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Clinical, histopathological and immunohistochemical study of keratoacanthoma

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Abstract

Keratoacanthoma (KA) is an epithelial tumor of the skin, classically considered as having a malignant transformation risk of 15%; however, many authors and the new *World Health Organization* (WHO) Classification of skin tumors consider KA as an incipient variant of the cutaneous squamous cell carcinoma (SCC). The aims of the study were to assess the clinical, histopathological (HP) and immunohistochemical (IHC) aspects of the KA and the role of these factors in malignancy occurrence. The studied group comprises 194 patients diagnosed with KA or malignant KA, hospitalized in the Clinic of Dermatology, Emergency County Hospital, Craiova, Romania, between 2006 and 2019. There were 83 males and 111 females, aged 34 to 90 years, 57.21% of the patients being from the rural environment. The histopathology diagnosed 51 KAs and 143 malignant KAs (SCCs). Clinical diagnosis had a limited value in detecting the absence or presence of malignancy in the KA lesion, due to a low accuracy (36.08% and 29.89%, respectively) and specificity (23.07% and 27.02%, respectively); therefore, the HP exam of the surgical excision specimen has a paramount importance in establishing the diagnosis. IHC analysis revealed that the immunostainings for apoptosis-associated proteins and keratinocyte proliferative activity [p53, B-cell lymphoma-2 (Bcl-2), Ki-67 and proliferating cell nuclear antigen (PCNA)] provide some arguments to differentiate between KA and SCC in the studied cases. The correlation of clinical, HP and IHC data lead to an accurate diagnosis of KA; moreover, the clinical, HP and IHC data sustain the idea that KA is a particular form of well-differentiated SCC, which require an active therapeutic attitude.

Keywords: keratoacanthoma, squamous cell carcinoma–keratoacanthoma type, epidemiology, histopathology, immunohistochemistry.

Introduction

Keratoacanthoma (KA) is an epithelial tumor derived from pilosebaceous follicle, with a classically described 15% risk of malignant transformation, with clinical and histopathological (HP) features common with squamous cell carcinoma (SCC). To underline the impossibility of an accurate differentiation between KA and SCC using only usual HP criteria, some authors reclassified KA as SCC–KA type [1–3]. The nosology of KA remains thus unclear, and some authors suggested the term of 'abortive malignancy' [4].

Regarding the histogenesis of the KA, the immuno-histochemical (IHC) study of keratin and filaggrin expression confirms that KA originates from the outer root sheath cells below the infundibulum [4, 5]. KA that develops on the mucous membranes, subungual, palms and feet derives from the ectopic sebaceous glands and from the surface of the epidermis of those areas [4, 6]. Wagner *et al.* consider that KA of the lip has the origin from the cells associated with outer root sheath [7].

Although the etiology and pathogenesis of KA were not yet well defined, many factors are involved in the development and tumor progression: physical factors [ultraviolet (UV) and X-ray radiations, thermal factors], chemical carcinogens, traumatic injuries, preexisting lesions, genetic and immunological factors, human papilloma viruses, some drugs (Sorafenib, Infliximab) [2, 4, 8, 9].

Along with other keratinocytic precancers, KA it is also a strong predictor of the risk for developing other cutaneous carcinomas and melanoma [4, 10].

Aim

This study's objectives were to evaluate clinical, HP and IHC features of KAs and malignant KAs hospitalized in the Clinic of Dermatology, Emergency County Hospital, Craiova, Romania. IHC study aims were the assessment of p53, B-cell lymphoma-2 (Bcl-2), Ki-67 and proliferating cell nuclear antigen (PCNA) immunoexpressions, to determine their role in tumor progression and malignant transformation of KA, and the utility of these antibodies in the differentiation between KA and SCC.

Patients, Materials and Methods

A clinical, HP and IHC retrospective study was performed on a group of 194 patients diagnosed with KA, hospitalized in the Clinic of Dermatology, Emergency County Hospital,

Craiova, between 2006 and 2019. Surgical excision and HP exam were performed for all patients.

All patients have given their consent for surgery and subsequent HP and IHC exams.

The biopsy specimens were processed using Hematoxylin–Eosin (HE) staining, while the expressions of p53, Bcl-2, Ki-67 and PCNA were immunohistochemically assessed [Labeled Streptavidin–Biotin (LSAB)/Horseradish Peroxidase (HRP) method] (Table 1).

Table 1 – *Antibodies used for immunohistochemical staining*

Antibody	Clone	Dilution	Pre-processing	Producer/Code
p53	DO-7	1:25	Five cycles MW in citrate buffer	DAKO/M7001
Bcl-2	124	1:50	Seven cycles MW in Tris-EDTA, pH 9	DAKO/M0887
Ki-67	MIB-1	1:100	Five cycles MW in citrate buffer	DAKO/M7240
PCNA	PC10	1:100	Seven cycles MW in citrate buffer	DAKO/M0879

Bcl-2: B-cell lymphoma-2; EDTA: Ethylenediaminetetraacetic acid; MW: Microwave; PCNA: Proliferating cell nuclear antigen.

The IHC study was performed in the Research Center for Microscopic Morphology and Immunology, University of Medicine and Pharmacy of Craiova, Romania, on 10 clinically diagnosed lesions then confirmed by usual HP exam (HE staining).

To assess cell proliferation in KA, we used two immunomarkers represented by the anti-Ki-67 and anti-p53 monoclonal antibodies; the positive immunoreaction was interpreted from the point of view of their importance in differentiating benign from malignant HP forms, and as prognostic factors. For p53 and Ki-67, as a positive control we used sections of colon carcinoma with known high immunoreactivity for these markers; for negative control, the primary antibodies were replaced with saline.

