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Tomas, Davor; Krušlin, Božo; Čupić, Hrvoje; Stanimirović, Andrija; Bošnjak, Berislav; Lovričević, Ivo; Belicza, Mladen

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CORRELATION BETWEEN BCL-2 AND BAX IN ATROPHIC AND HYPERTROPHIC TYPE OF ACTINIC KERATOSIS

Davor Tomas[1], Božo Krušlin[1], Hrvoje Čupić[1], Andrija Stanimirović[1], Berislav Bošnjak[2], Ivo Lovričević[3], Mladen Belicza[1]

[1]Ljudevit Jurak Department of Pathology and [3]Department of Surgery, Sestre milosrdnice University Hospital, Vinogradska 29, 10 000 Zagreb, Croatia

[2]PLIVA Research Institute Ltd., Prilaz Bruna Filipovića 25, 10 000 Zagreb, Croatia

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CORRESPONDING AUTHOR AND AUTHOR TO WHOM REQUESTS FOR REPRINTS SHOULD BE ADDRESSED:

Davor Tomas M.D.

Ljudevit Jurak Department of Pathology, Sestre milosrdnice University Hospital, Vinogradska 29, 10 000 Zagreb, Croatia

Phone: +385 1 3787 909, Fax: +385 1 3787 244, E-mail: dtomas@kbsm.hr

ABSTRACT

BACKGROUND: Recent investigations consider actinic keratosis (AK) as an earliest visible pattern of squamous cell carcinoma (SCC). We have analyzed the expression of apoptosis-related proteins *TP53*, *Bcl-2 and Bax* in 30 atrophic and 30 hypertrophic AK.

MATERIAL AND METHODS: Immunohistochemical analysis was performed following Microwave Streptavidin ImmunoPeroxidase protocol on DAKO TechMate^{TM} Horizon automated immunostainer. Monoclonal antibody for *TP53* and *Bcl-2* and polyclonal antibody for *Bax* (DAKO, Denmark) were used.

RESULTS: Expression of *TP53* showed no significant differences between two analyzed groups (χ^2-test, p=0.35636) whereas expression of *Bcl-2* and *Bax* protein was significantly higher in atrophic compared to hypertrophic AK (χ^2-test, p=0.01458 and p=0.00358, respectively). Comparison of *Bcl-2/Bax* ratio in two analyzed AK showed significantly higher value in hypertrophic compared to atrophic AK (Mann-Whitney U test, p=0.02272). Statistical analysis did not show any correlation between patient's sex and age, localization and size of the lesion with expression of investigated oncoproteins (ANOVA, p>0.05).

CONCLUSIONS: Our results may indicate higher resistance of keratinocytes on apoptotic stimuli in hypertrophic compared to atrophic AK. Thus, we suppose that keratinocytes in hypertrophic AK live longer and probably have higher propensity for additional mutations and conversion to overt SCC.

INTRODUCTION

Actinic keratosis (AK) is a common problem in the population, and one of the most common conditions treated by dermatologists. The main risk factors for the development of AK are increased sun exposure and increased susceptibility to sun exposure [1,2]. Working outdoors, having skin type I and/or a history of severe sunburns during childhood were found to be important factors in development of AK [3].

Processes of onset and progression of skin tumors and precanceroses such as AK are under control of various functional relationships between inactivated tumors suppressor genes and activated oncogenes [4-7]. The initial UV-induced damage in development of AK takes place in the DNA. The majorities of the UV-induced lesions in the DNA are repaired but if not, damaged keratinocytes undergo apoptosis. On this way apoptosis serves as a control mechanism, which prevents the propagation of keratinocytes with damaged DNA [8]. However, mutations may occur as a result of base mispairing of the cell and its DNA replicates before the DNA lesion is repaired. The genes involved in the repair and apoptotic process are also potential UV targets, especially tumor suppressor gene *Tp53* [9]. It is well known that *Tp53* gene plays a pivotal role in repair of UV-induced DNA damage and apoptosis. Consequently, *Tp53* alterations are early events in human UV-induced skin carcinogenesis [10]. They are present in more than 90% of squamous cell carcinomas (SCC) and are usually found in AK [11].

