

Apoptotic markers in photoinduced cutaneous carcinoma

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Abstract

Cutaneous carcinomas are malignant lesions, which most commonly occur on photo-aggressed site. The purpose of our study was to evaluate the immunohistochemical expression of three apoptotic markers (p53, Bax, and Bcl-2) in photoinduced basal and squamous cell carcinoma. The study was performed on 24 patients diagnosed with these forms of cutaneous carcinoma localized on photoexposed regions: 14 cases of basal cell carcinoma (BCC) and 10 cases of squamous cell carcinoma (SCC), classified accordingly *WHO* 2003. The immunohistochemical study performed on the three proteins involved in the apoptotic process revealed certain specific features in their manner of expression, which do not correlate or respect the critical determinant rule (Bcl-2/Bax>1). Basal cell carcinoma expresses higher levels of Bcl-2, with a better prognosis, a less aggressive evolution, and no metastasis. Squamous cell carcinoma, on the other hand, expresses lower levels of Bcl-2, but the clinical outcome is more aggressive, the tumor has a faster evolution and may metastasize. P53 protein respects the profile given in literature data, having a higher score in squamous cell carcinoma versus basal cell carcinoma. According to the tumor localization on photo-aggressed sites, we have considered that ultraviolet rays play an important role in initiation of carcinogenesis through still occult mechanisms that may induce these particular or rather "bizarre" expressions of apoptotic markers.

Keywords: carcinoma, skin, apoptosis, ultraviolet radiation.

Introduction

Cutaneous carcinomas are malignant lesions, which most commonly occur on photo-aggressed sites. The major risk factor for cutaneous cancer is the excessive exposure to ultraviolet radiation (UV). The pathogenesis of these proliferative lesions includes complex mechanisms regulating the apoptotic process. Apoptosis has an important role during the development of different organs and plays a fundamental role in maintaining the homeostasis of the tissues. Apoptosis regulates the number of cells in different tissues, eliminates potentially dangerous cells, such as reactive lymphocytes, neoplastic or viral infected cells [1]. The massive involvement of apoptosis in human pathology, especially in tumoral processes, justifies the great amount of studies performed in this field.

The purpose of our study was to evaluate the immunohistochemical expression of three apoptotic markers (p53, Bax, and Bcl-2) in photoinduced cutaneous carcinoma aspects, which may allow the quantification of tumors clinical evolving behavior and prognosis.

Materials and Methods

The study was performed on 24 patients diagnosed

with cutaneous carcinomas, admitted to the Dermatology Clinic of the Emergency University Hospital of Cluj-Napoca, Romania, between 2004–2006. The lesions were localized on photo-exposed skin (face, forearm, calf). By analyzing human tumor samples, the study was conducted according to the principles expressed in the Declaration of Helsinki. All patients provided written informed consent for the collection of samples and subsequent analysis. Tumor material, Hematoxylin–Eosin staining and paraffin-embedded blocks of previously microscopically diagnosed (Department of Pathology) BCC and SCC (classified accordingly *WHO* 2003) were analyzed. Clinical data were available for all patients. For each case, normal skin at the tumor border was present on the slides.

Immunohistochemistry

Serial sections of 5 μm were made from each sample. Standard immunohistochemistry staining, Avidin–Biotin Complex (ABC) method, with DAB chromogen was performed. We used primary antibodies from Dako (Glostrup, Denmark): monoclonal mouse anti-p53 (dilution 1:50; clone DO-7), monoclonal mouse anti-human anti-Bcl-2 (dilution 1:50; clone 124), and from Santa Cruz Biotechnology: rabbit polyclonal anti-mouse Bax antibody (Cat. No. sc-493). Control slides were included in each staining run.

Immunostaining evaluation

All the immunohistochemical stainings were evaluated by two persons independently and consensus was made when the opinions differed.

The immunoreactivity was qualitatively and semi-quantitatively assessed using an Olympus BX50 microscope. Slides were evaluated under microscopic high-power magnification (40× objective).

Due to unequal sample sizes, we could not assess a standard number of fields; therefore, we evaluated the entire tumor, for each case.

For p53, the positive reaction was represented by brownish staining restricted to the nucleus. For Bcl-2 and Bax, the positive reaction was represented by granular, brownish color of the cytoplasm.

The intensity and the extent of staining of tumoral cells were evaluated.