For Bcl-2 immunostaining, the positive control were sections of breast carcinoma. For the quantification of p53 immunoreactivity, we used the method described of Lee *et al.*; immunoreactivity was graded according to the intensity of immunostaining as negative (0), weak (+), moderate (++), and strong (+++), and the extent of immunostaining was categorized as less than 5%, 5–50%, and greater than 50% [10]. For Bcl-2, we used a qualitative evaluation of immunolabeled cells distribution; we have considered as diffuse distribution when over 50% of the cells were positive and focal distribution when 5–50% of the cells were labeled as positive; the immunostaining reaction intensity was interpreted as low, moderate, or intense.

We assess the distribution of positive PCNA and Ki-67 cells in the dysplastic areas of epithelium and/or in epithelial hyperplasia. Distribution immunostaining was considered diffuse when over 50% of the cells were labeled as positive, respectively focal when 5–50% of the cells were labeled as positive (any intensity).

The image acquisition was made using Nikon Eclipse E600 microscope equipped with camera and the Lucia 5 image processing software. The processing of obtained images was done using Adobe Photoshop *ver.* CS6 in **.jpeg* format.

Statistical analysis was performed using the MedCalc software, *ver.* 18.10.2; to establish the statistical correlation between different variables (gender, age, living environment, lesion's topography, the clinical diagnosis, and the results of the HP exam), the χ^2 (*chi*-squared) test was used.

Results

Clinical and morphological data

There were 83 (42.78%) males and 111 (57.21%) females, while 111 (57.21%) patients were from the rural environment; rural women are more commonly affected (75 cases) compared to men (36 cases) – statistically significant (p=0.0008). We also have noticed that women are also more commonly affected (86 cases – 60.13%) in the group with malignant transformation of KA (143 cases), the ratio being 1.5:1 [p=0.16; odds ratio (OR) 0.82–2.98, 95% confidence interval (CI)]; 62 women and 26 men were from rural areas (p=0.001; OR 1.5–6.2, 95% CI). By comparison, even if the distribution by gender is not significantly different for malignant KA, when adding the environment, females from rural areas are significantly more affected by the malignancy compared with men.

Patient's age ranged from 34 to 90 years (the average 67.56±11.91 years), with a median of 70 years; hence, the most cases of KA were observed in the 8th decade (37.62% of cases), but an increase in incidence was noticed starting from the 6th (20.61%) and 7th (21.64%) decade.

The mean age of KA cases was 64.27±13.11 years (age ranged from 34 to 84 years), while for the group with malignant KA, the mean age was 68.72±11.22 years (range 35 to 90 years) (Table 2). The same tendency in the increasing number of malignant transformed cases with age is available, thus in the 8th decade 76.71% of the cases present malignant transformation (Figure 1). Using *t*-test comparison for means resulted in a statistically significant difference, with a more advanced age at diagnosis for cases with malignant KA (p=0.02).

Table 2 – *Main epidemiological characteristics of the group*

	F *n* (%)	M *n* (%)	U *n* (%)	R *n* (%)	Mean age ± SD [years]
KA	25 (22.52)	26 (31.32)	28 (33.73)	23 (20.72)	64.27± 13.11
Malignant KA	86 (77.48)	57 (68.68)	55 (66.27)	88 (79.28)	68.72± 11.22
Overall	111	83	83	111	67.56± 11.91

F: Females; KA: Keratoacanthoma; M: Males; *n*: No. of cases; R: Rural; SD: Standard deviation; U: Urban.

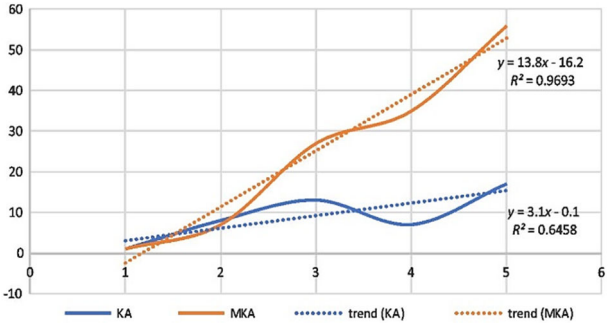

Figure 1 – *Age distribution and trends for keratoacanthoma (KA) and for malignant keratoacanthoma (MKA).*

The medical history ranged between three weeks to four years. For KA, history of the disease ranged between three weeks and 36 months (average 12.22±10.08 months), while for malignant KA, history ranged between one and 60 months (average 12.9±12.39 months), with no statistical significance (p=0.7).

The tumors' topography, both for benign and malignant KA is presented in the Table 3. Regardless of the gender and the presence or not of malignant transformation, in the studied group, the most frequent location of KA was at the cephalic extremity, but the location on the limbs was more often noticed in men; most KAs occurred on sun-exposed areas. Considering the tumor's location, the chest lesions present a significantly lower risk to be malignant compared with other locations (p=0.03; OR 0.12–0.95, 95% CI), but no other significant difference was noticed for the other topographies.

Table 3 – ***The influence of the lesion's topography and the risk of malignancy***

Topography	Cephalic region *n* (%)	Chest *n* (%)	Upper limbs *n* (%)	Lower limbs *n* (%)
KA	29 (24.16)	8 (47.05)	11 (26.19)	4 (26.66)
Malignant KA	91 (75.84)	9 (52.95)	31 (73.81)	11 (73.34)
Overall	120 (61.85)	17 (8.76)	42 (21.64)	15 (7.75)

KA: Keratoacanthoma; *n*: No. of cases.

The medium size of the lesion was 1.25±0.63 cm, ranging from 0.5 cm to 2.5 cm; for benign KA, the size ranged between 0.5 cm to 2.5 cm (average 1.4±0.67 cm), while for malignant KA, the size ranged between 0.5 cm and 2.5 cm (average 1.18±0.58 cm) (p=0.3, not significant).

