade 1                               | 12    | 464±236                           | 487±221                         |
|                                       |       | p=0.234                           | 39±81                           |
| Grade 2                               | 17    | 438±335                           | 345±275                         |
|                                       |       | p=0.142                           | 52±161                          |
| Grade 3                               | 10    | 210±342                           | 214±200                         |
|                                       |       | p=0.024                           | 0±0                             |
| Mann-Whitney                          |       | p=0.102                           | p=0.029                         |
| Tumor FIGO stage                      |       |                                   |                                 |
| Confined to uterus FIGO (II)          | 27    | 334±288                           | 334±271                         |
|                                       |       | p=0.087                           | 17±56                           |
| Regional spread (FIGO III)            | 8     | 406±400                           | 352±263                         |
|                                       |       | p<0.0001                          | 79±223                          |
| Mann-Whitney                          |       | p=0.862                           | p=0.923                         |
| Lymphovascular invasion               |       |                                   |                                 |
| Detected                              | 13    | 279±352                           | 319±299                         |
|                                       |       | p=0.009                           | 49±175                          |
| Not detected                          | 22    | 392±286                           | 352±249                         |
|                                       |       | p=0.144                           | 21±62                           |
| Mann-Whitney                          |       | p=0.130                           | p=0.583                         |

NA refers to not applicable and the significant relationships are bold.

Concomitant glandular and stromal expression of Bcl-2 was demonstrated in 26 cases and concomitant glandular and stromal expression of Bax was demonstrated in 17 cases. None of the cases showed pure stromal expression of either Bcl-2 or Bax molecules.
Figure 2. — Correlation of glandular and stromal Bcl-2 and Bax with estrogen and progesterone receptors. Association of Bcl-2 and Bax expression with expression of ER and PR in hyperplastic (green circles) and neoplastic (red circles) endometrial lesions at glandular (A, B, C and D) and stromal (E, F, G and H) levels. The green and red sloping lines refer to correlation fit through the data of endometrial hyperplasia and endometrial carcinoma, respectively.
maintained with different menopausal status, in different types of endometrial hyperplasia, in endometrioid carcinoma, in high grade carcinoma, and in tumors with different FIGO stages (Table 1).

The expression of Bcl-2 and Bax in non-invasive and corresponding invasive malignant cells of the same primary tumor was evaluated. Invasive and non-invasive tumor cells showed similar expression levels of both Bcl-2 (Wilcoxon’s signed rank test, \( p=0.61 \)) and Bax (Wilcoxon’s signed rank test, \( p=0.26 \)) molecules. The strong association between Bcl-2 and Bax expression was maintained in non-invasive \((R=0.482, p=0.003)\) and invasive \((R=0.384, p=0.023)\) tumor components.

The stromal expression of Bcl-2 and Bax molecules was measured irrespective to glandular expression. In general, stromal Bcl-2 expression and stromal Bax expression were always weaker than corresponding glandular expression (Wilcoxon’s Signed rank test, \( p<0.001 \) for both) and stromal expression of both molecules was down-regulated in neoplastic compared to hyperplastic endometrial lesions (Mann-Whitney, \( p=0.001 \) and \( p=0.028 \); respectively). In addition, it has been noted that stromal expression of both Bcl-2 and Bax was frequently lost in malignant endometrial lesions \((p<0.001 \) and \( p=0.04 \), respectively). Although glandular-Bcl-2 expression was strongly associated with glandular Bax expression in both non-neoplastic and neoplastic endometrial lesions, the stromal expression of both molecules was not correlated in either hyperplastic \((R=0.042; p=0.81)\) or malignant \((R=-0.052; p=0.75)\) endometrial lesions. Nonetheless, there was no recorded association of stromal expression of the two molecules in different menopausal status, in different types of endometrial hyperplasia, in different histological subtypes, and grades of endometrial carcinoma or in tumors with different FIGO stages (Table 1).