The staining intensity was classified as grade 0 (no color), 1 (low), 2 (moderate), and 3 (intense). The extent of staining (semiquantitative score), represented by the percentage of positive cells was also graded: grade 0 (no positive cells), 1 (1–25% positive cells), 2 (25–50% positive cells), 3 (>50% positive cells).

A staining index or immunohistochemical index (IHI) was calculated for each tumor specimen by multiplying the color intensity grade with the extent of staining grade: negative (0 points), low (1–3 points), moderate (>3–6 points), high (9 points).

Statistical analysis

Average values of the immunohistochemical index were determined. For evaluation of group differences, we used one-way ANOVA test. $P < 0.05$ was considered significant.

Results

The classic histopathological investigation identified 14 cases of basal cell carcinoma (BCC) (seven cases superficial forms and two deep infiltrative tumors) and 10 cases of squamous cell carcinoma (SCC) (eight cases well differentiated and two low differentiated).

In basal cell carcinoma, immunohistochemical analysis for p53 and Bax was positive in 100% (14) of cases, and Bcl-2 was positive in 85.71% (12) cases (Table 1). P53 protein was intensely expressed in 50% (7) of cases (Table 1; Figure 1A), but Bcl-2 and Bax showed mainly a moderate expression in this type of tumor (Table 1; Figure 1, B and C).

Table 1 – Distribution of immunohistochemical index (IHI) for p53, Bcl-2, and Bax in basal cell carcinoma

IHI	p53 (n=14)	Bcl-2 (n=14)	Bax (n=14)
Negative	–	2	–
Low	2	3	4
Moderate	5	7	6
High	7	2	4

A different pattern of immunostaining was observed within the superficial forms of BCC (high expression for

p53, and moderate for Bcl-2 and Bax) versus deep infiltrating cases [low expression for p53 and Bax, and negative for Bcl-2 (Table 1)].

The epidermis at the tumor borders expressed the apoptotic markers at different degrees of intensity: p53 was moderately positive in basal and suprabasal layers, Bcl-2 had a homogenous expression in the basal layer, whereas Bax was occasionally expressed in the basal layer and constantly expressed in the suprabasal layer.

The immunohistochemical investigation of apoptotic markers in SCC was positive for p53 and Bax in 100% (10) of cases, but Bcl-2 was positive in only 50% (5) of cases. The evaluation of staining intensity showed high expression for p53 in the great majority of cases – 90% (Table 2; Figure 2).

The same high intensity was observed in most of cases (80%) for Bax, whereas the staining for Bcl-2 showed mainly low intensity.

Table 2 – Distribution of immunohistochemical index (IHI) for p53, Bcl-2, and Bax in squamous cell carcinoma

IHI	p53 (n=10)	Bcl-2 (n=10)	Bax (n=10)
Negative	–	5	–
Low	1	2	1
Moderate	–	1	1
High	9	2	8

P53 protein was well expressed especially at the periphery of the tumoral masses, where nuclei appeared intensely stained.

The immunoreactivity for p53 protein was more intense within squamous cells carcinomas, where the apoptosis rate was higher and the expression of Bcl-2 protein lower. The pattern of staining was also different for each apoptotic marker. Bax protein showed a homogenous positive expression (mostly in differentiated carcinomas), whereas Bcl-2 was occasionally expressed at the periphery of the tumor, reacting with the peritumoral lymphocytic infiltrate.

We also compared the immunohistochemical index (IHI) of p53, Bcl-2, and Bax in BCC and SCC. The values of the immunohistochemical index were statistically significant only for the Bax antibody (average 5.23 in BCC versus 8.86 in SCC), whereas for p53 and Bcl-2 the values of immunohistochemical index were not statistically significant (for p53: average 7.11 in BCC versus 9.39 in SCC, and for Bcl-2: average 4.58 in BCC versus 2.57 in SCC) (Table 3).

Table 3 – Average of immunohistochemical index (IHI) values of p53, Bcl-2 and Bax values in basal cell carcinoma versus squamous cell carcinoma

IHI	p53	Bcl-2	Bax
BCC (n=14)	7.11	4.58	5.23*
SCC (n=10)	9.36	2.57	8.86*

* $P < 0.05$.

As a general observation, for p53 and Bax the immunohistochemical index was higher in SCC, whereas Bcl-2 had higher IHI values in BCC (Figure 3).

