ORIGINAL PAPER

RJME
Romanian Journal of Morphology & Embryology
http://www.rjme.ro/

P53, p16 and Ki67 immunoexpression in cutaneous squamous cell carcinoma and its precursor lesions

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Abstract

The incidence of cutaneous squamous cell carcinoma (CSCC) and its precursor lesions argues the research for validating markers that would define the biomolecular mechanisms behind the potential progression and aggressiveness of these lesions. In this study, we analyzed the expression of p53, p16 and Ki67 in 91 cases of CSCC and its precursors in relation with the histological prognostic parameters. The quantification of the immunohistochemical reactions indicated superior significant differences for the studied markers in squamous cell carcinomas compared to keratinocytic intraepithelial neoplasia (KIN). P16 and Ki67 immunostaining for Bowen's disease were similar to those from poorly differentiated carcinomas. In this study, we found significant differences in p53 expression in relation to tumor grading and p16 expression in relation to tumor staging. Ki67 showed higher values in high-grade and advanced stage carcinomas. Positive reactions in preinvasive lesions as well as in CSCC support the sequential development and p53 and p16 involvement from the early stages of skin carcinogenesis.

Keywords: KIN, CSCC, p16, p53, Ki67.

Introduction

The squamous cell carcinoma is the second worldwide most common type of skin cancer and usually develops on sun-exposed skin areas. The cellular mechanisms underlying the initiation and progression of skin tumors are of great importance for understanding the disease's mechanisms and its prognosis.

Both actinic keratosis (keratinocytic intraepithelial neoplasia, KIN) and Bowen's disease are direct precursors with potential of progression to cutaneous squamous cell carcinoma (CSCC) [1–3]. Histopathology is the gold standard for diagnosis of actinic keratosis, but it is rarely performed due to the reduced risk of progression to squamous cell carcinoma, even in high-risk populations, the progression rate being less than 1% per year [4]. Despite the low rate of progression, studies suggest that around 60% of cutaneous squamous cell carcinoma arise from pre-existing actinic keratosis, reinforcing the idea that these lesions are closely related [4, 5]. Moreover, actinic keratosis presents tumor markers identical to those present in CSCC [6]. For the Bowen's disease, which is considered a carcinoma *in situ*, the potential for progression to invasive squamous cell carcinoma is estimated to 3–5% [7, 8]. The risk of progression is 10% for location at the neck skin level in comparison to other locations where the average risk is 4% [7, 9]. Although there are no accurate epidemiological data about the Bowen's disease, the incidence has increased in recent decades, the lesions being more common among Caucasians [7, 8].

In this study, we investigated the expression of some proteins involved in tumor progression and prognosis of CSCC and its precursor lesions, respectively p53 and p16 oncoproteins and the proliferation marker Ki67.

Materials and Methods

We investigated a number of 91 precursor lesions and CSCC operated in the Clinics of Dermatology and Plastic Surgery, Emergency County Hospital, Craiova, Romania. Surgical excision pieces were fixed in 10% formalin, processed by the technique of paraffin embedding and Hematoxylin–Eosin (HE) stained. Lesions classification was performed according to the lesional degree for actinic keratosis [10–13] and according to the tumor grade and stage for CSCC [14], as recommended in the literature.

Subsequently, we performed serial sections which were immunohistochemically processed using a detection system based on amplification polymer (polymer-HRP Histofine, Nichirei, Japan, ready to use, code 414151F). In order to visualize the reactions, we used the DAB (3,3'-diamino-benzidine) chromogen (code 3467, Dako) and for reactions validation, we used positive and negative external controls (by omitting the primary antibody) (Table 1).