Most of the cases (50 out of 51 cases – 98.03%) were solitary KA; the typical form of solitary KA was present in 47 (90.60%) cases; in only one case, we have diagnosed the eruptive form of KA (Figure 2).

In the studied group, there was no case with spontaneous regression of the lesion, in all cases patients relating an increasingly growing of the lesion over time.

The HP exam revealed the KA aspect in 51 (26.29%) cases, while malignant transformation of KA diagnosed in 143 (73.71%) cases.

In 37 KAs, the clinical diagnosis was histopathologically confirmed, while in 14 cases only the HP exam established the correct diagnosis. Out of 147 clinically suspected KAs, in 110 cases the HP exam established other diagnosis. Thus, for the KA clinical diagnosis, the accuracy was 36.08%, with a sensitivity of 72.54% and a specificity of 23.07, with a positive predictive value of 25.17% and a negative predictive value of 70.21%.

In malignant KA, only eight cases were clinically suspected of malignant transformation, while the HP exam revealed malignancy in 135 cases. In one case, clinically suspected as having malignant transformation, the HP exam established the benign KA diagnosis. Therefore, the accuracy of clinical diagnosis of malignant KA was 29.89%, with a sensitivity of 88.88%, a specificity of 27.02%, a positive predictive value of 5.59% and a negative predictive value of 98.03%.

The HP features of KA depend on the stage of the tumor's evolution (Figure 3).

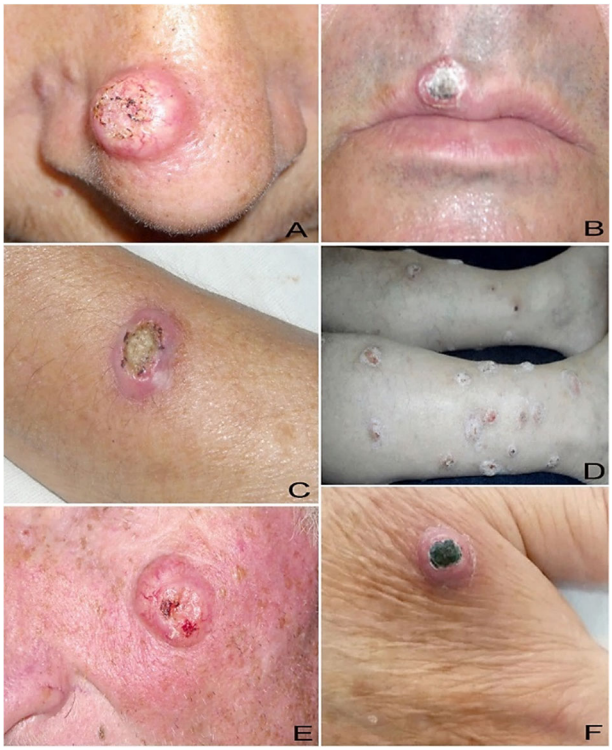

Figure 2 – ***(A) Typical solitary KA on the nasal pyramid; (B) KA on the upper lip mucosa; (C) Typical solitary KA on the left forearm with a tendency to central ulceration; (D) Eruptive-type, generalized KA; (E) KA on left cheek with malignant transformation to SCC; (F) Solitary KA on the dorsal right hand, traumatized, with malignant transformation. KA: Keratoacanthoma; SCC: Squamous cell carcinoma.***

In four cases, we have noticed a foreign body inflammatory giant cell reaction, which clinically correlates with the presence of lesions on the limbs, on chronic sun-exposed areas, the rural environment, and the 6th and 7th decade of life. The HP findings were consistent in 50 cases with solitary KA clinical form and in one case with multiple KA lesions.

HP exam of surgically excised lesions allowed the assessment of the percentage of malignancy, of the type of carcinoma and degree of tumor invasion (Table 4; Figure 4).

Table 4 – ***The distribution of malignant KA cases***

Malignant KA	*n*	%
KA with area of *in situ* carcinoma	4	2.82
KA with area of microcarcinoma	63	44.05
KA with area of well-differentiated SCC	40	27.97
KA with area of moderately differentiated SCC	31	21.67
KA with area of acantholytic SCC	5	3.49

KA: Keratoacanthoma; *n*: No. of cases; SCC: Squamous cell carcinoma.

Immunohistochemical results

Table 5 presents the results of the IHC study.

p53 immunoreactivity

The immunoexpression of p53 was detected in all cases of examined KA. In KA without malignancy, 60% of cases showed weak or moderate immunostaining, 40% of cases involved less than 5% of the cells; 60% of cases showed positive immunostaining in 5–50% of cells. In KA

with malignant transformation, in 60% of cases intensity of immunostaining was moderate and in 40% was intensely positive. Forty percent of cases involved over 50% of positive cells, 60% involved 5–50% of the cells. In the acantholytic carcinoma area, the p53 immunoexpression reached almost 70% (Figure 5, A and B).