Apoptosis is regulated by estrogen in normal and ectopic endometrial tissue [18]. In this study, glandular expression of ER and PR was demonstrated in 38 and 42 cases, respectively while stromal expression of ER and PR was detected in 25 and 22 cases, respectively. The expression was specifically nuclear in both glandular and stromal elements (Figures 1 E-H). The mean \((\pm \text{SD})\) histoscores of glandular ER and PR expression were 303.7 \(\pm\) 365.7 and 270.4 \(\pm\) 340.2, respectively and the mean \((\pm \text{SD})\) histoscores of stromal ER and PR expression were 136.3 \(\pm\) 233.9 and 105.6 \(\pm\) 210.5, respectively. There were conserved strong associations for glandular as well as stromal expression of ER and PR in both hyperplastic \((R=0.574, p<0.0001 \) and \( R=0.468 p=0.004 \); respectively) and neoplastic \((R=0.693, p<0.0001 \) and \( R=0.865 p<0.0001 \); respectively) endometrial lesions. The expression of both molecules showed a significant reduction in neoplastic compared to hyperplastic endometrial lesions (Mann-Whitney test, \( p<0.0001 \) for both). There was a strong association of glandular Bcl-2 expression with glandular expression of ER and PR in both hyperplastic and neoplastic endometrial lesions (Figures 2A and 2B). The strong association of glandular Bax expression with glandular expression of both hormone receptors in hyperplastic endometrial lesions was lost in malignant lesions (Figure 2C and 2D). Regarding stromal expression, there was a tendency toward stronger association of stromal Bcl-2 with stromal ER expression in malignant endometrial lesions (Figure 2E). Oppositely, the stromal expression of Bax was associated with stromal expression of ER in hyperplastic endometrial tissue with loss of this relationship in malignant endometrial lesions (Figure 2G).

Discussion

Apoptosis is regulated by several pro- and anti-apoptotic genes; the imbalance of which constitutes an important mechanism in carcinogenesis. It has been established that cyclic endometrial changes are controlled in part by the apoptotic molecules [1]. In vivo, the main anti-apoptotic molecule; Bel-2 forms a heterodimer complex with the main pro-apoptotic molecule; Bax and Bel-2/Bax ratio is the determinant for progression/regression of apoptosis in endometrial tissues [19]. Previous studies of apoptosis in endometrial lesions mainly assessed epithelial apoptotic molecules, but investigations of stromal expression of Bel-2 and Bax are generally deficient. In this study, the detailed association of epithelial and stromal expression of Bax and Bel-2 proteins was measured in neoplastic and non-neoplastic endometrial lesions.

Glandular expression of Bcl-2 and Bax proteins was demonstrated in all hyperplastic and in a considerable proportion of neoplastic endometrial lesions and there was a down-regulation of expression of both molecules in neoplastic compared to hyperplastic endometrial lesions and in high-grade compared to low-grade carcinomas (Table 1). Several studies reported the reduced expression of glandular Bcl-2 and Bax in neoplastic endometrial lesions; however interpretation of biological importance of these findings is controversial. Kounelis et al. [20, 21] showed reduced immunopositivity of Bcl-2 and Bax in endometrial carcinoma compared to hyperplasia and in low-grade and early-stage compared to high-grade and advanced-stage adenocarcinomas, while Porchi et al. [11] and Sakuragi et al. [19] showed no relationship of Bcl-2 and Bax expression with tumor stage, histological subtype or other histopathological prognostic factors. In one series, a slightly raised Bcl-2/Bax ratio was demonstrated in only 10% of the investigated endometrial carcinomas [22] and in another series, Bcl-2/Bax gene ratio was high in grade 3 tumours but confusingly low in grade 2 tumours [11]. Other reports showed that Bcl-2 immune-reaction was frequently lost in endometrial carcinoma with stronger expression of Bax in malignant lesions compared to hyperplasia and normal endometrial tissue [23, 24] which implies decreased...
Bcl-2/Bax ratio; a status that favors apoptosis rather than proliferation of malignant cells. Additionally, increased expression of Bax and decreased expression of Bcl-2 were reported in advanced endometrial carcinomas with strong expression of Bax protein in the third group of clinical staging [6]; an unexpected finding as Bax expression should induce regression of neoplastic growth. According to this literature, there was no consensus regarding the role of apoptosis in endometrial carcinogenesis.