We followed the semi-quantitative expression of p53 and p16 through an adapted scoring system that was awarded independently by two specialists, based on the staining intensity and percentage of positive cells [15]. Intensity of score was noted by 1 (low), 2 (moderate), and 3 (high). Cutoff value for positivity of reactions was set at 5%. The percentage of stained cells of was scored 1 (6–25% positive cells), 2 (26–50% positive cells), 3 (51–75%

positive cells), and 4 (>75% positive cells). Multiplication of the intensity score and of the percentage allowed us to calculate the final staining score (FSS), which was considered to be low for values between 1–4 and high for values of 6–12. Statistical analysis used average values and comparison tests (ANOVA, *chi*-square/Fisher and Pearson tests) in the automatically software SPSS10.

Table 1 – *Antibodies used: clone, dilution, retrieval and external positive controls*

Antibody	Clone/ Manufacturer	Dilution	Antigen retrieval	External control
p53	DO-7/Dako	1:50	Tris-EDTA buffer, pH 9	Tonsil
Ki67	MIB 1/Dako	1:100	Citrate buffer, pH 6	Tonsil
p16	DC-468/Dako	1:100	Citrate buffer, pH 6	HSIL uterine exocervix

EDTA: Ethylenediaminetetraacetic acid; HSIL: High grade squamous intraepithelial lesion.

Proliferation index of Ki67 (PI Ki67) represented the average number of marked tumor cells reported to the total number of cells on 10 microscopic fields (×40 objective), each field containing about 1000 cells.

Results

The analyzed casuistry included both precursor lesions for the CSCC represented in 28 cases by the actinic keratosis with variable degrees of severity and in two cases by the Bowen's disease and 61 cases of CSCC with various degrees of differentiation and tumor stages (Table 2).

Table 2 – *Distribution of the casuistry according to lesional degree and stage*

Lesional type		No. of investigated cases	Stage		
			I	II	III
Precursors lesions	KIN I	22	–	–	–
	KIN II	3	–	–	–
	KIN III	3	–	–	–
	Bowen's disease	2	–	–	–
Squamous carcinoma	Well differentiated	18	16	2	–
	Moderate differentiated	37	32	4	1
	Poorly differentiated	6	3	2	1

KIN: Keratinocytic intraepithelial neoplasia.

For the selected cases, we found a predominance of CSCC precursor lesions, respectively actinic keratosis and Bowen's disease to male patients, from the sixth decade of life. Cutaneous squamous cell carcinoma also prevailed in male patients in the seventh life decade.

Actinic keratosis cases presented positivity for all the investigated markers: p53 in 19 (67.8%) cases respectively for p16 in 16 (57.1%) cases and for Ki67 in 20 (72.1%) cases (Table 3). We have not found any differences in markers' expression depending to the morphological variant of the lesions.

The immunostaining for p53 was observed in 13 KIN I cases, in three KIN II cases and in three KIN III cases. The signal distribution was nuclear, frequently in basal and parabasal layers of the epidermis, with low or moderate intensity (Figure 1A). The average FSS of p53 in these cases was 1 for KIN I, 1.3 for KIN II and KIN III.

Table 3 – *Actinic keratosis: FSS / PI values of the investigated markers*

KIN degree	KIN I			KIN II			KIN III		
Markers	*FSS p53*	*FSS p16*	*PI Ki67 [%]*	*FSS p53*	*FSS p16*	*PI Ki67 [%]*	*FSS p53*	*FSS p16*	*PI Ki67 [%]*
FSS / PI	1	1.2	7±1.5	1.3	1.6	10	1.3	1.6	14.3±6

FSS: Final staining score; PI: Proliferation index; KIN: Keratinocytic intraepithelial neoplasia.

The immunostaining analysis of the p16 oncoprotein indicated positivity in 10 cases KIN I, in three cases of KIN II and in three cases KIN III. The signal distribution was nuclear and cytoplasmic, predominantly in basal keratinocytes, isolated or in small groups, as well as in rare cells from the upper layers of the epidermis, the immunostaining being heterogeneous (Figure 1B). The average FSS values for p16 in these cases was 1.2 for KIN I, and 1.6 for KIN II and KIN III.