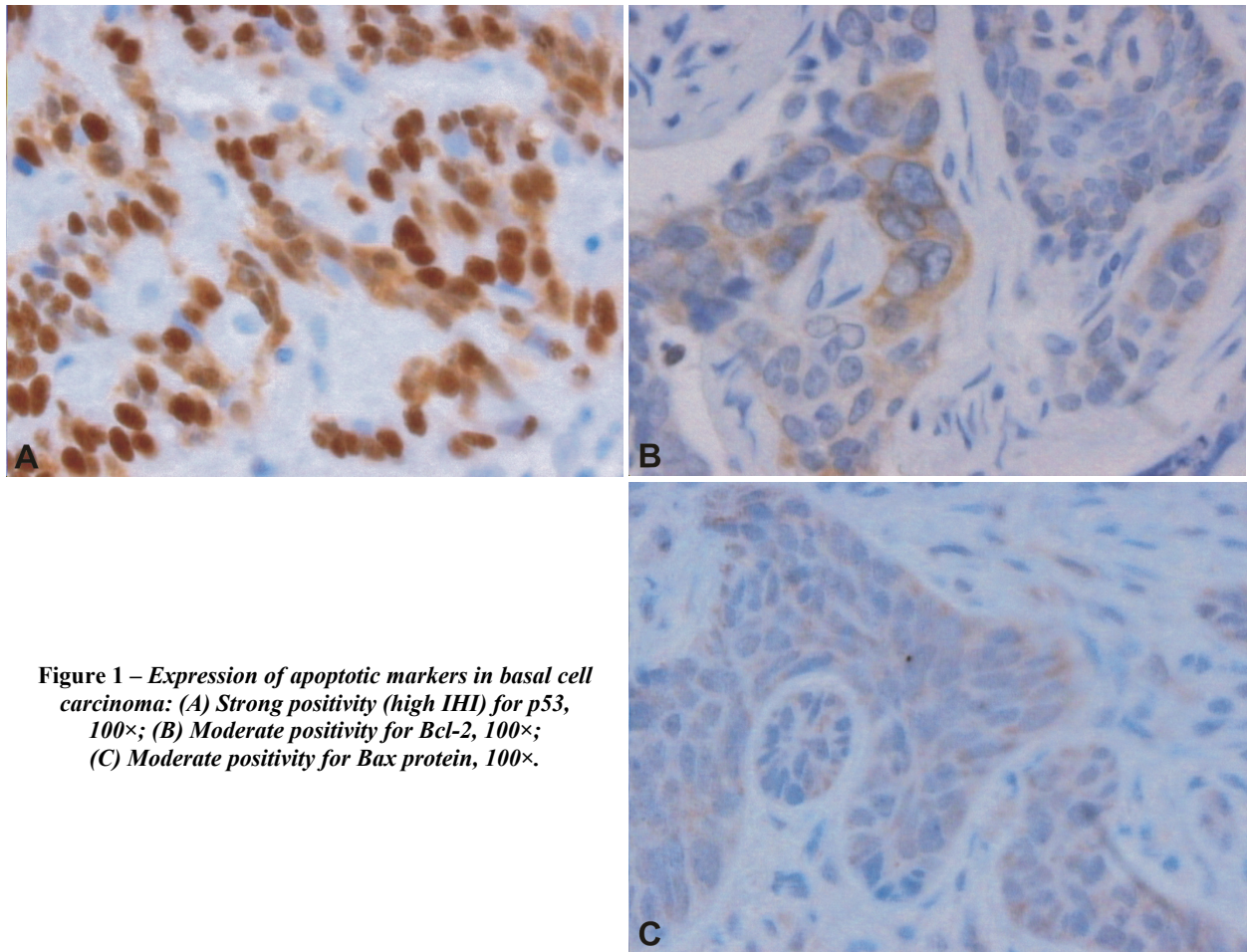


Figure 1 – Expression of apoptotic markers in basal cell carcinoma: (A) Strong positivity (high IHI) for p53, 100×; (B) Moderate positivity for Bcl-2, 100×; (C) Moderate positivity for Bax protein, 100×.

