Prognostic value of bcl-2 expression in patients with operable carcinoma of the uterine cervix

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Abstract

Aim—To evaluate the patterns of bcl-2 expression in early stage cervical carcinoma; to compare bcl-2 expression with clinicopathological findings; and to assess its prognostic value.

Methods—Wertheim radical hysterectomy specimens from 76 patients (FIGO stages Ia-IIb) with untreated non-metastatic invasive cervical carcinoma were studied. Expression of bcl-2 was detected immunohistochemically using a monoclonal antibody. A tumour was regarded as positive when more than 5% of the neoplastic cells exhibited bcl-2 immunoreactivity.

Results—Forty eight (63%) cervical carcinomas were scored as bcl-2 positive and 28 (37%) as bcl-2 negative. Most tumours showed heterogeneous cytoplasmic staining. Bcl-2 immunoreactivity did not correlate with tumour histology, tumour stage, presence of lymph node metastases, or involvement of the lymphovascular space. The five year survival rate for patients with bcl-2 negative tumours was 34% and was 71% for patients with bcl-2 positive tumours. On multiple regression analysis (Cox proportional hazards model), bcl-2 expression and vascular permeation were independent predictors of overall survival.

Conclusions—Bcl-2 expression seems to be associated with less aggressive behaviour in early stage cervical carcinoma. The transition to bcl-2 independence may play an important role in tumour progression.

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Keywords: bcl-2; cervical cancer; immunohistochemistry; prognosis.

In recent years it has become clear that carcinogenesis cannot only be explained by the increased stimulation of cell growth, but can also be caused by loss of growth suppression, changes in programmed cell death (apoptosis) and alterations in immune surveillance. Apoptosis and mitosis are closely inter-related, and there is some evidence that both in normal and neoplastic tissues very complex multifactorial control mechanisms (such as p53, c-myc, c-fos, bax, bcl-2 expression) determine whether cells will progress to mitosis or apoptosis.¹ The regulation of apoptosis is central to morphogenesis during fetal development and to the maintenance of tissue homeostasis during

adulthood and seems to be of importance for neoplastic transformation in some organs. One of the most important genes in this process is bcl-2. This gene is unique among proto-oncogenes because it has the ability to block programmed cell death without promoting cell proliferation.² The 25 kilodalton bcl-2 protein is located at the mitochondrial outer membrane, in the nuclear envelope, plasma membrane, endoplasmic reticulum, and in chromosomes.³⁻⁷ Bcl-2 expression was first detected in follicular lymphomas with a balanced chromosomal translocation t(14;18)(q32;21), but later bcl-2 overexpression was also found in lymphomas and epithelial malignancies lacking this translocation.

When overexpressed, bcl-2 produces dramatic extension of cell survival. Overexpression of bcl-2 is associated in a number of tumours with less aggressive malignant behaviour. The aim of the present retrospective study was to elucidate the functional role of bcl-2 in carcinoma of the uterine cervix using archival paraffin wax embedded tissue specimens and to compare bcl-2 expression with clinicopathological findings.

Methods

PATIENTS AND SPECIMENS

Wertheim radical hysterectomy specimens from 76 patients with untreated invasive carcinoma of the uterine cervix were studied. All operations were performed between 1979 and 1995 in the Departments of Obstetrics and Gynaecology of the Antwerp University and of the General Hospital Saint Camillus/Saint Augustinus. Patients underwent a preoperative blood test (full blood count, clotting parameters, electrolytes, liver function tests, urea creatinine, carcinoembryonic antigen), chest x ray, computed tomography scan of the abdomen and pelvis, and an examination under anaesthesia (including cystoscopy and sigmoidoscopy). The median age of the patients at the time of diagnosis was 53 years (range 24-82). Tumours were staged according to FIGO criteria.⁸ Six patients had FIGO stage Ia tumours, 37 stage Ib, 29 stage IIa, and four stage IIb. Histological subtyping of tumours followed WHO guidelines (1981).