The Ki67 immunostaining showed positivity in 15 KIN I cases, two KIN II cases and three KIN III cases. The immunostaining was nuclear in rare cells from the basal layer and only rarely in the upper layers of the epidermis (Figure 1C). The average value of PI Ki67 in these cases was 7±1.5% for KIN I, 10% for KIN II and 14.3±6% for KIN III.

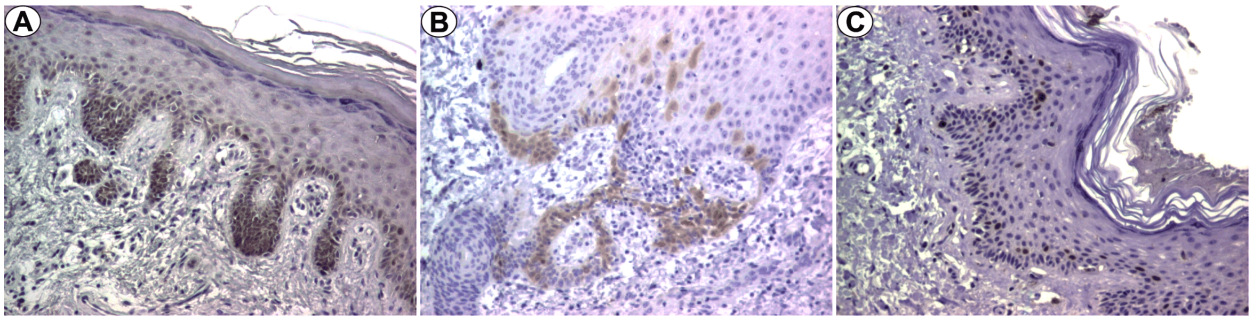

Figure 1 – *P53 (A), p16 (B) and Ki67 (C) immunostaining in KIN lesions, ×100.*

There was no statistically significant relation between the KIN degree and p53, p16 and Ki67 expression.

The two cases of Bowen's disease were positive for p16 and Ki67, both of which were negative for p53. Ki67 expression analysis revealed nuclear positivity, immunostaining being distributed throughout the entire thickness of the epidermis, with increased intensity and an average PI of 86% (Figure 2A).

Also, the nuclear and cytoplasmic immunostaining for p16 was distributed throughout the entire thickness

of the lesion, with an average FSS of 10.5 (Figure 2B, Table 4).

Table 4 – *Bowen's disease: FSS / PI values for the investigated markers*

Markers	FSS p53	FSS p16	PI Ki67 [%]
FSS / PI	0	10.5	85

FSS: Final staining score; PI: Proliferation index.

p16 and Ki67 immunomarkers for Bowen's disease were similar to those of low differentiated carcinomas with significant differences for both markers compared to KIN lesions (p=0.000, Fisher's exact test).

Squamous carcinomas were positive for all investigated markers in different proportions. Thus, the p53 marker was identified in 53 (86.8%) cases, p16 in 43 (70.5%) cases and Ki67 in 54 (88.5%) cases.

