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RESEARCH

# Apoptosis Phenomena in Squamous Cell Carcinomas and Adenocarcinomas of the Uterine Cervix

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**Abstract** To investigate the relationship between apoptosis and histologic types in invasive squamous cell carcinoma and adenocarcinoma of the uterine cervix. The present study involved the assessment of surgical specimens from 74 women with cervical carcinomas FIGO stage IB1 (54 squamous cell carcinomas and 20 adenocarcinomas). The study samples were obtained from selected paraffin blocks containing specimens from patients submitted to surgical procedures. The respective medical charts of patients were reviewed and epidemiologic, clinical and disease-related data were collected. Cervical specimens were assessed by the immunohistochemistry technique using the Bcl-2 protein as a marker. The reactions were considered positive when the cells became stained in brown color. Bcl-2 positive cells were counted in 10 fields under a high magnification (400x) using light microscopy, in the slides area containing squamous carcinoma and adenocarcinoma of the cervix. The total cell count was expressed as the number of positive Bcl-2 cells per $mm^2$. No significant difference in the number of cells marked by the Bcl-2 protein was found for the variables age, tumor diameter, angiolymphatic invasion or number of lymph nodes affected. Comparison of the number of cells marked by the Bcl-2 protein in the two histological groups revealed a statistically significant difference, with squamous tumors presenting a greater number of marked cells. Squamous cervical tumors present a greater number of positive Bcl-2 cells per $mm^2$, suggesting that that the rate of cell death in squamous cell carcinomas of the cervix is lower than in adenocarcinomas.

**Keywords** Apoptosis · Uterine cervix · Squamous cell carcinoma · Adenocarcinoma · Bcl-2 protein

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## Introduction

Cervical cancer is one of the most prevalent diseases in women and causes considerable morbidity [1]. Indeed, after breast cancer, it is the most common cancer in women worldwide, although there is a discrepancy in prevalence between developed and developing countries [1]. In developed countries, there has been a steady decline in incidence and mortality, whereas in developing countries there is a more stable and possibly increasing pattern of cervical cancer. This rise is more likely to be due to the lack of screening and infectious cofactors than to ethnic differences [2, 3].

Invasive cervical cancer can be divided into two major histological types: squamous cell carcinoma (SCC) and adenocarcinoma (AC). In terms of proportions, 80–85 % of cases are SCC and 10–15 % are adenocarcinomas. The other tumors include adenosquamous carcinomas, small cell carcinomas and undifferentiated carcinomas [4–7].

Compared with SCC, adenocarcinoma of the cervix is rare. Due to its relative rarity, only a few large studies have addressed risk factors in adenocarcinoma. Furthermore, only a few studies have been published comparing risk factors for both tumor types. SCC arises from squamous epithelia on the ectocervix whereas adenocarcinoma stems from the glandular epithelia in the ectocervix or endocervix. In recent years, strong pathological, cell and molecular evidence has been

accumulated implicating human papillomavirus (HPV) as the primary surrogate for the development of both cervical neoplasias. The cervical carcinogenesis and risk factors of both types overlap, but there are also some differences [8–10].

The incidence of SCC has been declining in many developed countries since the introduction of screening of cervical smears in the 1940s by Papanicolaou, while the incidence of AC is increasing (having almost doubled). This increase appears to be occurring mainly in young women [5–7].

The reason for this phenomenon remains unclear but is partially due to the cohort effect and to the fact that screening may be less effective in detecting adenocarcinomas. Some researchers believe that the rise is related to the difficulty in detecting AC precursor lesions in cervical cytological examinations (cytology screening is undoubtedly more effective for the detection of SCC than for AC) and also due to increased human papillomavirus infection, especially type 18 [5]. There was a long-held belief that AC of the uterine cervix was associated with the use of oral contraceptives. Although prolonged use of oral contraceptives has been implicated in the emergence of cancer of the uterine cervix, this does not seem to be an exclusive risk factor for AC [5, 10].

There is a great deal of debate in the literature with regard to the prognosis for AC cases in relation to SCC cases. Some studies have described worse prognosis for AC compared to SCC. According to these reports, adenocarcinoma has relatively more aggressive biological behavior as compared to its squamous cell counterpart. Adenocarcinoma is bulky and expansive with less favorable prognosis, earlier local extension and lymph node metastasis [6, 7, 11, 12]. The treatment of adenocarcinomas is challenging because of its tendency for remote spread and its resistance to radiation and systemic therapies [13].