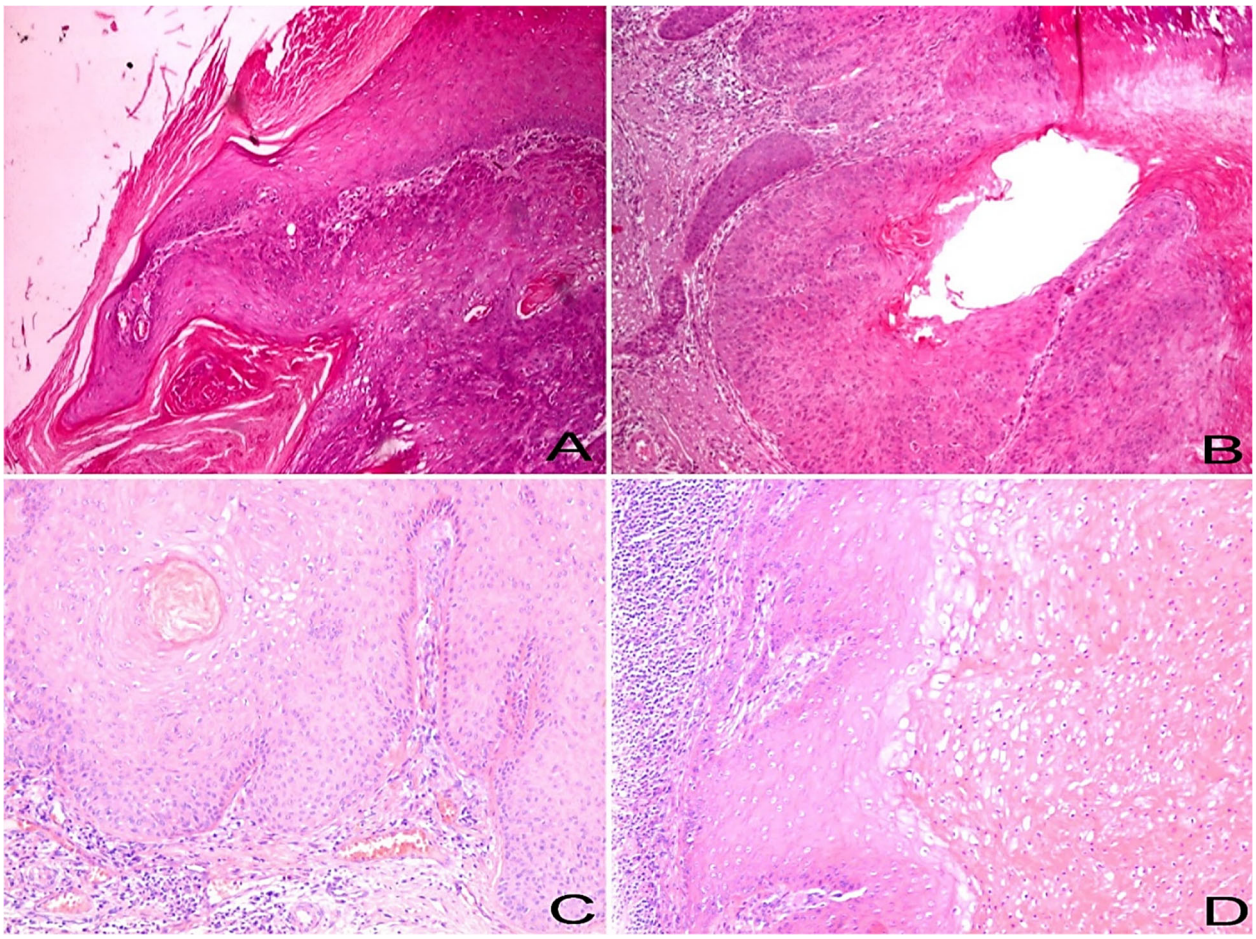

Figure 3 – *(A) Typical solitary KA: epidermal lip extended above the stratum corneum; (B) KA with dysplasia, adjacent epithelium shows acanthosis, hypergranulosis and premature cornification; large keratinocytes with eosinophilic cytoplasm, atypical cells and mitoses was observed; (C) KA with area of dysplasia, intraepithelial keratosis bodies; (D) KA with dysplasia and abundant inflammatory infiltrate in the superficial dermis. HE staining: (A, B and D) ×40; (C) ×100. HE: Hematoxylin–Eosin; KA: Keratoacanthoma.*

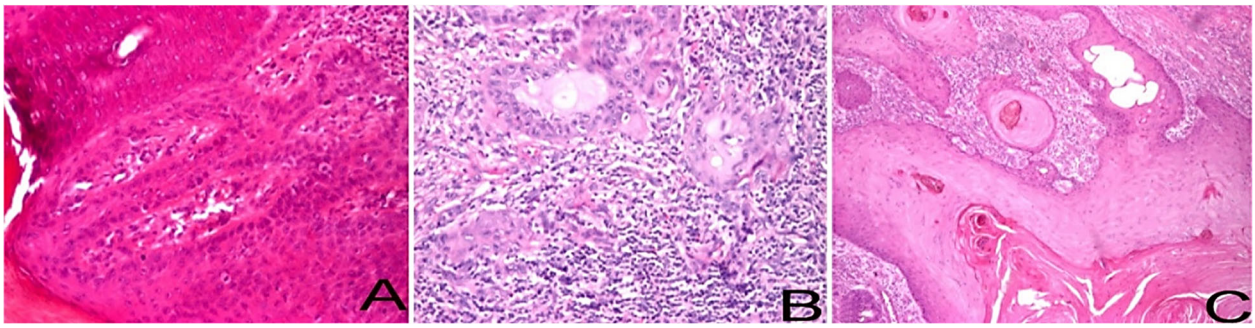

Figure 4 – *(A) KA with area of in situ carcinoma; (B) KA with area of microcarcinoma; (C) KA with invasive SCC. HE staining: (A and C) ×40; (B) ×100. HE: Hematoxylin–Eosin; KA: Keratoacanthoma; SCC: Squamous cell carcinoma.*

Table 5 – *The intensity and extent of immunostaining for p53, Bcl-2, Ki-67 and PCNA*

Immunomarker	KA								Malignant KA							
	Intensity of immunostaining				*Extent of immunostaining*				*Intensity of immunostaining*				*Extent of immunostaining*			
	0	*+*	*++*	*+++*	*0*	*<5%*	*5–50%*	*>50%*	*0*	*+*	*++*	*+++*	*0*	*<5%*	*5–50%*	*>50%*
p53	0	2	2	1	0	2	3	0	0	0	3	2	0	0	3	2
Bcl-2	2	3	0	0	2	2	1	0	1	3	1	0	1	2	2	0
Ki-67	0	3	2	0	0	1	3	1	0	4	1	0	0	2	2	1
PCNA	0	1	4	0	0	0	5	0	0	2	3	0	0	0	4	1

Bcl-2: B-cell lymphoma-2; KA: Keratoacanthoma; PCNA: Proliferating cell nuclear antigen.