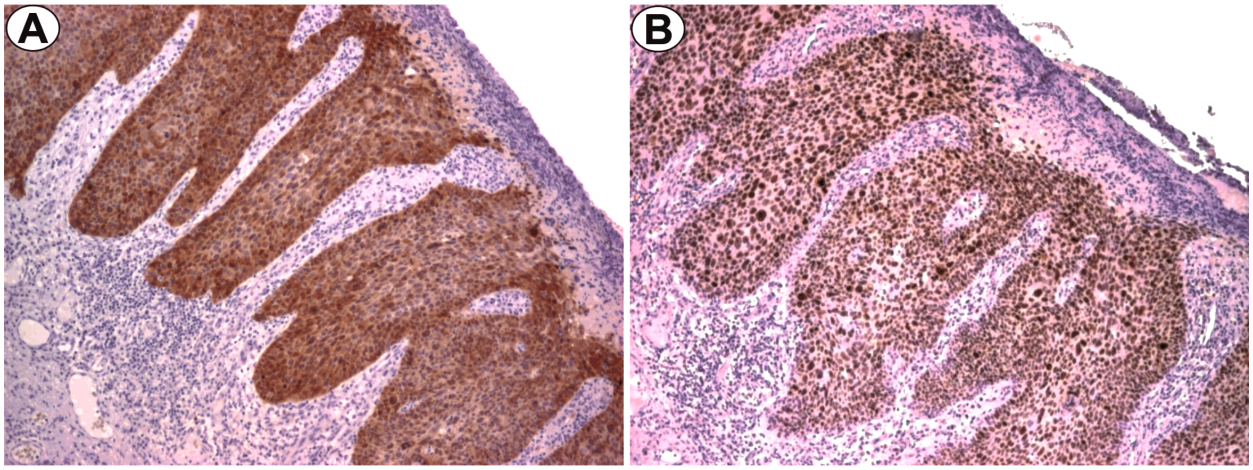

Figure 2 – *P16 (A) and Ki67 (B) immunostaining in Bowen's disease, ×40.*

We found positivity for p53 oncoprotein in all (100%) poorly differentiated tumors, regardless of tumor stage, in 34 (91.2%) of the moderate differentiated cases and in only 13 (72.2%) of well-differentiated tumors. Depending on the tumor stage, we identified positivity in all tumors in stage III and stage II and in 43 (84.3%) stage I tumors. In well differentiated tumors, p53 marker was present in nucleus of tumoral cells from the periphery of tumoral islands and only rarely isolated inside tumor cells islands, with low or medium intensity; the average number of labeled cells was 16.5±5.6, the average p53 FSS being 1.7 (Figure 3A). For moderately and poorly differentiated CSCC, p53 immunostaining was present in the nucleus, both at the periphery of the neoplastic tumor islands as well as randomly inside them, with moderate to high intensity; in these cases the average number of labeled cells was 42.9±12.3, respectively 68.3±20.8, and the p53 FSS average values were 4.8 respectively 7 (Figure 3B).

The statistical analysis indicated significant differences in the expression of p53 in CSCC compared to the KIN lesions (p=0.000, *chi*-square test), as well as in moderate/ poorly differentiated carcinomas compared to the well-differentiated lesions (p=0.000, *chi*-square test) (Figure 3C, Table 5). We did not find any differences in p53 expression in relation to the tumor stage (p=0.059, *chi*-square test).

Table 5 – *Cutaneous squamous cell carcinoma: FSS / PI for the analyzed markers*

Degree / Stage	Well differentiated			Moderate differentiated			Poorly differentiated		
	FSS p53	*FSS p16*	*PI Ki67 [%]*	*FSS p53*	*FSS p16*	*PI Ki67 [%]*	*FSS p53*	*FSS p16*	*PI Ki67 [%]*
Stage I	1.7	2.6	13.3± 4.5	4.8	4.7	40± 5.2	7	7.3	51.6± 7.6
Stage II	2.5	5	17.5	8.2	7.5	54.5± 3.3	12	9	67.5
Stage III	–	–	–	9	9	60	12	12	85

FSS: Final staining score; PI: Proliferation index.

P16 expression analysis revealed positivity for all poorly differentiated tumors, regardless of the tumor stage, for 29 (78.3%) of the moderately differentiated and only for eight (44.4%) well-differentiated tumors. Depending on the tumor stage, we remarked positivity in all (100%) stage III tumors and stage II tumors and for 33 (64.7%) of the stage I tumors. In well differentiated tumors, p16 marker was cytoplasmic and nuclear, heterogeneous, more intense in tumor cells at the island's periphery and only rarely, isolated in inside the tumor cells islands; the average number of labeled cells was 22.5±7.4, the average p16 FSS being 2.6 (Figure 3D). For moderately and poorly differentiated forms of CSCC, p16 was present both in the periphery and as well as random in islands of neoplastic cells with moderate to high intensity; in this cases, the average values of the number of labeled cells was 43.6± 8.5 and 61.6±14.3, respectively, and the average values of the p16 FSS were 4.7 and 7.3, respectively (Figure 3E).