However, other studies have found no difference between these two types of tumor [14, 15]. Fregnani et al. [10] concluded that the AC group showed less aggressive histological behavior than did the SCC group, but no difference in the disease-free survival rates was noted. Nevertheless, these studies are not fully comparable since the populations analyzed and the treatments carried out differed, thereby hampering the drawing of any conclusions.

The clinical behavior of invasive cervix carcinomas is diverse and includes tumors of both relatively slowly and rapidly progressing diseases [5]. The development and progression of cervical tumors is likely associated with alterations in apoptosis (programmed cell death), disturbance in immune surveillance, increased cell growth or loss of growth suppression (uncontrolled proliferation) [16, 17].

Unfortunately, most previous studies on cervical cancer have not focused on or involved large series of adenocarcinoma. It is possible that the proliferative and/or apoptotic properties of adenocarcinoma may differ to those of squamous cell carcinoma, thus accounting for its different biological behavior.

Therefore, the present study was designed to investigate the relationship between apoptosis and histologic types in invasive squamous cell carcinoma and adenocarcinoma with the expression of Bcl-2, as detected by immunohistochemical staining.

## Material and Methods

A retrospective study of surgical specimens from 74 women with invasive neoplasia of the cervix, comprising 54 squamous cell carcinomas and 20 adenocarcinomas, was performed. All patients included in the study were attended at Santa Casa São Paulo Hospital between January 2003 and May 2008. This research was approved by the Research Ethics Committee of the hospital.

After obtaining the medical records of the patients selected for the study, the paraffin blocks for each case were requested. Histological sections were evaluated under light microscope and reviewed by the same pathologist to confirm the histopathological diagnosis of cervical neoplasia (adenocarcinoma or squamous cell carcinoma).

Only cases with histology of squamous cell carcinoma and cervical adenocarcinoma stage IB1 tumors [18], submitted to surgical procedure were included (since these cases had histology specimens with larger tumor volume for use in immunohistochemical reactions). Cases with other stages or those with stage IB1 not managed surgically were excluded. Patients undergoing treatment prior to surgery (radiotherapy or chemotherapy) were also not considered.

The respective medical charts for each case were subsequently reviewed, from which epidemiological, clinical and disease-related data were collected. Only epidemiological data uniformly present in all patients was studied.

### Immunohistochemistry

With regard to the immunohistochemical technique used to identify the Bcl-2 protein, paraffin blocks containing areas most representative of cervical invasive lesion were selected.

The presence of the Bcl-2 protein was confirmed by immunohistochemical study using the streptavidin-biotin-peroxidase complex method. All immunohistochemical reactions were performed at the same time. For these reactions, 3 μm-thick sections were cut. After deparaffinization in toluene (twice for 10 min each) and in alcohol solutions (5 min in 100 %, 5 min in 95 % and 5 min in 75 % alcohol), sections were washed in tap water. The deparaffinized sections were treated with hydrogen peroxide to block endogenous peroxidase activity and then washed in phosphate-buffered saline (PBS).

Sections were incubated with primary polyclonal antibodies to Bcl-2 protein, prepared in rabbits (diluted 1: 1000; DAKO, M887- California, USA), for 18 h (overnight) at 4 °C. Sections were washed 3 times in PBS and incubated for 1 h with biotinylated goat anti-rabbit immunoglobulin (DAKO, K0492 - California, USA) at 37 °C. Sections were washed again 3 times in PBS and incubated with streptavidin-biotin-peroxidase complex for 30 min, to label the antibodies.

The antigen- antibody complex was visualized by incubating for 5 min with diaminobenzidine (0.6 mg/ml buffer) and hydrogen peroxide. Sections were washed in sterile distilled water, counterstained with Harris' haematoxylin and mounted for light microscopy. The reactions were considered positive in the presence of cells stained a brown color (Fig. 1). Appropriate positive (cases of follicular lymphoma) and negative (lymph nodes) controls were included in each case.