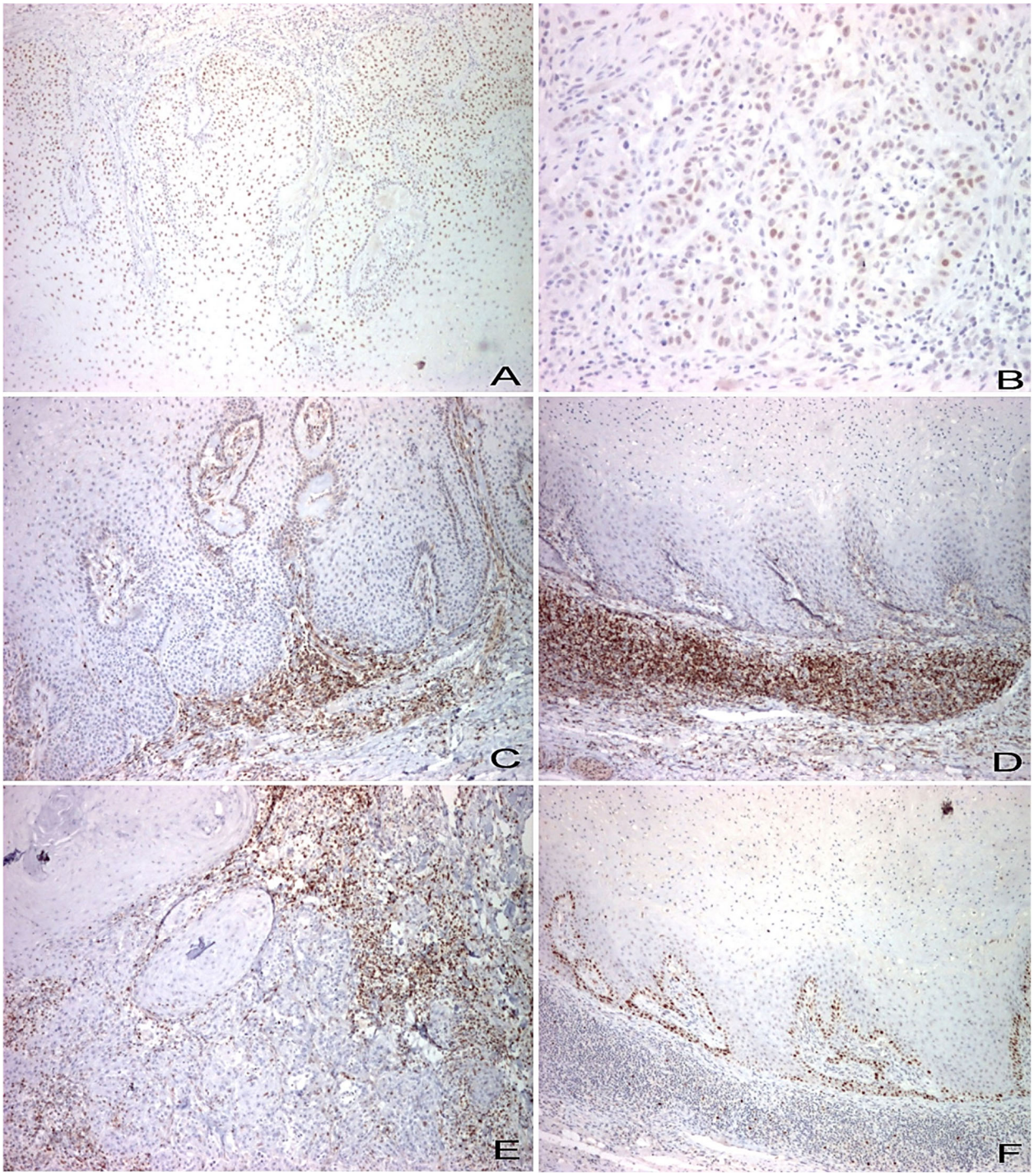

Figure 5 – *(A) KA with pseudoepitheliomatous hyperplasia: moderately positive diffuse p53 immunostaining in 1/2 and here and there in 2/3 of the epidermis; (B) Malignant KA: strongly positive diffuse p53 immunostaining in the acantholytic SCC; (C) KA with pseudoepitheliomatous hyperplasia: weakly positive Bcl-2 immunostaining in the basal keratinocytes, and intensely positive immunostaining in the inflammatory infiltrate of the dermis; (D) KA without pseudoepitheliomatous hyperplasia: weakly positive diffuse Bcl-2 immunostaining in the basal keratinocytes, and intensely positive immunostaining in the inflammatory infiltrate of dermis; (E) KA with area of acantholytic SCC: isolated weakly positive Bcl-2 immunostaining in the tumor cells and strongly positive in the inflammatory infiltrate of dermis and in the intratumoral lymphocytes; (F) KA without pseudoepitheliomatous hyperplasia: positive nuclear Ki-67 immunostaining only in the keratinocytes of the basal layer. LSAB/HRP method: (A, C–F) ×40; (B) ×100. Bcl-2: B-cell lymphoma-2; HRP: Horseradish Peroxidase; KA: Keratoacanthoma; LSAB: Labeled Streptavidin–Biotin; SCC: Squamous cell carcinoma.*

Bcl-2 immunoreactivity

Positive cytoplasmic immunostaining for Bcl-2 was noticed in seven out of the 10 studied KA. All KA that expressed Bcl-2 showed cytoplasmic immunostaining with diffuse, focal or isolated cells pattern. The intensity of immunostaining was weak in 85.71% of cases and moderate in one case; in 57.14% of the cases, the ratio of the immunopositive cells was less than 5%.