The statistical analysis showed significant differences of the p16 expression in CSCC compared to KIN lesions (p=0.000, *chi*-square test) and CSCC found in II/III stage compared with those in stage I (p=0.029, *chi*-square test) (Figure 3F, Table 5). We did not find differences in p16 expression in relation to the degree of tumor differentiation (p=0.091, *chi*-square test).

We found positivity for Ki67 in all poorly and moderately differentiated tumors regardless of tumor stage and only in 11 of the well-differentiated tumors (61.1%). Depending on the tumor stage, we noticed positivity for all stage III and stage II tumors and 44 of the stage I tumors (86.2%). In well-differentiated tumors, the average PI of the Ki67 was 13.3% and 17.5% for stage I and II tumors (Table 5). Ki67 marking was present nuclear in rare cells at the periphery of the tumor islands and only rarely, isolated inside tumor cells islands with low or medium intensity (Figure 3G). Also, the immuno-staining was present in peritumoral inflammatory cells.

For moderately and poorly differentiated CSCC, Ki67 immunostaining was also nuclear, both in the periphery as well as random in neoplastic islands cells with moderate to high intensity; Ki67's PI average values in these cases were 42.1±7.3 for moderately differentiated carcinoma, respectively 62.5±14.4 for those poorly differentiated forms (Figure 3H).

Ki67 immunostaining was significantly higher in CSCC (p=0.000, ANOVA test) compared to KIN lesions, as well as in moderately/poorly differentiated CSCC and in its advanced stages compared with well-differentiated lesions (p=0.000, ANOVA test) (Figure 3I). In this study, the Pearson's test indicated a positive linear relation of the distribution of the percentage values for the three analyzed markers, which was statistically significant in the case of p53 and Ki67. We found no statistical association of markers' expression in relation to gender and age.