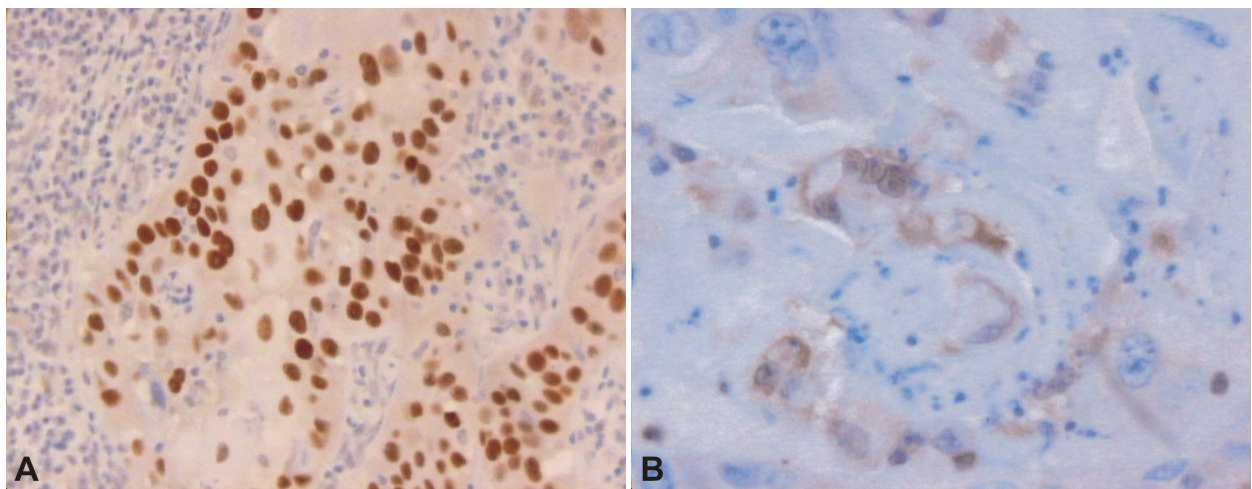


Figure 2 – Expression of apoptotic markers in squamous cell carcinoma: (A) Intense positivity for p53 in a moderate differentiated tumor, 50×; (B) Moderate expression of Bax protein, 100×.

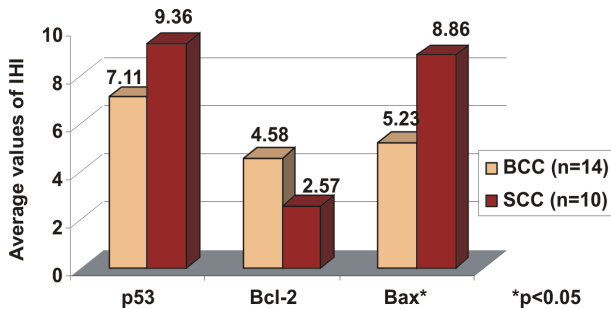


Figure 3 – Expression of p53, Bcl-2, and Bax in basal cell carcinoma versus squamous cell carcinoma.

Discussion

Photoinduced cutaneous carcinomas are frequent tumors that raise significant public health issues, despite their low mortality rate [2]. Regardless of their presentation, as either a benign proliferation (acanthoma) or a malignant tumor, solar imprint is always present. The origin of photo induced keratocytic tumors resides in epidermal or appendageal keratinocyte [2, 3], but it is their development on a photo aggressed tegument that might modify both their clinical and histological behavior. At the beginning of their evolution there is always a history

of lesion, due mainly to the solar radiation, though, other carcinogens may be involved (ionizing radiation, local irritants or arsenic ingestion) [2, 3].

The most common forms are represented by BCC (8090/3 *WHO* classification, 2003) [2] and SCC (8070/3 *WHO* classification, 2003) [2], the latter occasionally showing aggressive growth and metastatic potential [2]. In both neoplasms, the UV mark is present, even if they comprise a large spectrum of variants (depending on the type of lesion and stage of development) and a various clinical appearance. The triage of patients is based on clinical aspect, but the diagnosis is usually certified by routine histopathology [2, 3].

BCC is clinically diagnosed after its typically appearance as a pearly papule or ulcerated nodule, with telangiectasia [2, 4]. On routine stained sections, a common histological feature links the different types of basal cell carcinoma: strands of “germinative cells” (with a “palisade” arrangement at the periphery) that invade the dermis [2]. The superficial forms are clinically more aggressive, have a sclerotic stroma, and usually indistinct borders [2, 3]. In the underlying dermis, evidence of solar elastosis is always found.

SCC has a suggestive clinical pattern too, as shallow ulcers with elevated surroundings that present degrees of actinic alterations and a keratinous crust [2]. Most differentiated forms resemble normal keratinizing epithelium, while spindle-squamous cells characterize the undifferentiated neoplasm [2, 3]. After the degree of anaplasia and the extent of penetration these tumors can be graded in three large categories: “well”, “moderately” and “low differentiated” ones [2, 3].

