Acta Derm Venereol 2002; 82: 423–427

Taylor & Francis
healthsciences

INVESTIGATIVE REPORT

Bcl-2-related Proteins, α-Smooth Muscle Actin and Amyloid Deposits in Aggressive and non-Aggressive Basal Cell Carcinomas

ÖNDER BOZDOGAN[1], EMEL ERKEK[2], PINAR ATASOY[1], MUKADDER KOÇAK[2], AHU BIROL[2] and MUZAFFER ÇAYDERE[3]

Departments of [1]Pathology and [2]Dermatology, Kırıkkale University Faculty of Medicine and [3]Ministry of Health, Ankara Training and Research Hospital, Turkey

Aberrant expression of bcl-gene products has been implicated in the development of non-melanoma skin cancers. Recently, altered expression of α-smooth muscle actin has been proposed as predictive of tumour behaviour in basal cell carcinomas. The purpose of this study was to compare the aggressive and non-aggressive basal cell carcinomas in terms of bcl-gene products and α-smooth muscle actin expression. Fifty excisional biopsy samples were studied by immunohistochemical technique for the differential expressions of bcl-2, bax, bcl-x and α-smooth muscle actin. Bcl-2, bcl-x and bax were expressed in 34 (68%), 38 (76%) and 41 (82%) specimens, respectively. Immunoreactivity for α-smooth muscle actin was noted both in tumour nests (64%) and within the stroma (54%). There was a significant difference between aggressive and non-aggressive basal cell carcinomas in terms of bcl-2 and stromal α-smooth muscle actin immunoreactivity. Non-aggressive basal cell carcinomas display a concordant expression of bcl-family proteins, whereas aggressive tumours reveal a discordant pattern. An increased expression of stromal α-smooth muscle actin with a concomitant decrease or loss of bcl-2 expression may be highly suggestive of aggressiveness in basal cell carcinoma. ***Key words: basal cell carcinoma; amyloid; apoptosis; bcl-2; bax; bcl-x; myofibroblast.***

(Accepted July 18, 2002.)

Acta Derm Venereol 2002; 82: 423–427.

Emel Erkek M.D., Neyzen Tevfik sokak, Ferah apt. 12/12, Maltepe 06570, Ankara, Turkey. E-mail: emelerkek@hotmail.com

Current evidence indicates that the differential expression of anti-apoptotic versus pro-apoptotic bcl-family proteins is an important determinant of the inherent susceptibility of a given cell to respond to apoptotic signals (1–3), and genetic alteration of this pathway might play a role in the pathogenesis of various tumours, including non-melanoma skin cancers (3–5). Basal cell carcinoma (BCC) is the most commonly diagnosed malignant skin tumour in white races (6, 7). BCCs are generally slow-growing tumours that require months to years to double in size, despite high mitotic rate (1, 7–9). Apoptosis plays a considerable role in the kinetic homeostasis of BCCs, leading to elimination of the bulk of the tumour (1, 7, 10) and hypothetically to the formation of amyloid deposits within the tumour and stroma (10).

The classifications of BCC are complex and lack uniformity of terminology and clear definition (11). During the past decade, the determination of clinicopathological prognostic parameters in predicting aggressive behaviour has been the focus of investigations. Based on these studies, BCC has been classified as aggressive (A-BCC) and non-aggressive (NA-BCC) by well-described clinicopathological criteria. Recently, immunohistochemical markers such as actin have received significant interest in connection with BCC. Actin is the predominant component of contractile microfilaments (12, 13). α-smooth muscle actin (α-SMA) is found exclusively in contractile muscle cells, myoepithelial cells and myofibroblasts (12–14). It has been suggested that an altered expression of α-SMA in BCC might be predictive of aggressive invasion (12, 13).

The present study was designed to question the relationship between amyloid deposits and the expression of bcl-2-related markers in BCC and to compare A-BCCs and NA-BCCs in terms of bcl-family proteins and α-SMA immunoreactivity.

MATERIALS AND METHODS

Collection of samples

Fifty formalin-fixed, paraffin-embedded excisional BCC samples were retrieved from the archives of the Pathology Departments at Kırıkkale University Faculty of Medicine and the Ministry of Health Ankara Training and Research Hospital. Sections of each biopsy sample were stained with H&E, histopathologically reviewed by two of the authors (ÖB and PA) and grouped as A-BCC (infiltrative, morpheaform or micronodular growth pattern; spiky, jagged and irregular cell group contours; loss of palisading; significant pleomorphism; invasion to subcutaneous tissue) and NA-BCC (nodular or superficial growth pattern; smooth, rounded cell group contour; prominent palisading; no pleomorphism; location in reticular dermis). Tumours with mixed growth patterns were classified according to the predominant component comprising $>50\%$ of the neoplastic compartment. Predominantly micronodular growth pattern was classified as A-BCC.