Postoperatively, all 25 patients with lymph node metastases and the six patients with negative lymph nodes but evidence of lymphovascular space involvement received adjuvant external field pelvic radiotherapy. The doses varied from 1.5 to 2 Gray daily, given in a four field technique, with a maximum of 50 Gray in total. Follow up examinations, consist-

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Table 1 Relation between classic prognostic factors and bcl-2 cytoplasmic immunoreactivity

Classic prognostic factors	n	bcl-2 positivity (n (%))	p value (χ^2 test)
Histology			
Squamous carcinoma	59	38 (64)	NS
Adenosquamous carcinoma	6	2 (33)	
Adenocarcinoma	7	5 (71)	
Other	4	3 (75)	
Tumour grade			
I	15	11 (73)	NS
II	35	21 (60)	
III	26	16 (62)	
Lymphatic permeation			
Absent	40	28 (70)	NS
Present	36	20 (56)	
Vascular permeation			
Absent	51	34 (67)	NS
Present	25	14 (56)	
Lymph node status			
Negative	51	34 (67)	NS
Positive	25	14 (56)	

ing of physical examination and vaginal vault smears, were done at regular intervals. Patients developing symptoms suggestive of metastatic disease were investigated further. Follow up data were available in all 76 patients. Median observation time was 39 months (range four to 203).

bcl-2 IMMUNOSTAINING

Sections, 5 μ m thick, were cut from the formalin fixed, paraffin wax embedded tumour specimens and deparaffinised routinely. They were then treated with 0.3 % hydrogen peroxide to block endogenous peroxidase activity. The slides were placed in a 0.01 citrate buffer and heated in the microwave oven at 700 W for 2 $\times$ 5 minutes. Bcl-2 expression was detected by using a standard immunohistochemical procedure, incubating the slides for 30 minutes with monoclonal mouse anti-human bcl-2 (clone 124, isotype IgG1; Dako, Glostrup, Denmark) diluted 1 in 40 in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA). After three washes with PBS, the slides were incubated with secondary biotinylated rabbit anti-mouse antibodies (Dako) (diluted 1 in 350) for 30 minutes. Then they were exposed to avidin biotin peroxidase complex (Dako). Diaminobezidine tetrachlo-

ride (DAB) (Sigma, Poole, Dorset, UK) was used as the chromogen. Slides were counterstained with Mayer's haematoxylin. For negative controls, the primary antibody was omitted and the anti-bcl-2 antibody was substituted with an unrelated monoclonal antibody of the same concentration but directed against an unrelated antigen (monoclonal mouse anti-human CD68 antibody, isotype IgG1; Dako). Normal lymphocytes served as an internal positive control.

EVALUATION OF IMMUNOSTAINING

Bcl-2 immunostaining was scored as the percentage of cells exhibiting definite immunoreactivity in at least 500 neoplastic cells encountered in 10 randomly selected high power fields ($\times 400$). A specimen was regarded as positive when more than 5% of the neoplastic cells showed bcl-2 immunoreactivity.

STATISTICAL ANALYSIS

All numerical data are expressed as median (range). The relation between bcl-2 immunoreactivity and the clinicopathological findings was evaluated using the χ^2 test. To evaluate prognostic relevance, survival was computed from the time of surgery until death or date of the last follow up visit. The Kaplan-Meier method was used for univariate analysis and the log rank test was used to assess differences between groups.⁹ The adjusted hazard ratios and their 95% confidence intervals (CI) were calculated by means of a multiple regression analysis based on the Cox proportional hazards model.¹⁰ For all statistical analysis a p value <0.05 was regarded as statistically significant.