In KA without malignancy, the intensity of immunostaining was weak in 60% of cases, in 40% of cases immunostaining was negative. In 75% of the positive

cases, the proportion of positive cells was less than 50%, and in 25% of cases the proportion of positive cells was 5–50%. We did not notice significant differences between immunoexpression of Bcl-2 in KA with mild dysplasia and pseudoepitheliomatous hyperplasia; in all cases, the immunostaining was weak or moderate, diffuse positive in basal keratinocytes and strongly positive in inflammatory infiltrate of the dermis.

In malignant KA, positive immunostaining for Bcl-2 was noticed in 80% of cases; 75% of the cases presented weak positive diffuse immunostaining in basal keratinocytes, weak positive isolated immunostaining in tumor cells, negative immunostaining in carcinoma area and strongly positive in the inflammatory infiltrate of the dermis and intratumoral lymphocytes.

We did not notice significant differences in terms of the intensity and the proportion of positive cells immunostaining between malignant and nonmalignant cases. We have noticed in lesions with intense inflammatory infiltrate that Bcl-2 immunoexpression was intensely positive in lymphocytes in the upper dermis and, in some cases, in intratumoral lymphocytes (Figure 5, C–E).

Ki-67 immunoreactivity

Ki-67 positive nuclear immunostaining was noticed in all examined cases. The immunoexpression was weak and moderate in 60% of KAs, 75% of the cases having less than 50% positive cells. In malignant KA, in 75% of cases we have noticed the same proportion of positive cells with weak positive immunostaining.

In KA without pseudoepitheliomatous hyperplasia, we have noticed positive immunostaining only in keratinocytes from periphery of the tumor; in KA with pseudoepitheliomatous hyperplasia, the number of positive cells increased up to 1/3 of the epidermis, with diffuse distribution. In malignant KA, we found a diffuse immunostaining in about 30% of the tumor cells from the carcinoma area (Figures 5F and 6A).

PCNA immunoreactivity

Proliferating cell nuclear antigen (PCNA) immunoexpression was found in all cases, the intensity of immunostaining being moderate in 70% of the cases and weak in 30% of the cases; in 90% of the cases, the extent of the immunostaining was less than 50%. We have noticed a peripheral pattern of immunostaining in forms of moderate dysplasia; in forms of moderate dysplasia and pseudoepitheliomatous hyperplasia, the proportion of positive cells increase, with diffuse distribution, affecting between 1/2 and 2/3 of the epidermis. In the presence of carcinoma, we have noticed a diffuse immunostaining distribution in the carcinoma area (Figure 6, B–D).