An attractive finding in this study is the frequent loss of stromal expression of Bcl-2 and Bax in malignant endometrial lesions. Out of the 39 investigated tumours, only nine cases showed stromal expression of Bcl-2 and/or Bax proteins, of which five were grade I, six were limited within uterine corpus, and eight with no recorded lymphovascular invasion. This could imply a more significant role of stromal Bcl-2 and Bax in early progression of endometrial cancer compared to the controversial role of the epithelial molecules. In support for this claim, there were always strong associations of epithelial but not stromal Bcl-2 and Bax expressions in different hyperplastic and malignant endometrial lesions and their subcategories including invasive malignant cells (Table 1), which reflects a coordinated expression between these two molecules at the epithelial level but a distorted Bcl-2/Bax ratio at the stromal level. Previous data confirmed the expression of Bcl-2 and Bax in endometrial stromal cells and showed that stromal expression is lower compared to the glandular counterpart and is lower in neoplastic tissue compared to normal endometrial tissue [20, 23].

Apoptosis of endometrial tissue is controlled by estrogen under physiological and pathological conditions [1, 6]. According to the present data, glandular but not stromal Bcl-2 and Bax expression showed consistent strong associations with ER and PR expression in both hyperplastic and neoplastic endometrial lesions (Figures 2 A, B, E, and F). Alternatively, the epithelial and stromal expression of Bax showed strong correlations with ER and PR in hyperplastic but not in neoplastic endometrial lesions (Figures 2 C, D, G, and H). Taken together, these data suggest that epithelial Bcl-2 and Bax expression is controlled by estrogen in hyperplastic endometrial lesions and that hormonal control of Bax but not Bcl-2 is disrupted in neoplastic endometrial lesions particularly at the stromal level. This is compatible to previous findings about hormonal control of epithelial Bcl-2 and Bax expression in endometrial hyperplasia [6]. Vereide et al. [25] reported that glandular but not stromal Bcl-2 and Bax expression was markedly reduced in response to progesterone therapy of endometrial hyperplasia which also implies a hormonal control of these apoptotic molecules at epithelial but not stromal cells.

Initiation of endometrial carcinoma is related in most instances to mutations of PTEN, p53, k-ras, β-catenin, and HER2/neu genes [26]. The role of apoptosis in endometrial carcinogenesis is controversial. The findings of this work about the consistent strong association between epithelial expression of Bcl-2 and Bax proteins in different benign and malignant endometrial lesions and their sub-categories implies that imbalance of epithelial Bcl-2/Bax ratio has a limited role in initiation of endometrial cancer. In contrast, there was no association between stromal Bcl-2 and Bax expression in hyperplastic or neoplastic endometrial lesions and the hormonal control of Bax at the stromal level seems to be disrupted in neoplastic endometrial lesions. This implies that imbalance of stromal Bcl-2/Bax ratio, which could be initiated by disruption of hormonal control of Bax may help early progression of endometrial carcinoma. Previous data confirmed that stromal cells of endometrial carcinoma contribute directly to the biosynthesis of estrogen [14], a finding that ensures the importance of the stroma in progression of endometrial carcinoma.

Conclusion

The role of epithelial Bcl-2/Bax ratio in initiation of endometrial cancer seems to be limited. Instead, stromal Bcl-2/Bax imbalance could help progression of endometrial carcinoma at least in a subset of this disease. The hormonal control of apoptosis in hyperplastic endometrial lesion could be disrupted after malignant change particularly at the stromal level. Detailed molecular investigations of tumor microenvironment are required to elicit the exact role of these molecules in endometrial carcinogenesis.

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