### Morphometry

Each section prepared by the immunohistochemical method was evaluated under light microscope (Axioskop 40 – Zeiss) fitted with a microcamera and screen. For the morphometric analysis, the calculation of the area in $mm^2$ was performed with the aid of a Neubauer chamber, where it was established that each area of the 400x magnification represented 0.094 $mm^2$. The number of positive cells was counted at 400x magnification and a 10-field count was performed in the areas of cervical neoplasia in each section, by two investigators concurrently who were blind to the clinical details and identity of the patients. All labeled cells were counted in these areas. So, necrosis, bleeding, connective tissue cells were excluded from the count and only the tumor cells were counted. The cell counts were expressed as numerical densities, i.e. number of positive cells per square millimeter of epithelium [19].

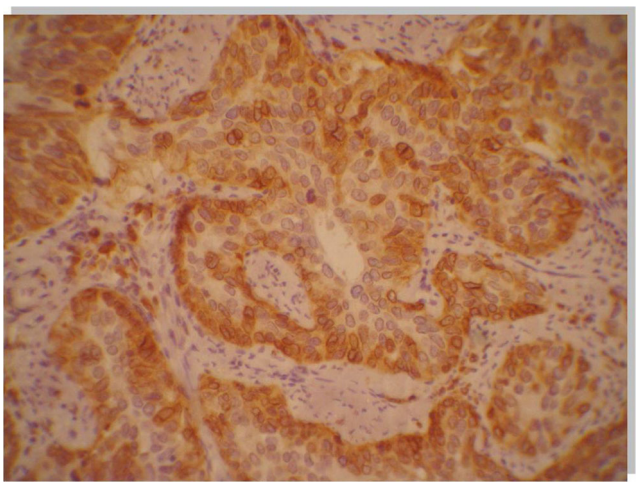

**Fig. 1** Photomicrography of an histological section of a case of cervical cancer (adenocarcinoma) showing cells labeled by bcl-2 protein in brown color

### Statistical Analysis

For the statistical analysis of results, the Qui-square, Fisher's exact, Mann–Whitney test, T-Student and Pearson's correlation coefficient tests were applied as appropriate. Levels < 0.05 were considered significant.

## Results

The mean age of the 74 IB1 stage patients included to the present study was 46.1±11.8 years, ranging from 25 to 76 years with a median of 43 years. The diameter of patients' tumors varied from 1 to 4 cm and had a mean of 1.94±0.96 cm and median of 2 cm.

Patients underwent the following surgical procedures: three (4.07 %) conizations, three conizations followed by total abdominal hysterectomy (TAH) (4.07 %), three TAH (4.07 %), two conizations followed by Wertheim-Meigs surgeries (2.7 %) and 63 Wertheim-Meigs surgeries (85.1 %).

The pelvic lymph nodes were dissected in 65 patients (87.8 %) while 9 patients (12.2 %) did not undergo this procedure. Surgical procedures found between 1 and 36 lymph nodes, a mean of 10.1±6.3 lymph nodes and median of 8 lymph nodes. Lymph nodes were compromised by neoplasia in six out of the 65 women (9.2 %). The number of lymph nodes affected ranged from 1 to 4 in each patient with cervical neoplasia. Angiolymphatic invasion was detected in 24 (32.4 %) of the 74 women treated.

Of the 74 selected stage IB1 cases, 54 (73 %) were SCC and 20 (27 %) adenocarcinomas. Comparisons of the histological groups found no significant differences between them (homogenous groups) for the variables age ($p$=0.872; Student's $t$ test), tumor diameter ($p$=0.912; Student's $t$ test), presence of invaded lymph nodes ($p$>0.999; Fisher's exact test) or angiolymphatic invasion ($p$=0.406; Qui-squared test).

The expression of bcl-2 protein was predominantly within the cytoplasm of the cervical epithelial cells. The number of cells marked by the Bcl-2 protein in our total sample ranged from 45 to 2070 cells per $mm^2$, with a mean of 311.7±380.6 and median of 203.8 cells per $mm^2$. No significant difference in number of marked cells was found for the variables age, tumor diameter and invaded lymph nodes (Table 1).

**Table 1** Pearson's correlation coefficient matrix between Bcl-2 and the other variables

| Correlation matrix | Age (years) | Tumor diameter (cm) | Number of invaded lymph nodes |
|---|---|---|---|
| Coefficient (Bcl-2) | 0.061 | −0.084 | −0.075 |
| p | 0.605 | 0.476 | 0.551 |

In SCC cases, the number of cells marked by the Bcl-2 protein varied from approximately 46 to 1834 marked cells, with a mean of 333.2±358.4 and median of 222.4 cells per $mm^2$. For adenocarcinoma these values varied from approximately 45 to 2070 cells per $mm^2$, with a mean of 253.5±439.9 cells per $mm^2$ and median of 130.8 cells per $mm^2$.