In both cancer forms apoptosis is usually apparent [2, 3].

The term “apoptosis” was introduced by Kerr *JFR et al.*, in 1972, defining the cease of the vital functions of the cell during the cell cycle, due to an internal mechanism, which does not depend on any kind of external factors [5].

The regulation of apoptosis lies under the strict control of certain proteins, including p53 and Bcl-2 gene families. Bcl-2 proteins act as anti- or pro-apoptotic regulators, implicated in several cellular activities. Bax, the Bcl-2-associated X-protein, is the first described protein of the Bcl-2 gene family. It is an apoptosis promoter, entering into competition with proper Bcl-2 [6, 7]. Bax is involved in p53-mediated apoptosis, its expression being upregulated by this tumor suppressor protein [7]. By favoring cells to live longer, Bcl-2 is an anti apoptotic factor; yet, with time, its action has an opposite effect, the accumulation of genetic changes resulting in increased cells susceptibility of malignant transformation [7]. The production of tumors usually originates in the suppression of apoptosis (suggested by the negative correlation between the expression of Bcl-2 – apoptosis inhibitory protein, and Bax protein – apoptosis promoting protein) [8, 9]. Still, some tumors (*e.g.*, breast) do not behave as is generally consented, the expression of Bax being positively correlated with Bcl-2 and not correlated with p53, or histological grade [5, 7].

A tumor development, the histopathological subtypes and grades [10], depend on critical determinant, the ratio between proliferation and apoptosis rate, thus Bcl-2/Bax

ratio; according to data published in literature this is >1 in most cases of carcinomas (bronchus, colon, esophagus) [11].

p53 tumor suppressor gene, the guardian of the genome, is frequently mutated/inactivated at early stages of UV-induced skin cancers; its loss account for the overexpression of p53 protein that facilitates malignant progression [2, 12–15]. In normal conditions, high levels of p53 protein are induced by UV radiation (DNA-damaging agent) to repair the deoxyribonucleic acid (DNA); yet, excessive unrepaired DNA damage leads p53 protein to trigger apoptosis by activation of Bax and/or down-regulation of Bcl-2 expression [6, 12]. Identical p53 gene mutations were found in all types of photo induced cutaneous lesions [15, 16]. However, recent studies demonstrated increased levels of p53 protein in tumors without p53 gene mutations [15, 16] or different p53 mutations in BCC adjacent areas of clonally expanded non-neoplastic keratinocytes [13]. Experimental data acknowledged the fact that mutations of p53 can induce mutant-specific effects and that alteration of p53 function is critical in modeling gene expression patterns in several forms of cancer [17]. P53 mechanism is complex due to the numerous mutations in different tumor types and their disparate effects in clinical stages; furthermore, we can add the lack of randomized prospective studies, heterogeneous experimental designs, sample types or techniques [17, 18].

In order to clarify all the aspects regarding the p53 protein abnormal regulation and distribution in skin or other human cancers, or its associations with clinical outcome, further research is still needed [17–21].

The accumulation of p53 that might serve as a surrogate biomarker for TP53 mutation is under research focus, trying to confirm its use in the clinical diagnosis, prognosis and treatment of cancer [18]. Encouraging results concerning the survival prediction in skin cancer were associated with mutations of TP53 [18, 22]. In several retrospective studies, abnormal p53 protein expression and somatic mutations were correlated with poor survival or lack of response to therapy [17, 23–27]. Nevertheless, as Robles AI and Harris CC [18] have put it “it would be naïve to expect that a ubiquitously mutated gene would have the same clinical value regardless of context”. A parallel between TP53 mutation and worsened survival was found in head and neck squamous tumors, besides others (hematopoietic/lymphoid systems, breast and liver); yet many other neoplasm forms do not present this association (bladder, brain, lung, colon, esophagus, ovary) or they are scarcely studied (pancreas, prostate gland, rectum, and stomach) [18].

It is known so far that ultraviolet (UV) radiation is the major etiological factor in both BCC and SCC [2, 12, 15]. In skin carcinogenesis caused by UV radiation, induction of DNA damage represents the trigger; it will entail a multistep process including cell cycle arrest, DNA repair, mutation, and transformation [2, 12]. Sunlight serves as both a tumor initiator and a tumor promoter for p53-mutated cells by influencing their clonal expansion; thus, normal individuals will carry an important number of keratinocytes predisposed to cancer [13].