 ISSN 0001-5555

Clinical features were reviewed from patient records. All patients were Caucasians and their ages ranged from 30 to 77 years (mean 58.3). Eighteen patients were males and 32 were females. Duration of the disease varied from 1 to 20 years (mean 7.3; median 5.0). Clinically, 42 patients (84%) had noduloulcerative, 5 (10%) had superficial and 3 (6%) had pigmented BCC. Histologically, 26 (52%) specimens were nodular, 16 (32%) were infiltrative, 5 (10%) were superficial and 3 (6%) were micronodular. Nineteen (38%) samples were classified as A-BCC and 31 (62%) as NA-BCC.

Immunohistochemical (IHC) analysis

Five-micron-thick sections were obtained by microtome and transferred into adhesive slides. The sections were kept in the autoclave at 37°C for 16 h and at 60°C for 20 min. They were then deparaffinized and dehydrated by immersion in xylene twice for 10 min and into alcohol twice for 2 min. The specimens were incubated in 3% H_2O_2 for 5 min to inhibit activation of endogenous peroxidases. All preparations were transferred into antigen retrieval solution (DAKO; Glostrup; Denmark; pH 6) and placed in the microwave oven (750 watt) twice for 5 min. Using the Shandon Sequenza manual staining device for standardization, the classical avidin-biotin-peroxidase method and DAB chromogen were applied for immunohistochemical analysis of bcl-2 (DAKO-N 1587, prediluted), bax (DAKO-A 3533; dilution 1: 100), bcl-x (DAKO-A 35-10; dilution 1: 50) and α-SMA (Dako-1A4, prediluted). Lymph node, mammary carcinoma, normal skin and uterine leiomyoma specimens served as positive controls for bcl-2, bax, bcl-x and α-SMA, respectively. In addition, the immunostaining in lymphocytes (bcl-2), epidermis (bcl-2, bax and bcl-x), pericytes around the blood vessels and, when found, arrector pili muscles (α-SMA) served as internal positive controls. Mayer's haematoxylin was used as counterstain and slides were examined by light microscopy. The amount (the percentage of positive cells) of immunostaining for bcl-2, bax, bcl-x and tumoral α-SMA was analysed semiquantitatively as (–) if $<5\%$; (+) if $<20\%$; (++) if $20-50\%$; (+++) if $>50\%$. Stromal α-SMA immunoreactivity was assessed as (–) if no staining was found; (+) if only a few myofibroblasts showed positivity around the tumour islands; (++) if $<50\%$ of the islands were surrounded by α-SMA-positive cells and (+++) if $>50\%$ of the islands were diffusely surrounded by α-SMA-positive cells.

Detection of amyloid and keratin

Crystal violet and Congo red (followed by polarized light) stains were used to confirm amyloid deposits. The amount of amyloid was semiquantitatively recorded as: (–) no amyloid; (+) very small, just detectable; (++) moderate, easily detectable; (+++) abundant. In amyloid-positive cases, streptavidin-biotin-peroxidase and DAB chromogen were applied for immunohistological demonstration of cytokeratin (Dako-AE1/AE3-prediluted), microscopically assessed either as (–) or (+) within amyloid-positive areas.

Statistics

The results of the study were statistically analysed using the SPSS 6.0 program (Windows, Microsoft, USA). The Mann-Whitney U-test was utilized to compare numerical data. The Pearson correlation coefficient test was utilized to analyse categorical data that can be evaluated in numerics. A p-value of $\alpha \leq 0.05$ was considered significant.

RESULTS

The globular and amorphous amyloid deposits were found within the tumour islands, within the fibrous stroma and in the superficial dermis in 15 cases (30%). Cytokeratin was (+) in 13 of 15 amyloid-positive specimens (86.7%).