Comparison of the number of cells marked by the Bcl-2 protein between the two histological groups revealed a statistically significant difference, showing that squamous tumors have a larger number of marked cells ($p$=0.018; Mann–Whitney test).

## Discussion

Although cancer exhibits highly heterogeneous characteristics, all malignant tumors acquire the property of growth beyond the limits imposed on normal cells. Clonal expansion of a transformed cell depends on loss of control of its proliferative capacity and a growing incapacity to die by apoptosis. Therefore, despite the huge variability of cancers, evidence shows that resistance to apoptosis is a hallmark of the majority of malignant tumors [16].

Apoptosis and necrosis are two fundamental types of cell death. The key biochemical mechanism unique to apoptosis is the internucleosomal cleavage of DNA upon activation of a Ca21/Mg21-dependent endogenous endonuclease. It is commonly accepted that apoptosis, which is distinct from necrosis, is an essential feature of the turnover and development of cells in normal and neoplastic tissues [20, 21].

Apoptosis can be triggered by external or internal stimuli of intercellular tension. These different means of initiation culminate in activation of proteases known as a caspases. These are present in cell cytoplasm in the form of inactive zymogens (pro-caspases), being activated by their deds (death effector domains) and card (caspase recruitment domain) domains and considered the central executors of the apoptosis process [22, 23].

In recent years, it has become clear that carcinogenesis and tumor progression cannot be explained simply in terms of the enhanced stimulation of cell growth but that loss of growth suppression and changes in apoptotic cell death are also involved. The significance of the apoptotic pathway in the development and progression of human malignant tumors has become a major topic of discussion over recent years. One of the proteins that play a regulating role in programmed cell death is encoded by the Bcl-2 gene [24, 25].

The Bcl-2 family of related proteins is one of the key regulators of the apoptotic process. This consists of two opposing groups of proteins: death antagonists (Bcl-2, Bcl-$X_L$, Mcl-1) and death agonists (Bax, Bak, Bcl-Xs). Apoptosis occurs through competing dimerization between the two protein groups, the relative proportions of which ultimately control the sensitivity or resistance of cells to apoptosis stimuli [23, 25].

The bcl-2 oncoprotein, found at the inner mitochondrial membrane, is known to inhibit apoptosis and has been shown to prevent apoptosis as opposed to promoting cell proliferation. By a mechanism which remain unclear, Bcl-2 prevents programmed cell death by suppressing the physiological mechanisms which normally maintain homeostatic balance between cell production and demise, enabling the growth of neoplasias. Bcl-2 hyperexpression also contributes to the emergence of mutation which affects proto-oncogenes and cancer suppressing genes [5, 16, 17, 25]. Excess Bcl-2, although not directly causing cancer, can be conducive for the action of other oncogenes [23].

Other studies have shown that the protective effect of Bcl-2 is also necessary in cell biology, preventing apoptosis in a host of different types of cell both directly (blocking caspase complexes) and indirectly (blocking release of the mitochondria components able to activate caspases 3, 6 and 7 into the cytoplasm) [23].

Overexpression of Bcl-2 and its prognostic significance have been reported in several epithelial cancers such as breast cancer by Kallel-Bayoudh et al. [26], among others.

However, the data on carcinoma of the uterine cervix are limited and the results conflicting. It was therefore decided in the present study to perform a comparative analysis of stage IB1 squamous cell carcinomas and adenocarcinomas of the uterine cervix for immunohistochemical expression of the Bcl-2 protein. Although the proportion of malignant cells showing morphological features of mitosis or apoptosis in histologic sections seemed to be small, the involvement of these two processes appeared to be more extensive when assessed immunohistochemically [16].

Only IB1 stage cervical tumors were selected since we sought to achieve a homogenous study group, where this represented the majority of the stage I patients in the sample of our hospital. Of the 74 IB1 cases selected, 54 (73 %) were SCC and 20 (27 %) adenocarcinomas. This proportion of the two histological types reflects the data from the literature cited earlier. In our study, all cases of cervical cancer were considered positive for Bcl-2 marker. However, we observed subjective differences in the intensity of the reaction. Thus, instead of grouping the cases by staining intensity, we decided to count the number of labeled cells per mm2. We consider this sort of evaluation more objective than the simple staining intensity.