In BCC induction, the major target gene is p53. As a result, in human BCCs, p53 mutations are found in almost

56% of cases, even in small early lesions and the “UV genetic signature” (characteristic pattern CC to TT tandem mutations) is seen in 65% of them [2, 12, 21, 23]. In SCC, recent evidence [2, 12, 16, 22, 25, 28, 29] confirmed the importance of p53 for tumor evolution; in this case, the tumor suppressor protein p53 is generally overexpressed and inactivating mutations represent about 50%). A study performed on p53 protein, in a SCC located on the lower lip showed a positive immunohistochemical expression of p53 in 100% of the analyzed cases [8]; moreover, in other different studies, expression of p53 is significantly associated with tumor grade and survival status [16, 22, 24, 25, 28].

Thus, in our study, we detected highly expressed p53 protein in most BCC and SCC cases, this protein practically being an indirect malignancy marker. In several cases of BCC and SCC, scientific literature data confirms the constant presence of p53 protein; in other cases, only variable but high levels of p53 expression were obtained: 0–92% in BCC and 0–72 % in SCC [21–26, 30–32]. The increased p53 immunoreactivity found in skin carcinoma (BCC, SCC), implies the possibility to use p53 as a method of differentiation between malign and benign lesions [21, 24, 25, 27, 33]. In numerous BCC studies (Yan L *et al.*, 1999; Stratigos AJ *et al.*, 2005), high levels of p53-protein expression were acknowledged, in positive correlation with Bcl-2 and negative association with Bax [8, 34]. On the other hand, our data showed that the presence of mutant p53 protein in photo induced skin carcinomas was in a positive manner correlated with apoptosis and negatively with Bcl-2. Same result was reached by Ouhtit A *et al.* (2000), who came with a possible explanation stating that UV irradiation of mouse skin induces both apoptosis (mediated by the p53/p21/Bax/Bcl-2 pathway) and hyperplasia, also the two phenomena being closely linked and tightly regulated; in consequence the dysregulation of these two events can trigger cutaneous cancer development [15]. Studies on most aggressive types of breast carcinoma presented the same inverse relationship between p53 and Bcl-2 [7]. Kim MY *et al.* (2002) discussed the possibility that BCCs distinct features might be affected by both p53 molecular mutations (epidemiology and type) and their repair process (*via* UV-induced DNA lesions) [26].

The rate of tumor proliferation and apoptosis influences the tumor growth, but may also determine other aspects regarding the aggressiveness, metastasizing potential, prognosis, therapy efficiency [2, 3, 7].

As we have seen before, p53 protein was present in all BCC cases from our study, underlying the malignancy of the lesion. Superficial forms of BCC expressed a highly positive p53 protein but a low level Bcl-2 protein. Infiltrating forms of BCC had no expression of Bcl-2, and low to medium expression of Bax protein and p53. The decreased Bcl-2/Bax ratio may be associated with a better prognosis, according to the critical determinant rule, and it is concordant up to a point with tumor clinical profile, due to the BCC slow growth rate (high apoptotic activity) and lack of metastasis [2, 3]. We can also forward the idea that BCCs different histological types have different degrees of aggressiveness, because superficial forms of BCC, which express medium to

high positive Bax protein, might have a slower rate of growth than deep infiltrating forms [7, 12]. Moreover, it must be pointed out that p53/Bax/Bcl-2 pattern of BCCs deep infiltrating forms is identical with that found in many cases of breast cancer: a positive correlation between Bax and Bcl-2 and inverse relationship between p53 and Bcl-2 [7]. Regarding the correlations found between Bcl-2 expression and BCC histological variants, these are concordant with the results of a research on ovary that showed the same positive correlations between Bcl-2 expression and tumor types [10]. It is also interesting to mention the results of Rossen K *et al.* (1998) that observed that BCC does not immunohistochemically express detectable amounts of Bax [9]. The authors stated the possibility that BCC apoptosis does not involve Bax protein and that the apoptotic pathway either bypasses the regulation of the Bcl-2 gene family or may be regulated by less common members of this gene family [9].