Eight of the 19 A-BCC specimens (42.1%) showed bcl-2 expression, 17 expressed bax (89.5%) (Fig. 1) and 15 expressed bcl-x (78.9%) (Fig. 2). Among the 31 NA-BCC specimens, bcl-2, bax and bcl-x immunoreactivity was detected in 26 (83.9%), 24 (77.4%) and 23 (74.2%) samples, respectively. Intratumoral α-SMA positivity was found in 13 of 19 A-BCCs (68.4%) and 19 of 31 NA-BCCs (61.3%). Fifteen (78.9%) A-BCC samples and 12 (38.7%) NA-BCC samples expressed stromal α-SMA (Fig. 3). Coincidental intratumoral and stromal α-SMA expression (Fig. 4) was noted in 11 A-BCC (57.9%) and 6 (19.4%) NA-BCC specimens.

All bcl-related markers and α-SMA showed diffuse intracytoplasmic positivity. Bcl-2 immunoreactivity was significantly stronger and more diffuse in NA-BCC

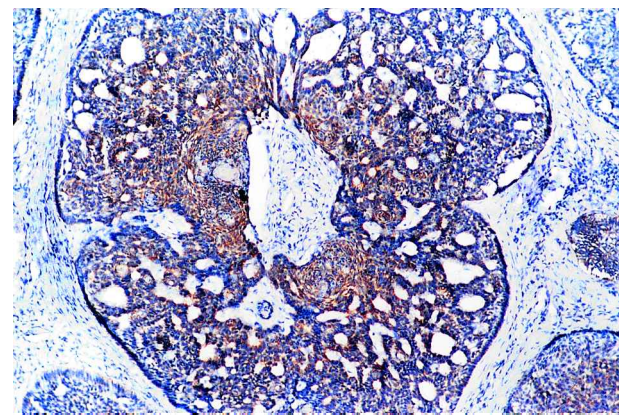

Fig. 1. Diffuse bax immunostaining in a micronodular basal cell carcinoma (bax, IHC $\times 10$).

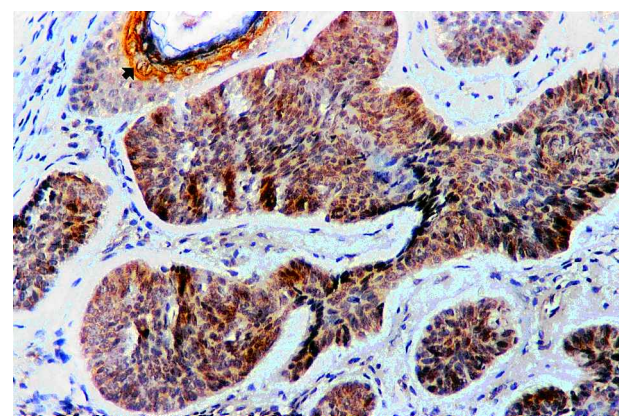

Fig. 2. Bcl-x immunostaining in a micronodular basal cell carcinoma. Adjacent hair follicle also shows positive staining (arrow) (bcl-x, IHC $\times 20$).

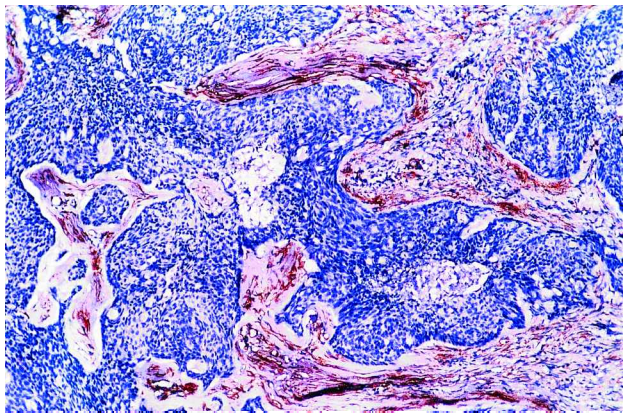

Fig. 3. α-SMA positive myofibroblasts surrounding the neoplastic islands. Although nodular configuration is predominant, irregularities in cell group contours and loss of palisading are clearly visible in some areas (SMA, IHC $\times$ 10).