Except *Tp53*, other two genes *bcl-2* and *bax* play major roles in the control of apoptosis. The *bcl-2* gene belongs to a family of proto-oncogenes that are unrelated directly to cell proliferation, but participate in tumorigenesis by promoting cell survival via inhibition of apoptosis [12]. Oltvai et al. discovered the pro-apoptotic

gene bax in 1993 and it is a first gene in apoptotic cascade initiated by *Tp53* gene [13,14]. The *bcl-2/bax* ratio is considered to be a marker of a cell's susceptibility to apoptotic stimuli [13, 15-18).

AK was initially considered to be precancerous, because it presents with continuous, preserved basement membrane without invasion into the dermis [19]. However, AK shares many similarities with squamous cell carcinoma: causative factor (UV-light), clinical and pathological properties (i.e. localization, epidermal thickening, atypical keratinocytes) as well as molecular abnormalities (i. e. DNA aneuploidy, loss of heterozygosity) [20,21]. Thus, recent investigations consider AK as an earliest visible pattern of SCC, but there is still no generally accepted agreement about this new postulation [22-25].

MATERIAL AND METHODS

Patients

Our study group included consecutive specimens of 30 patients with atrophic type and 30 patients with hypertrophic type of AK who underwent surgery in the period from January 1st to December 31st, 2003. Clinical information about the patients (age, sex, lesion size and location) was obtained from the department charts. Specimens were fixed in 10% buffered formalin, embedded in paraffin, cut at 5 μm thickness and routinely stained with hematoxylin and eosin. The diagnosis of atrophic and hypertrophic type of AK was histologically confirmed in all cases and each specimen was re-evaluated by three pathologists (D.T., B.K. and H.Č.).

Immunohistochemical analysis

Immunohistochemical determination of *TP53*, *Bcl-2* and *Bax* proteins was performed following Microwave Streptavidin ImmunoPeroxidase (MSIP) protocol on DAKO TechMate™ Horizon automated immunostainer. We used monoclonal antibody DO-7 H7123 for *TP53*, monoclonal antibody 124 H7124 for *Bcl-2* and polyclonal antibody 057 A3533 for *Bax* (purchased from DAKO, Copenhagen, Denmark). Dilution of antibodies for *TP53* and *Bcl-2* was "ready to use" (manufacturer diluted both antibodies 1:50). Antibody for *Bax* protein was diluted 1:500. Positive control for *TP53* staining was colon carcinoma tissue and for *Bcl-2* and *Bax* staining breast carcinoma tissue.

The positive cells were scored in whole lesion at X400 magnification. To evaluate the level of *TP53*, *Bcl-2* and *Bax* protein expression, the percentages of positive-staining cells and the staining intensity were graded on a scale of 0 – 3. Staining percentage was labeled as: 0 = 0% positive cells; 1 = 1 – 10% positive cells;

2 = 11 – 25% positive cells; and 3 – 26% or more positive cells. Staining intensity was denoted as: 0 = no staining; 1 – weak staining; 2 – moderate staining; 3 – strong staining. For each sample, the staining percentage and staining intensity scores were multiplied to give staining index. Immunohistochemical staining index (ISI) was labeled as: 0 – zero; 1-3 = low; 4-6 = moderate; and 9 = high.

Statistical analysis

Statistical analysis was performed using Mann-Whitney U test, χ^2-test and ANOVA test. The level of significance was set at $p < 0.05$ in all cases.

RESULTS

The age range of the patients with atrophic type of AK was between 57 and 88 years (median age 71.2). There were 15 females and 15 male patients (M/F = 1:1). Twenty-one (70.0%) lesions were localized on the face, 4 (13.3%) were located on the helix and 5 (17.7%) were on the neck. Lesions size varied between 0.2 and 1 cm (median size 0.8 cm).