Results

bcl-2 IMMUNOSTAINING

Bcl-2 immunostaining was typically cytoplasmic. In normal squamous and columnar epithelium, bcl-2 immunoreactivity was observed at the basal layer and sometimes in the stroma. A heterogeneous distribution of bcl-2 expression was observed in the neoplastic cells of the tumours. There was striking intra- and intertumour variability: in some tumours only very scanty bcl-2 immunostaining was observed whereas in others large areas of bcl-2 positivity were found (fig 1). Bcl-2 immunoreactivity was rarely found at the leading edge of the tumour.

bcl-2 EXPRESSION IN RELATION TO CLINICOPATHOLOGICAL FINDINGS

Forty eight (63%) cervical carcinomas were bcl-2 positive and 28 (37%) were bcl-2 negative (table 1). The mean age of patients in the bcl-2 negative group was 55 years and in the bcl-2 positive group was 51 years (NS). A reflection of the quality of surgery is given by mean number of lymph nodes removed: 24 in the bcl-2 negative group and 27 in the bcl-2 positive group (NS). The relation between clinicopathological factors and bcl-2 immunoreactivity is summarised in table 1. There was no significant relation between bcl-2 expression and tumour histology, tumour differentia-

Figure 1 Strong bcl-2 immunoreactivity in moderately differentiated squamous carcinoma of the cervix.

Figure 2 Kaplan-Meier curves for overall survival according to bcl-2 expression (logrank test, $p < 0.001$) in all patients.

tion, FIGO stage, presence of lymph node metastases, or lymphovascular space involvement.

bcl-2 EXPRESSION IN RELATION TO SURVIVAL

Two women died from causes other than cervical cancer, with no evidence of disease; 42 women (55%) were in complete remission; 34 women (45%) had a recurrence, and 22 of the latter group (29% of the total study group) died of their tumours. Using univariate analysis, bcl-2 negativity ($p < 0.001$), presence of vascular permeation ($p = 0.002$) or lymphatic permeation ($p = 0.014$), and lymph node metastasis ($p = 0.051$) were significant markers of poor overall survival. The five year survival rate for the entire study population was 57% (95% CI 40–71%). The five year survival rate for patients stratified according to FIGO stage was: 75% (95% CI 13–96%) for stage Ia; 64% (41–80%) for Ib; 28% (2–67%) for IIa; and 38% (1–81%) for IIb. The five year survival rate was 73% (46–88%) for patients without and 42% (22–61%) for patients with lymphatic permeation, was 69% (47–83%) for patients without and 33% (11–57%) for patients with vascular permeation, and was 66% (43–81%) for patients with negative and 41% (18–62) for patients with positive lymph nodes. The five year survival rate was 90% (47–99%) for patients with grade I tumours, 60% (35–78) for those with grade II tumours, and 39% (16–62%) for those with grade III tumours. The five year survival rate for patients with bcl-2 negative tumours was 34% and for patients with bcl-2 positive ones was 71% (logrank test, $p < 0.001$) (fig 2). Subgroup analysis showed that both for the lymph node negative (logrank test, $p < 0.001$) and lymph node positive groups (logrank test, $p < 0.001$), patients with bcl-2 positive tumours had a better prognosis.

The best fitting model for overall survival, according to Cox proportional hazards regression analysis (deviance of 156, likelihood ratio on 3 DF = 17.751, $p < 0.001$), included bcl-2 expression, FIGO tumour stage and vascular permeation (table 2). Multiple regression analysis adjusted for stage and vascular permeation demonstrated a significantly shorter overall survival (adjusted hazard ratio = 0.293

Table 2 Multivariate analysis for overall survival

	Coefficient	Standard error
bcl-2 expression	-1.229	0.427
FIGO stage	0.169	0.335
Vascular permeation	1.155	0.452

Deviance = 156 and likelihood ratio on 3 DF = 17.751; $p < 0.001$.

(95% CI 0.127–0.676)) for patients with bcl-2 negative tumours compared with those with bcl-2 positive ones.

Discussion

The role of apoptosis in tumour development is currently one of the hot topics in oncology. Apoptosis is a distinct mode of programmed cell death that is responsible for the deletion of cells in normal and pathological tissues.² Failure of tumour cells to undergo programmed cell death can result in uncontrolled accumulation of cells. In this retrospective study we found a strong relation between apoptosis, as evaluated by bcl-2 expression, and prognosis in patients with cervical carcinoma. No relation was found between bcl-2 overexpression and clinicopathological factors. However, this may be because of the limited number of patients studied, as there was a trend towards a positive correlation between bcl-2 expression and lymph node status or lymphatic tumour permeation.