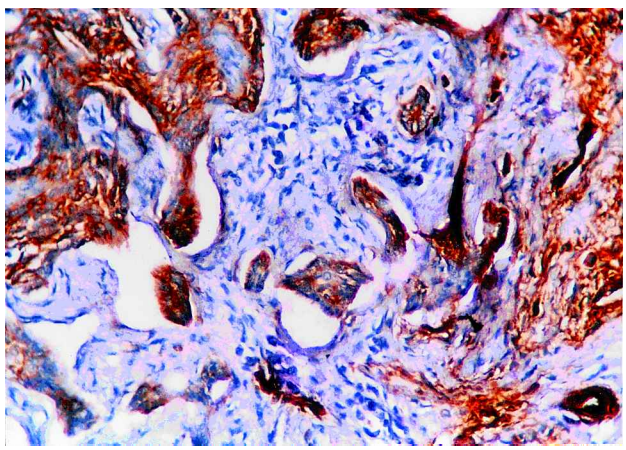

Fig. 4. Strong tumoral and stromal α-SMA positivity in infiltrative basal cell carcinoma. Separation artefact was noted around the cell groups (α-SMA, IHC $\times$ 20).

samples compared with A-BCC samples. Bax and bcl-x expression overlapped in BCC samples; both showed a strong expression at the periphery of tumoral islands, particularly at tumour nests close to the epidermis. The distribution of bcl-x protein within BCC was associated with areas of squamous differentiation. Intratumoral α-SMA immunostaining was pronounced at the periphery of neoplastic islands.

Statistical analysis revealed a significant negative correlation between bcl-2 and stromal α-SMA expression ($r = \_ 0.475$, $p = 0.001$). There was no correlation between amyloid and bcl-family proteins. When the two major groups of BCC (A-BCC versus NA-BCC) were compared, there was a statistically significant difference for bcl-2 ($U = 126.00$, $p = 0.001$) and stromal α-SMA ($U = 131.5$, $p = 0.001$) between the two groups.

DISCUSSION

There are strong indicators that the bcl-family is involved in the regulation of programmed cell death (3). Bcl-2 is an anti-apoptotic protein that promotes cell viability without promoting cell proliferation (15, 16). Overexpression of bcl-2 can counter the physiologically relevant signals of apoptosis (1, 8, 9, 17). Bcl-2 has been shown to be highly expressed in BCC. Using the Western-Blot technique, Delehedde et al. (18) showed a 5.5-fold increase in bcl-2 expression in BCC compared to normal skin. The frequency of bcl-2 staining in BCC varies from 39% to 100% in published series (6, 15, 19). In the present study, 68% of BCCs expressed bcl-2 protein. Consistent with the previous data (15–17), we detected a significantly decreased bcl-2 labelling in A-BCCs (42%), while NA-BCCs overexpressed (84%) the bcl-2 protein. Current evidence indicates that bcl-2 expression is more frequent in preneoplastic and preinvasive lesions than their invasive and malignant counterparts in various tissues. The possible outcome of bcl-2 overexpression is the accumulation of neoplastic cells with heightened life span and that remain continuously exposed to many physical or chemical agents, principally to UV rays, resulting in subsequent mutagenic events, including the p53 mutation (15–17). The accumulation of UV-induced genetic alterations along with a spontaneous and/or UV-induced down-regulation of bcl-2 may result in aggressive invasion in BCC (1, 2, 17, 19, 20).

Bcl-x is a bcl-2 independent regulator of apoptosis (3). Its two splicing products bcl-x_L (long) and bcl-x_S (short) have opposite effects; bcl-x_L inhibits apoptosis and bcl-x_S promotes cell death and inhibits bcl-2 function (1, 3, 21). The predominant form of bcl-x expressed by keratinocytes *in vitro* and *in vivo* is the long form (18, 20). Malignant keratinocytes in BCC have been shown to express bcl-x (18, 21). In our study, bcl-x was expressed in 76% of BCCs. The over-expression of bcl-x in BCC may contribute to the longevity of tumoral cells by protecting them from spontaneous or UV-induced apoptosis (22). In aggressive BCCs when the bcl-2 expression is decreased or lost, cell-survival function may be maintained and primarily performed by bcl-x.

Bax is a principal molecular inducer of apoptosis (1–3). It forms heterodimers with homologous anti-apoptotic proteins and inactivates them (19). In 1998, Rosen et al. (23) reported that BCCs did not express bax protein. Later, Tomkova et al. (24) reported that bax staining was absent in 70.6% of BCCs studied. Recently, Delehedde et al. (18) demonstrated by immunohistochemical technique that bax was expressed in the vast majority of BCCs and that it was expressed 2-fold in BCCs compared to normal skin by Western-Blot analysis. Our study revealed bax expression in 82% of BCCs. Although not significant, the occurrence of bax positivity was greater in A-BCC than in NA-BCC. As a trancriptional activator of bax, p53 protein signals the induction of apoptosis (6), and bax over-expression in A-BCCs could be a consequence of p53