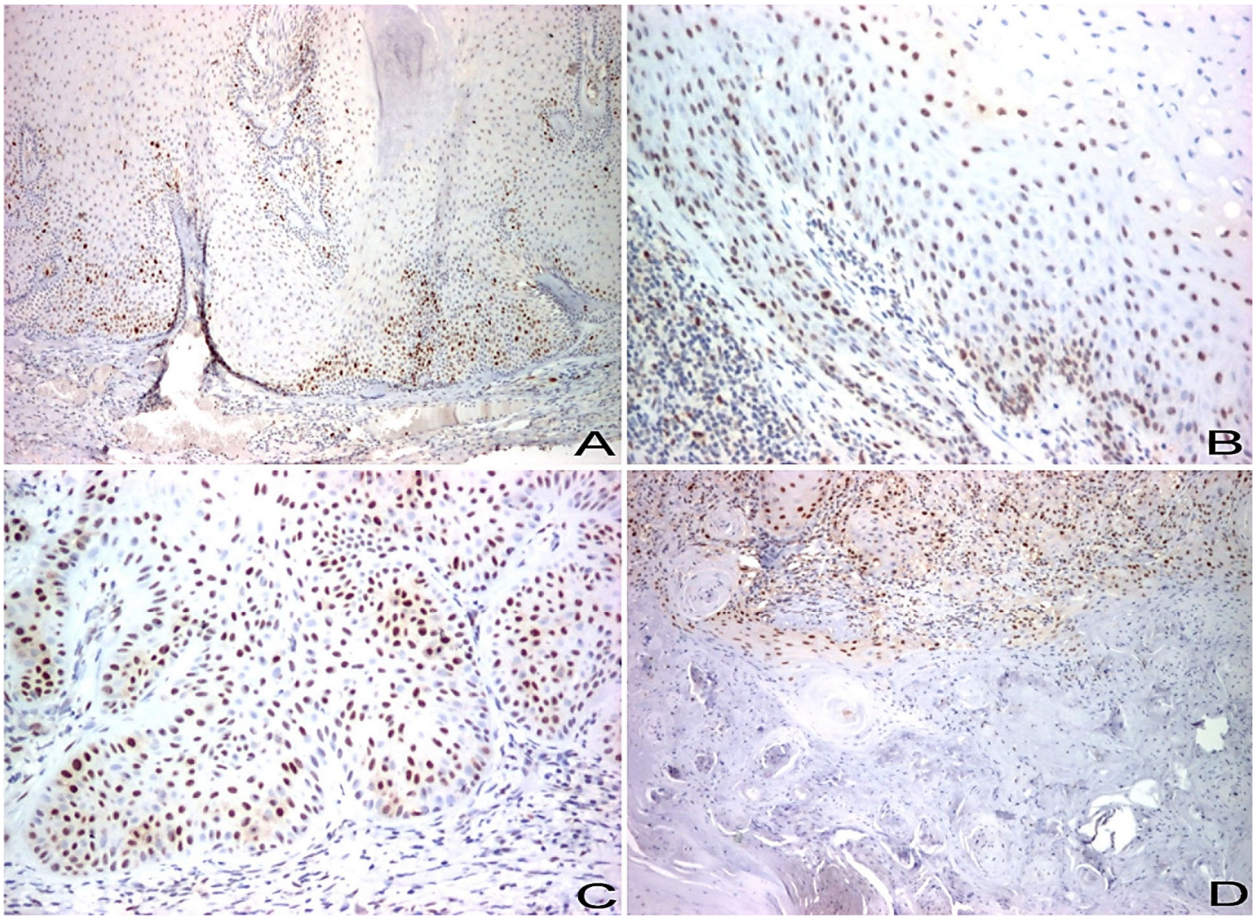

Figure 6 – ***(A) KA with pseudoepitheliomatous hyperplasia: positively diffuse nuclear Ki-67 immunostaining in the basal and parabasal layer to 1/3 of the epidermis; (B) KA without hyperplasia: positively PCNA immunostaining in the basal and parabasal layer (lower 1/3 of the epithelium); (C) KA with pseudoepitheliomatous hyperplasia: positively diffuse PCNA immunostaining bottom 1/2 of the epithelium; (D) Malignant KA: positively diffuse PCNA immunostaining in the area of acantholytic SCC. LSAB/HRP method: (A) ×40; (B, C and D) ×100. HRP: Horseradish Peroxidase; KA: Keratoacanthoma; LSAB: Labeled Streptavidin–Biotin; PCNA: Proliferating cell nuclear antigen; SCC: Squamous cell carcinoma.***

Discussions

Clinical and histopathological aspects in keratoacanthoma

KA was classically considered as an epithelial benign tumor; however, due to the possibility of malignant transformation controversies concerning the classification of KA as a cutaneous precancer, pseudo-cancer or even a specific form of early SCC still exist [3, 4, 11, 12].

Since 2018, in *World Health Organization* (WHO) Classification of skin tumors proposed of the *International Agency for Research on Cancer* (IARC), KA is considered like a well-differentiated variant of SCC [13].

Positive diagnosis of the lesion is based on clinical suspicion, but we have found that history, tumor size and even tumor aspect has little influence on differentiation between malignant and non-malignant lesions; the only statistically significant arguments were in favor of age (an increasing incidence of malignancy with age) and gender (women from rural areas appear to be at risk of malignancy compared to men).

In our study, the HP evaluation of the excised KAs established the absence of malignancy ("benign" KA) in only 26.28% of the cases; in all other cases, the pathologist found malignant transformation in the KA lesion. Therefore, we have found a low accuracy for the clinical diagnosis, both for the KA and malignant transforming KA (36.08% *vs* 29.89%), the clinical diagnosis having a very low specificity in predicting the presence or the absence of malignancy (23.07% *vs* 27.02%). With such low positive predictive values (25.17% *vs* 5.59%), the clinical diagnosis cannot predict the lesion's evolution; therefore, all KAs require HP confirmation to exclude malignancy.

Moreover, for an accurate HP diagnosis, the KA requires examination of the entire lesion since the diagnosis is based on KA architecture and cell characteristics. Consequently, for clinician is necessary to excise the entire lesion; in cases in which this is not possible, a fusiform excision should be performed including altogether the center of the lesion, tumor's periphery, central area and deep portion with subtumoral tissue. Biopsy samples of the surface of the lesion are not useful and recommended because HP changes of the tumor base are of foremost importance to differentiate between KA and invasive SCC [4, 14–16].

HP features of KA depend on the evolutive stage of the tumor; HP features in favor of KA diagnosis are the presence of a relatively symmetric epithelial neoplasm, with a large irregular crater, filled with keratin and epithelial "lipping", as well as the lack of prominent atypia and mitotic figures [3, 14, 15].

In our histologically assessed lesions, we have noticed large keratinocytes with eosinophilic cytoplasm, atypical cells, and mitoses. Area of pseudoepitheliomatous hyperplasia was noticed around and at the base of the crater that formed folds inside the crater and the adjacent dermis. Depending on the section, those extensions presented as buds, cords, or isolated islands, centered by orthokeratotic pearl. In the upper and middle dermis, we noticed a lymphoplasmacytic and histiocytic perivascular inflammatory infiltrate, sometimes abundant; this inflammatory infiltrate was noticed in all cases, being most abundant in the underlying tumor. In 6.89% of the cases, we have seen a foreign body inflammatory reaction, with giant cells, that clinically correlated with the presence of lesions on the limbs, on chronic sun-exposed sites, in the rural people at the 6th and 7th decade of life. The reactional inflammatory infiltrate occurs in stromal ever since the formation of the crater. As the tumor grows, infiltrate becomes more abundant, especially under the lesion, consisting of lymphocytes, histiocytes and a variable number of neutrophils, plasma cell and eosinophils.

During the involution stage, the infiltrate invades the base of the tumor and organizes intraepidermal neutrophilic and eosinophilic microabscesses [6, 14]; the involution phase seems to be immunologically mediated, cytotoxic T-lymphocytes (predominantly) invading the tumor [17].

Recently, Aguiar *et al.* have reported a case of *centrifugum marginatum* KA associated with accumulation of mast cells located in the center of the tumor, suggesting the involvement of mast cell-like modulator directly or indirectly in its pathogenesis [18].