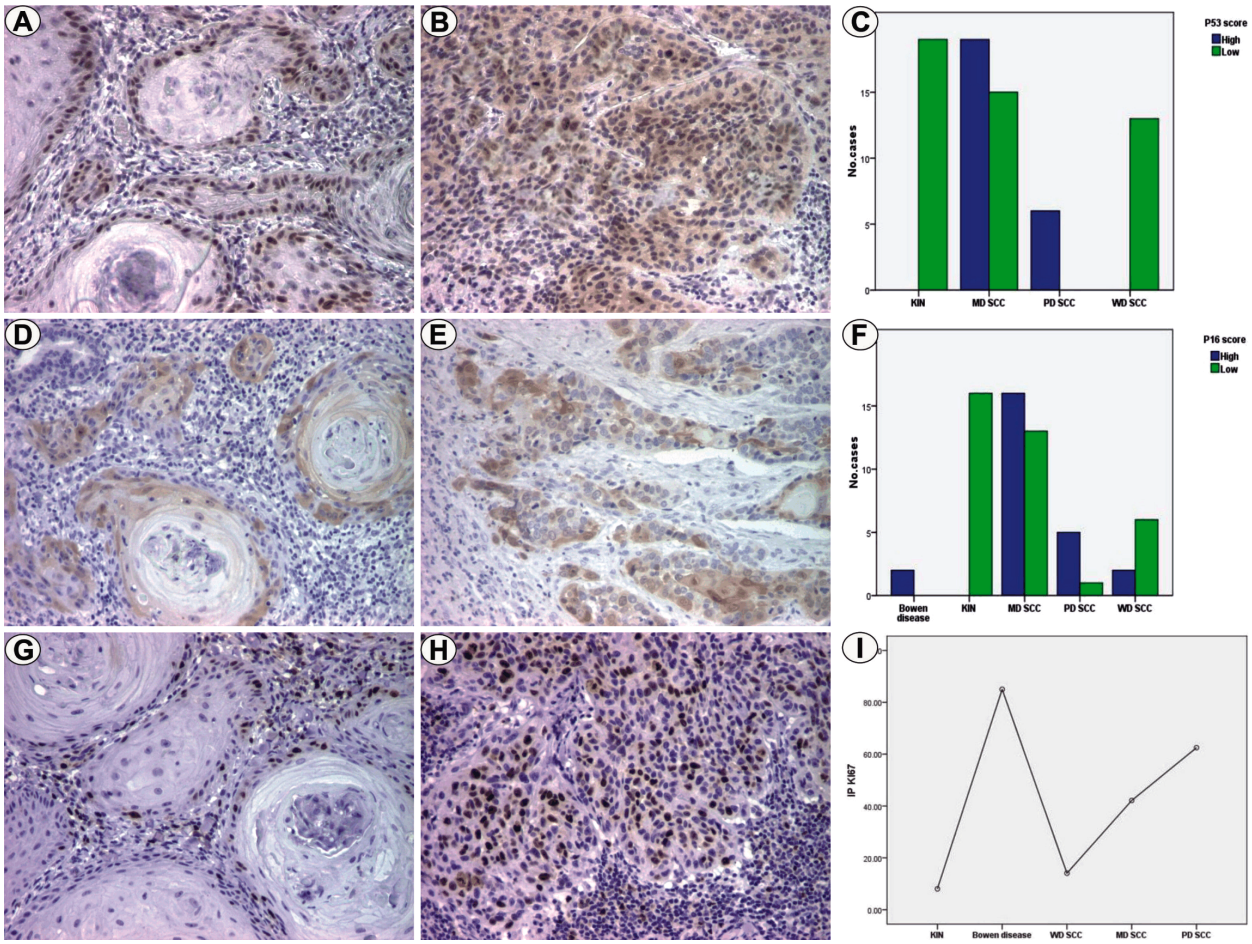

Figure 3 – ***Cutaneous squamous cell carcinoma (CSCC): (A) Well differentiated CSCC, p53 immunostaining, ×100; (B) Poorly differentiated CSCC, p53 immunostaining, ×100; (C) P53 immunostaining scores distribution; (D) Well differentiated CSCC, p16 immunostaining, ×100; (E) Poorly differentiated CSCC, p16 immunostaining, ×100; (F) P16 immunostaining scores distribution; (G) Well differentiated CSCC, Ki67 immunostaining, ×100; (H) Poorly differentiated CSCC, Ki67 immunostaining, ×100; (I) Ki67 immunostaining values distribution.***

Discussion

Like other types of cancer, CSCC development is a multistage process that involves the sequential acquisition of genetic alterations. Activation of proto-oncogenes and inactivation of tumor suppressor genes are critical molecular events that lead to neoplastic transformation. P53 tumor suppressor gene is a classic example of these genes, as it suffered changes in 50–90% of human malignancies, including skin cancer [16, 17]. Also, inactivation of the p16 through deletion, mutation or methylation has been observed in a wide range of human cancers [18–20]. Ki67 antigen, a non-histone protein with a high molecular weight, is usually accepted as the most reliable proliferating cells marker [17], which is correlated with tumor growth, metastatic potential and decrease of the overall survive [16, 21].

The investigation of p53 expression for the 91 analyzed cases revealed the presence of reaction in 67.8% of preinvasive lesions and in 86.8% cases of CSCC as well as the absence of expression in the two cases of the Bowen's disease that we investigated. The extensively presence of p53 expression in preinvasive and CSCC indicates that the oncoprotein plays an important role in skin carcinogenesis by intervening since the early stages of the disease, but the lack of expression in Bowen's disease may mean that other changes are needed. Studies in the literature report similar results [22–26]. Also, some studies have shown that only p53 positive actinic keratosis can progress to CSCC [23, 27]. However, other studies indicated significantly low differences in p53 expression for actinic keratosis associated with CSCC compared to those not associated, which would support the hypothesis of a high risk of malignant transformation for precursor

lesions with low p53 expression [24]. However, for some premalignant lesions, such as Bowen's disease, expression of p53 was not correlated with the Ki67 score [28]. In our study, p53 immunostaining showed significant differences in carcinomas compared with KIN lesions, and in moderately/poorly differentiated carcinomas and Bowen's disease compared to the well-differentiated lesions. In a study conducted by Talghini *et al.* p53 expression indicated significant differences in the cases of actinic keratosis, Bowen's disease and squamous cell carcinomas that were analyzed, p53 positive cell percentages being 26.6%, 41.8% and 54.6% [29].