Table I. *The occurrence of bcl-2, bax, bcl-x, α-SMA and amyloid positivity in aggressive (A-BCC) and non-aggressive (NA-BCC) basal cell carcinomas*

Type	No. of cases	Bcl-2	Bax	Bcl-x	α-SMA tumoral	α-SMA stromal	Amyloid
A-BCC[1]	19	8 (42.1%)	17 (89.5%)	15 (78.9%)	13 (68.4%)	15 (78.9%)	4 (21.0%)
NA-BCC	31	26 (83.9%)*	24 (77.4%)	23 (74.2%)	19 (61.3%)	12 (38.7%)*	11 (35.5%)
Total	50	34 (68%)	41 (82%)	38 (76%)	32 (64%)	27 (54%)	15 (30.0%)

[1]Including micronodular basal cell carcinoma. *$p \leq 0.001$ significance has been measured between A-BCC and NA-BCC. All other values are non-significant.

overexpression observed in A-BCCs (17). Moreover, enhanced bax expression could be responsible for the higher apoptotic index encountered in A-BCCs (6, 17). Our findings indicate that bax is overexpressed and is involved in the apoptotic process that occurs in BCCs.

In our study, both intratumoral expression and stromal α-SMA expression were detected in BCCs. Stromal α-SMA immunoreactivity was significantly higher in A-BCC than in NA-BCC. Similar findings have been published previously (12–14). Actin within the stroma surrounding BCC nests is a marker for myofibroblasts that play a significant role in invasion, as they secrete stromolysin-3, a metalloproteinase that degrades the stromal matrix (12, 13). Degradation of the stromal matrix may enhance the stromal-tumour communication that is essential for invasion (12). Although the pathophysiologic mechanism responsible for the myofibroblastic reaction is obscure, it has been proposed that the induction of cytokines from BCC cells (such as basic fibroblast growth factor) may be responsible for stromal α-SMA expression (12, 13). In this study, co-expression of stromal and intratumoral α-SMA was approximately 3-fold higher in A-BCCs than in NA-BCCs (57% vs. 19%), supporting the view that the same BCC-derived cytokines that induce the stromal myofibroblastic response have an autocrine effect on the individual BCC cells, increasing tumoral actin synthesis and leading to enhanced cellular motility and invasion (12, 13). A striking finding in our study was a significant inverse correlation between stromal α-SMA and bcl-2 expression, indicating that A-BCCs reveal an increased expression of stromal α-SMA along with a depressed bcl-2 expression.

The frequency of amyloid deposits in BCC varies from 28% to 75% in the literature (6, 10, 25–27). In this study, amyloid deposits were found in 30% of BCCs. Although the origin of amyloid in BCC is obscure, it has been speculated that the deposits reflect apoptosis of tumour cells and that the precursor proteins may be epidermal tonofilaments (6, 10, 15, 28). Supporting this hypothesis, amyloid in BCC has been shown to immunoreact with anti-keratin antiserum (10, 28). It has been proposed that amyloid deposits reflect the apoptotic rate in BCCs (10). Olsen & Westermark (27) could not detect a correlation between amyloid deposition and apoptosis in BCC. Wang et al. (6) studied 53 BCC samples for amyloid deposits and the expression of p53 and bcl-2 proteins and could not demonstrate a correlation between amyloid deposition, apoptosis and bcl-2/p53 protein expression. Likewise, there was no correlation between amyloid deposits and bcl family proteins in our study. However, the absence of a statistical correlation cannot exclude the possibility of bax-mediated amyloid deposition. Amyloid deposits might not be a marker of the current apoptosis status, rather they may reflect the cumulative effects of previous apoptosis. Alternatively, other apoptotic pathways, such as the Fas/FasL system, that directly stimulate caspases and bypass the action of bcl family proteins might be involved in amyloid deposition in BCC.

In conclusion, the results of the present study support a keratin-origin for amyloid in BCC and suggest a concordant expression for bcl-2, bcl-x and bax in NA-BCCs. A-BCCs display a discordant expression of bcl-family proteins, with a spontaneous decrease or loss of bcl-2 expression. An increased expression of stromal α-SMA along with a depressed bcl-2 expression might reflect increased aggressiveness in BCC and immunohistochemical examination of BCCs for these two markers may help clinicians in predicting tumour behaviour.

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