The age range of the patients with hypertrophic type was between 59 and 81 years (median 72.9 years), 18 patients were female and 12 patients were male (M/F = 1.5:1). The most common location was the face (73.3%), subsequent location was helix (16.7%) and only 3 (10.0%) lesions were removed from neck. Size of lesions varied between 0.3 and 1 cm (median size 0.8 cm).

Statistical analysis revealed no statistically significant difference between two types of AK considering patient's sex and age, localization and size (Mann-Whitney U test, p>0,05).

Immunohistochemical staining index for *TP53*, *Bcl-2* and *Bax* protein in atrophic and hypertrophic AK is shown in Table 1.

Twenty-eight investigated atrophic AK (93.3%) were positive for *TP53*. Among them, minority (8, or 26.7%) had low ISI, 10 (33.3%) had moderate ISI and 10 (33.3%) had high ISI. Twenty-nine of 30 hypertrophic AK (96.7%) were positive for TP*53* and 1 (3.3%) was negative. Among positive hypertrophic AK, 13 (43.3%) had low ISI, 5 (16.7%) had moderate ISI and 11 (36.7%) had high ISI. In positive specimens most of the atypical epidermal keratinocytes were positive. The positive cells were diffusely spread in lesion (Figure 1A and 1B).

Immunohistochemical staining for *TP53* showed no statistically significant difference between two analyzed groups (χ^2-test, p=0.35636).

IHC staining for *Bcl-2* oncoprotein was detected in all investigated atrophic and hypertrophic AK (100%). Twelve (40.0%) atrophic and 23 (76.7%) hypertrophic AK had low ISI while 12 (40.0%) atrophic and 4 (13.3%) hypertrophic AK had moderate ISI. Only 6 (20.0%) atrophic and 3 (10.0%) hypertrophic AK had high ISI. In both types of AK the positive cells were predominantly located in the lower epidermis (Figure 1C and 1D).

Five (16.7%) atrophic and 15 (50.0%) hypertrophic AK were immunohistochemicaly negative for *Bax* protein. Fourteen (46.6%) atrophic and 13 (43.3%) hypertrophic AK had low ISI. Moderate ISI had 11 (36.7%) atrophic and only 2 (6.7%) hypertrophic AK. None of analyzed actinic keratosis had high *Bax* ISI. In positive cases, immunoreactive cells were predominantly located in the basal and suprabasal layers of epidermis and positivity diminished toward surface of the lesion (Figure 1E and 1F).

Expression of *Bcl-2* and *Bax* protein were statistically significantly higher in atrophic type compared to hypertrophic type AK (χ^2-test, p=0.01458 and p=0.00358, respectively).

Comparison of *Bcl-2/Bax* ratio in two analyzed AK showed statistically significant higher value in hypertrophic type compared to atrophic type AK (Mann-Whitney U test, p=0.02272) (Table 2).

Statistical analysis found no correlation between patient's sex and age, localization and size of the lesion with ISI of investigated oncoproteins (ANOVA, p>0.05).

DISSCUSION

We analyzed the expression of apoptosis-related oncoproteins in two types of AK. The *Tp53* tumor suppressor gene is essential in maintaining the genomic integrity of cells through its role in allowing repair of DNA damage or apoptosis. Nearly half of all human cancers contain mutation in *Tp53* gene, although these mutations generally occur late in the tumorigenesis process [26]. Non-melanoma skin cancers are exception because of mutations that can be found in normal-appearing, chronically sun-exposed skin of patients with skin cancer and in premalignant lesions [27]. Studies have reported the presence of wild type *Tp53* in acute ultra-violet exposure and both wild type and mutated *Tp53* in AK and in normal skin with or without chronic sun exposure [28-30]. Several authors have suggested that the transient elevation of wild type *Tp53* in normal epidermis following ultra-violet exposure is a reaction to damage to the genome, which arrests the cell cycle in G1 for repair purpose or, if that unsuccessful, induces apoptosis [31,32]. However, ultra-violet exposure may also cause specific mutations in the *Tp53* gene that lead to its malfunction and expansion of mutated keratinocytes and progression to skin cancer [31]. Thus, *TP53* immunopositivity should be analyzed with caution, because immunoreactivity may be related to overexpression or stabilization of wild type *TP53* or to the accumulation of mutant *TP53* proteins. Conversely, negative immunoreactivity does not ultimately exclude presence of *Tp53* truncated or missense mutations [33].