P16 analysis indicated the presence of expression in all studied categories of lesions. Thus, we observed the positivity of the reaction in 22 (57.1%) of the cases of actinic keratosis, in both (100%) Bowen's disease cases and in 25 (70.5%) of the investigated squamous cell carcinoma. Blokx *et al.* reported while analyzing p16 expression in CSCC and actinic keratosis that high-grade KIN lesions expresses significantly more often p16 compared to low grade KIN and CSCC [30]. Hodges & Smoller reported the expression of p16 in 100% of actinic keratosis, staining being weak to moderate, located in the lower half of the epidermis, as well as p16 expression in 100% of squamous cell carcinomas with moderate to intense staining [3]. On the contrary, other studies have shown the absence of p16 marking in non-Bowenoid actinic keratosis [31] and, respectively, positivity in 60% of squamous skin carcinomas [32]. However, since most injuries of actinic keratosis do not progress to *in situ* or invasive carcinoma, the authors pointed out that overexpression of p16 appears to be necessary but not sufficient for tumor progression, in this transformation being needed the involvement of other factors [3]. In our study, p16 immunostaining indicated significant differences in advanced stages carcinomas and Bowen's disease lesions compared to KIN lesions. There are other studies that found high expression of p16 in Bowen's disease compared to KIN lesions [33], but no statistical association relationship between p16 and the skin carcinomas histological type or grade [32].

Ki67 expression analysis indicated the presence of Ki67 expression in all studied lesions categories. We observed the reaction positivity in 13 (72.1%) cases of actinic keratosis, in both (100%) Bowen's diseases cases and in 25 (88.5%) of the investigated CSCC. Also, immunostainig was significantly higher in the case of CSCC and Bowen's disease, compared to KIN as well as in advanced and high grade CSCC compared with the well-differentiated cases.

Ki67 expression has been reported in both precursor lesions as well as in CSCC. In CSCC, the Ki67 immunostaining PI values were between 15–84% [34], the highest expression being reported in low differentiated tumors, confirming the link, at least partially, between the aggressive behavior of neoplasia and cell proliferation [16, 17]. Although some studies have not identified any differences between Ki67 expression in preinvasive lesions and skin carcinoma [29, 35], or in relation to histological type and tumor differentiation degree [32], most authors stresses the usefulness of marker investigation for biopsy fragments.

Also, in this study, we observed a positive linear relation for the investigated markers. Following the analysis of Ki67 and p16 expression in different histological types of CSCC, Conscience *et al.* indicated p16 overexpression in 40% of CSCC, which is associated with high rates of Ki67 positivity [32]. One study concluded that the proliferative activity in squamous cell carcinoma is associated with p53 immunoexpression [36]. Other studies report that p16 and p53 are frequently overexpressed in CSCC and KIN and that p16 expression is independent from the p53 one, both proteins being part of parallel control pathways of keratinocytes response to DNA damage [30].

Conclusions

P53, p16 and Ki67 immunostaining are useful for differentiation between KIN lesions and CSCC, as well of the malignant aggressive lesions. Positivity of the analyzed markers in an increased proportion of preinvasive lesions and CSCC support the lesional continuum dynamic concept between these lesions as well as the intervention of two oncoproteins – p53 and p16 still since the early stages of skin carcinogenesis. On the other hand, the absence of their expression in a proportion of these lesions, suggests that in the progression from preinvasive to the invasive lesions is necessary the involvement of other biomolecular mechanisms.

Conflict of interests

The authors confirm that there are no conflict of interests.

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Received: December 4, 2015

Accepted: August 26, 2016