In SCC, Bax protein was also positive in all cases. The Bcl-2/Bax ratio was sub unitary, due to the low expression of Bcl-2. Bax protein is better expressed when Bcl-2 has a lower expression level and codifies for a more intense apoptotic process in SCC than in BCC. In case of SCC aggressive or infiltrative forms, literature data show that Bax and Bcl-2 are not reliable diagnostic and prognostic markers. A threatening pattern might be caused by other genetic modifications or tumor progression events [12, 28], knowing that in SCC alterations of several signaling pathways (RAS, p53, p14, and p16) are present [12].

Hence, the possibility that these particular aspects described in our study may originate in other mechanisms involved in photocarcinogenesis and triggered by UV radiations [7, 21].

The absence of Bcl-2 positivity in half of our SCC cases and the discrete expression of this marker in BCC cases may witness a higher proliferative activity, exceeding apoptosis [7]. The expression model of the apoptotic markers and the critical determinant ratio, which decreases in BCC due to the increase of Bax expression or the decrease of Bcl-2 protein, may be characteristic for cutaneous photoinduced carcinomas [7]. The Bcl-2 positive BCCs (80–100%) are associated with a lower malignancy degree and a better prognosis than Bcl-2 positive SCCs (25–50%).

The values obtained in our study were statistically assessed. The average differences are statistically significant only in the case of Bax protein. The Bax average is smaller in BCC than it is in SCC, and the difference is statistically significant for $p < 0.05$. In case of p53 and Bcl-2 proteins, average differences were also obtained, but they were statistically insignificant, probably due to the small number of cases taken into study.

According to the illustrated chart, pro-apoptotic Bax protein quantifies apoptosis in both BCC and SCC, but the process is conditioned by Bcl-2 protein. Thus, Bcl-2 protein allows the initiation of apoptosis at a much higher level in SCC compared to BCC; in SCC, Bcl-2 protein is less expressed, not respecting the critical determinant rule. Even though there are differences regarding the degree of expression of the apoptotic markers, generally the values of Bcl-2/Bax ratio may be correlated with data from specialty literature [7, 35–37].

If in neuroblastoma, small cells pulmonary carcinoma or undifferentiated lymphomas, Bcl-2 high expression is associated with a bad prognosis and an aggressive evolution [7, 18], this rule does not apply for our cases of photoinduced cutaneous carcinoma. Paradoxically, even though BCC expressed moderate to high levels of Bcl-2, the tumor is generally considered to have a good prognosis, a less aggressive evolution without metastasis. SCC on the other hand, expressed lower levels of Bcl-2, but it is known to be more aggressive, with a faster evolution and may metastasize. These contradictory patterns may suggest that apart from the studied elements, there are still undetected or insufficiently studied factors that influence the tumoral behavior [18]. UV-rays, for example, trigger new mechanisms (oxidative stress, release of pro-inflammatory cytokines, molecular alterations in protein structure) overlapping those of cutaneous carcinogenesis process. Moreover, the cross talk between Bcl-2, Bax and p53 may regulate apoptosis in a specific manner, neoplasm dependent [7].

However, our findings are well correlated with extended studies (Basu A and Haldar S, 1998) on different malignancies (breast, ovary, testis) [7].

Recent advances show us only a glimpse from these yet not elucidating factors. The conversion of the Bcl-2 molecule from a cell death protector, to death promoter is one good sample. This occurs by substituting its homologous BH3 domain with the corresponding domain of Bax. The mutant Bcl-2 with the substitution of BH4 domain subdues apoptosis in mammalian cells and fails to rescue *e.g.*, yeast from Bax induced lethality [7]. Future research of these markers will require considering the genomic and epigenomic features of cancer [18].

Even if immunohistochemistry is a current method, often applied, it is not a good readout for mutations (*e.g.*, TP53 mutation). In order to raise its accuracy and therefore to provide reliable clinical information, it must be incorporated to panels of biomarkers for specific subtypes of cancer [18].

The quantification of the apoptotic markers correlated to other molecular markers may bring new research perspectives in the field of cutaneous carcinogenesis and may represent a scientific support for modern treatments.

☐ Conclusions

The immunohistochemical study performed on the p53, Bax and Bcl-2 proteins involved in the apoptotic process of photo induced cutaneous carcinomas (BCC and SCC) revealed certain specific features in their manner of expression, which do not correlate or respect the critical determinant rule, even though they are concordant with other literature data. The contradictory patterns of apoptotic markers may be induced by other occult mechanisms involved in cutaneous carcinogenesis and triggered by ultraviolet rays.

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