Although there are many investigations of *Tp53* expression in AK, there is no general agreement in the literature about the intensity of *TP53*-positive staining of AK. The results vary markedly, from 0% up to 100%, but different authors reported positivity in approximately 63% to 100% of analyzed AK specimens [34-39]. In the

largest series, Shimitzu reported immunohistochemical positivity of *TP53* in 58 (85%) from 68 specimens [39]. Our results of prominent *TP53* immunopositivity in AK are consistent with reports in the literature [34-39]. In our study, statistical analysis found no significant differences between the expressions of *Tp53* protein in two investigated AK types. We did not determine whether the immunopositivity was a related to mutated *TP53* or accumulation of the wild type protein, but both possibilities are feasible and may even coexist. The positivity of *TP53* protein in both types of analyzed AK was located in nucleus and diffusely dispersed through lesions, which is consistent with literature data [34-39].

Bcl-2 family member proteins, including the anti-apoptotic *Bcl-2* protein and the pro-apoptotic *Bax* protein, are important regulators of apoptosis [39]. The relationship between *Bcl-2* and *Bax* and their capacity for heterodimerization are thought to determine the apoptotic status of the cell [13]. Thus, altered expression of these genes contributes to neoplastic cell expansion by prolonging cell survival [15-18].

The anti-apoptotic *Bcl-2* oncoprotein is normally expressed in basal keratinocytes and has been shown to be deregulated in skin cancers [41]. Several authors investigated *Bcl-2* expression in AK, but there was no general agreement in the literature about the intensity of *Bcl-2* positivity [36,37,42-46]. The majority of these investigations have been performed on relatively small number of AK specimens (up to 10) [42-45] except three recent investigations, two performed by Stanimirović *et al.* [36,37] and one performed by Feinmesser *et al.* [46]. Feinmesser analyzed *Bcl-2* expression in 20 AK and revealed positivity in only 35% of cases [46]. In our study, all investigated AK showed *Bcl-2* expression but the majority was weakly positive, and only 41.7% of investigated AK had stronger immunoreaction

than unaffected perilesional skin which is consistent with results in the literature [36,37,46]. Comparison of *Bcl-2* expression in atrophic and hypertrophic AK revealed statistically significant stronger expression in atrophic type. The strongest positivity of *Bcl-2* protein in both types of analyzed AK was located in cytoplasm of basally located keratinocytes. In lesions with the most intensive immunoreaction, positive keratinocytes were also observed in the upper epidermal layers. Nakagawa and Tucci reported same pattern of *Bcl-2* immunopositivity in AK [42,45].

The pro-apoptotic *Bax* protein is normally expressed in the suprabasal cells in the normal epidermis [47,48]. *Bax* opposes *Bcl-2* function and enhances susceptibility of cell to undergo apoptosis [13]. Some studies have reported that is also related to keratinocyte differentiation [16]. However, other studies found that apoptosis and terminal differentiation of keratinocytes are distinct processes [49].