Bcl-2 overexpression has been reported to be a marker of good prognosis in patients with B cell lymphomas,¹¹ non-small cell lung cancer,¹² colorectal carcinomas,¹³ breast cancer,¹⁴ and ovarian carcinoma.¹⁵ To the best of our knowledge this is the first study demonstrating that bcl-2 is an independent prognostic factor in patients with early carcinoma of the uterine cervix. Levine *et al*¹⁶ stated that spontaneous apoptosis in cervical tumours may be a predictor of response to radiotherapy. In the present study bcl-2 expression was an important independent prognostic factor in patients who were treated primarily by radical surgery. Subgroup analysis revealed that both in patients who received adjuvant radiotherapy (lymph node positive) or in patients who were only treated surgically (lymph node negative), bcl-2 overexpression remained a significant marker of good prognosis. This suggests that bcl-2 expression plays a crucial role in the regulation of apoptosis and disease progression in patients with operable cervical carcinoma. Greater understanding of the processes involved may lead to the development of improved medical treatment strategies.

Apoptosis occurs spontaneously in malignant tumours and its extent is enhanced by well established treatment modalities, such as irradiation, cytotoxic chemotherapy, heating, and hormone ablation.¹ The regulation of this process is very complex and involves numerous genetic and epigenetic factors. Recent findings at the molecular level indicate that the interaction between certain oncogenes, such as c-myc, p53, bax, bcl-2 and its homologues, are mainly responsible for the regulation of spontaneous and treatment induced apoptosis. Probably the

most prominent regulator of programmed cell death is the bcl-2 protein family. This family contains antiapoptotic proteins (for example, bcl-2, bcl-X_L and mcl-1) and proapoptotic proteins, such as bax. As a result of heterodimer (or oligomer) formation between bax and bcl-2 or other suppressors of programmed cell death, such as bcl-X_L and mcl-1, the actions of these proteins are neutralised.¹⁷ For oncogenes other than those in the bcl-2 family the regulation is sometimes so complex that it seems to be contradictory. For example, c-myc can stimulate mitosis or apoptosis,^{18,19} depending on the available stimuli. One of these stimuli (regulators) is bcl-2, which suppresses the cell death promoting function of c-myc without affecting c-myc mitogenic function.^{20,21} Liang *et al.*²² found that high levels of bcl-2 expression occurred in cervical carcinoma cells lacking functional p53. Silvestrini *et al.*,²³ Miyashita *et al.*²⁴ and Liang *et al.*²² all reported high levels of bcl-2 expression in p53 negative tumours. Haldar *et al.*²⁵ and Miyashita *et al.*²⁴ showed that overexpression of p53 can induce down regulation of bcl-2 expression at both the protein and mRNA level. This indicates that functional p53 can transcriptionally repress bcl-2. Human papilloma virus (HPV) infection, which plays a crucial role in cervical carcinogenesis, often leads to the inactivation of wild type p53 by HPV E6.^{26,27} This may explain the important role of bcl-2 in the clinical behaviour of cervical carcinoma.

Bcl-2 transfection has been demonstrated to confer resistance to anti-cancer agents via a non-conventional drug resistance pathway.²⁸ In vivo and in vitro studies have demonstrated a role for the bcl-2 protein in the prevention of apoptosis induced by anti-cancer drugs and radiotherapy.^{29,30} Previous data from our laboratory¹⁴ and others³¹ strongly suggest that bcl-2 expression may be a predictor of response to chemotherapy or endocrine therapy in patients with breast cancer. There are several published observations about the parallel increase in bax concentrations opposing and neutralising the high level of bcl-2 expression in some transfectants and solid tumours, which may explain resistance to treatment by bcl-2 negative and probably bax negative tumours.^{17,32}

Our results show a strong correlation between bcl-2 expression and prognosis in cervical carcinoma. The expression of other genes involved in the regulation of apoptosis in cervical cancer could, in future studies, shed more light on the understanding of the association between bcl-2 overexpression in primary tumours and beneficial clinical outcome.

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