No significant difference was found on analysis of the number of cells marked by the Bcl-2 protein in terms of the variables age, tumor diameter or invaded lymph nodes. However, a statistically significant difference was found in the number of cells marked by the Bcl-2 protein between the SCC and AC cases, demonstrating that squamous tumors have a larger number of marked cells.

Therefore, it is likely that the frequency of apoptosis might have an important prognostic value in cervical carcinomas. Few studies are available in the literature comparing cases of SCC and cervical adenocarcinomas regarding the apoptosis phenomenon employing the Bcl-2 protein. The studies which are available report conflicting results.

Kokawa et al. [27] obtained cervical tissues from 19 patients with invasive squamous cell carcinoma and from nine patients with invasive endocervical adenocarcinoma. In SCC, most cells were immunonegative for Bax and Bcl-2. By contrast, strong immunostaining, specific for Bax, was observed in almost all of the neoplastic cells in AC, whereas no immunostaining of Bcl-2 was detected in these cells. These authors also observed that the intensity and frequency of bcl-2 immunoreactivity were similar in SCC and AC, and verified that the expression of Bax was dominant in AC. The authors suggested that the high incidence of apoptosis in AC is correlated with increased expression of Bax but is not associated with the expression of Bcl-2. Taken together with the high frequency of proliferating cells, these results indicate that AC of the cervix might activate the rapid turnover of cells, which in turn may be responsible for the higher frequency of local recurrence and the poorer prognosis of AC.

Dimitrakakis et al. [28] examined the expression of bcl-2 in cervical carcinomas and their premalignant lesions in order to assess the value of this protein as a prognostic indicator. A significant difference between the expression of bcl-2 in premalignant lesions and in carcinomas was found in their study (greater in CIN lesions). The positivity of bcl-2 was significantly higher in line with the grade of CIN. Similar rates of bcl-2 expression were seen in cervical tumor subtypes [SCC, 18/63 (29 %) versus AC, 5/18 (28 %)]. In cervical cancer patients, expression of bcl-2 was correlated to a greater than 5-year survival.

One hundred and eleven patients with primary cervical cancer were included in the study designed by Tjalma et al. [24] These authors confirmed Bcl-2 expression in 76 (68 %) cases. However, expression was heterogeneous and varied from strongly positive to negative within the same tumor. The authors noted that the number of Bcl-2 positive cells was significantly higher in SCC than in AC. Also, those patients with a positive Bcl-2 expression had a longer survival than did negative patients.

In contrast to the results of our study, the comparative analysis of Bcl2 protein by Yao et al. [29] in 74 cases of cervical carcinoma (46 SCC and 28 adenocarcinoma) and 15 normal cervical epithelial tissues, found positivity rates of 74, 71 and 20 %, respectively. No significant difference was detected between the two histological tumor types in terms of marker expression, although a difference between them compared to normal cervical tissue was identified.

Therefore, it has been postulated that the rate of cell death in squamous cell carcinomas of the cervix is low, as is the rate of cell proliferation and hence the progression of carcinomas is slow. By contrast, the incidence of apoptotic cell death is significantly elevated in cases of adenocarcinomas. It is widely accepted that prolonged cell survival with inhibition of apoptosis is associated with carcinogenesis. Hence, it is possible that neoplastic cells in AC are subject to a high rate of cell death as well as cell proliferation, with an active turnover of cells. High rates of mitosis and apoptosis in AC indicate the active kinetics of cancer cells. This situation might explain why AC progresses more rapidly and recurs more frequently than SCC [27].

Apoptosis in routine clinical practice represents a promising target for potential therapeutic use of cell death or as a means of better understanding the mechanisms of resistance to radiotherapy and chemotherapy. Elucidation of some of the molecular mechanisms of apoptosis will pave the way for modulation of these processes. The strategies are based on inducing the death of tumor cells through blocking of the genes with antisense oligonucleotides and conventional drugs, or modifying the function of these genes using recombinant molecules [30].

Several clinical and pre-clinical trials using drugs targeting the Bcl-2 family are underway. Reduction in Bcl-2 and Bcl-Xl activity would be sufficient to induce cells to begin apoptosis, showing great promise for the future [21].

**Conflict of Interest** The authors declare that they have no conflicts of interest.

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