Only few authors paid attention to the expression of *Bax* protein in AK [37,45]. The first investigation was performed by Einspahr *et al*., which compared expression of *Bax* protein in 13 SCC, 16 AK and 14 normal-appearing skin samples, and concluded that Bax protein was significantly increased in SCC compared to AK. However, they did not specify how many samples of AK were immunohistochemically positive and which type of AK investigated [38]. In a recent study by Feinmesser and his group *Bax* immunopositivity was found in 18 (90%) from 20 investigated AK but only in 2 (11%) of 18 cases AK immunopositvity was strong [46]. They also did not specify which type of AK was used in investigation. In our study immunohistochemical staining showed that *Bax* expression was present in 40 (66.7%) AK specimens. In the atrophic type AK *Bax* expression was observed in 25 (83.3%) specimens, whereas only 15 (50%) hypertrophic AK showed immunohistochemical positivity of *Bax* protein. ISI was predominantly low in both

investigated type AK. Statistical analysis revealed significantly stronger immunoreactions on *Bax* protein in atrophic type compared to hypertrophic type AK. In positive cases immunoreactions was located in the cytoplasm of basally and suprabasally located keratinocytes and decreased gradually toward the more superficial layers, which is consistent with literature data [46].

However, simple determination and comparison of *Bcl-2* and *Bax* immunopositivity is not sufficient for better characterization and understanding of apoptotic processes in two investigated types of AK. Some investigations revealed that cell susceptibility on apoptotic stimuli was directly dependent on *Bcl-2/Bax* ratio in cell cytoplasm [15,17,18]. Raisova *at al.* in their study on melanoma cell culture showed that increased *bcl-2/bax* ratio characterized cells resistant on apoptotic stimuli [15]. Cho and his group confirmed higher resistance on apoptosis induced by ultra-violet radiation in keratinocytes from *bax* (-/-) mice, which also had higher incidence of several tumors compared to control [17]. In a recent study Adhami, contrary to Raisova, showed that decreased *bcl-2*/*bax* ratio characterized cells that initiated apoptotic process [18]. In our investigation, *Bcl-2/Bax* ratio was statistically significantly higher in hypertrophic compared to atrophic type of AK.

Our results may indicate higher resistance of keratinocytes on apoptotic stimuli in hypertrophic compared to atrophic AK. Thus, we suppose that keratinocytes in hypertrophic AK live longer and probably have higher propensity for additional mutations, which may lead to malignant transformation and conversion to overt SCC. Furthermore, we propose a hypothesis that hypertrophic AK is the earliest visible pattern of SCC, whereas atrophic type is still precancerosis but further studies at the molecular level are needed to clarify this issue.

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Table 1 Immunohistochemical staining index (ISI) for *TP53*, *Bcl-2* and *Bax* protein in atrophic and hypertrophic actinic keratosis (AK).

	ATROPHIC AK			**HYPERTROPHIC AK**		
	Tp53	*Bcl-2*	*Bax*	*Tp53*	*Bcl-2*	*Bax*
ISI 0	2 (6.7%)	0 (0%)	5 (16.7%)	1 (3.3%)	0 (0%)	15 (50.0%)
ISI 1	8 (26.7%)	12 (40.0%)	14 (46.6%)	13 (43.3%)	23 (76.7%)	13 (43.3%)
ISI 2	10 (33.3%)	12 (40.0%)	11 (36.7%)	5 (16.7%)	4 (13.3%)	2 (6.7%)
ISI 3	10 (33.3%)	6 (20.0%)	0 (0%)	11 (36.7%)	3 (10.0%)	0 (0%)
TOTAL POSITIVE	28 (93.3%)	30 (100%)	25 (83.3%)	29 (96.7%)	30 (100%)	15 (50.0%)

Table 2 Comparison of *Bcl-2/Bax* ratio in two analyzed AK by Mann-Whitney U Test

	ATROPHIC AK	HYPERTROPHIC AK	MANN-WHITNEY U TEST
Mean	0.694	0.455	p=0.022726
Median	0.500	0.083	

Figure 1 Microphotographs of immunohistochemical staining for *TP53* (A), *Bcl-2* (C) and *Bax* (E) protein in atrophic type of actinic keratosis and for *TP53* (B), *Bcl-2* (D) and *Bax* (F) protein in hypertrophic type of actinic keratosis (All microphotographs were made under high magnification, 400X).