In our study, we have not found KA in spontaneous regression phase. Perivascular and perineural invasion was not noticed in the studied cases, these being described especially in wide and deep infiltrative lesions of the head and neck, and the prognosis was not affected by this process [19]. Vascular invasion was not associated with metastatic disease [20].

HP differential diagnosis includes adnexal tumors and especially SCC, but the differentiation between these two neoplasms may be difficult in some cases [4, 14, 15, 21]. Cellular and nuclear atypia are more common in SCC, with its basal membrane discontinuous.

Venkei & Sugar, analyzing the HP aspects, classifies KA in three categories: KA type A and B shows benign HP and cytological characters, while KA type C (proliferating KA) may be considered as an early SCC [4, 14].

The term 'squamous cell carcinoma–keratoacanthoma type' was introduced for otherwise classical KAs that reveal a peripheral zone formed by squamous cells with atypical mitotic figures, hyperchromatic nuclei, and loss of polarity of some degree. These marginal cells may also penetrate the surrounding tissue in a more aggressive pattern. This definition indicates the HP difficulties of distinguishing benign KA from SCC, as well as the aggressive nature of the KA in some cases, with locally destructive and metastatic potential [1].

There is still controversy regarding the metastatic potential of KA, reported by some authors, but recent studies add arguments that deny this behavior [15, 20, 22]. Considering the potential of malignancy, some authors believe that KA is a low-grade malignancy SCC, with a rare tendency to metastasize [14, 23].

The transformation of KA into SCC can be spontaneous, especially if the lesion is located on senile skin or with actinic degeneration, or because of immunosuppression. The actual percentage of malignant transformation is difficult to be establish, ranging between 15–50% of the cases, influenced by the HP interpretations. Particular attention should be given to relapsed KA lesions that seem to bear a high percentage of transformation in SCC [14].

In this study, we report a higher percentage of KAs transforming into SCC, of 73.71% of cases. We support the idea that the tendency of KA to regress spontaneously over a period of several months should be considered only as a possibility, but the therapeutic approach must but an active one, and not expectation. Most of the cases living in rural environment are associated with chronic sun exposure and recurrence of cutaneous microtraumas. The patients aged over 70 years old have an increased malignancy risk of KA. Only in 9.09% of the cases of malignant KAs the clinical aspect of the lesion was suggestive of malignancy, in most of the cases only the HP diagnosis demonstrating the progress of KA into a SCC.

HP aspects noticed in the group of patients with malignant KAs located on chronic sun-exposed skin show that these tumors tend to persist and progress into invasive SCC. Arising of the invasive process is announced by the emphasizing of the pseudoepitheliomatous hyperplasia, the presence of cells with atypical mitosis and hyperchromatic nuclei, the appearance of discontinuities in the basement membrane and the increase of inflammatory reaction. We have noticed that there is no positive correlation between the appearance of the tumor, time of evolution, patients' gender and arise of malignancy. The patients only have in common the old age and lesions located on chronic sun-exposed skin. Age and the immunosuppression induced by UV exposure are factors that interfere with the malignancy.

KA with areas of *in situ* carcinoma represented 2.82% of the cases with malignant transformation. Characteristic of these lesions was the presence of areas of dysplasia and carcinoma *in situ*, the basement membrane is intact, in the dermis observing a chronically lymphoplasmacytic inflammatory infiltrate. Malignant transformation was not suspected clinically, lesions having an evolution of two to six months, 1 cm in size, but they were ulcerated and often traumatized. The lesions were located on the cephalic extremity and back of the hand, at people with chronic sun exposure, with other precancerous skin lesions associated (actinic keratoses, actinic cheilitis).

KA with areas of microcarcinoma represented 46.85% of the malignant cases, in 61.19% of cases diagnosed in women, 65.85% of them coming from rural areas. HP appearance of early SCC lesions corresponds with giant KA clinical aspect, localized on the back of the hand and evolving for about a year. In six cases, the clinical diagnosis was basal cell carcinoma (BCC) or SCC; for the remaining cases, the clinical diagnosis was KA, cutaneous horn, or papilloma. The lesions evolution ranged between one month and two years, most of them being located cephalic (55.22%).

HP discovery of the microcarcinoma area was not clinically correlated with certain objective or subjective aspects, suggestive of malignancy, in most of the cases.

In cases with microcarcinoma area, we have noticed that the limits of the tumors' extensions are less obvious, with irregular appearance and more abundant inflammatory infiltrate; in one case, we noticed an intense inflammatory reaction to keratin. The images with higher resolution showed cells with increased volume and irregular outline, large and hyperchromatic nuclei, bi- or trinucleate cells, few mitoses.

KA with well-differentiated SCC areas represented 27.97% of all malignant cases. This type of KA occurs in both sexes on photo-exposed skin, patients age ranging from 35 to 90 years; the lesions had between 0.5–2.5 cm, some of them ulcerated, and evolving between one month to one year. The level of invasion is different in the middle and deep dermis, or hypodermis; in two cases, located on the lip, the invasion was until the deep dermis, to the proximity of the striate muscle fibers. Inflammatory infiltrate was abundant, sometimes we have noticed an inflammatory reaction with foreign body giant cells. The surgically resection limits were clear in 100% of the cases, in one case the limit of surgical resection corresponding to an area of pseudoepitheliomatous hyperplasia.

KA with moderately differentiated SCC areas represented 21.67% of the malignant cases; the lesions had 0.5–2 cm in size, with evolution ranging between one month and one year. The histopathology revealed the aspect of the KA with area of invasive SCC and abundant stromal inflammatory infiltrate. The invasion level of SCC was different, in the middle and deep dermis (11 cases) and in hypodermis (nine cases); in two cases, the invasion could not be determined precisely, the tumor fragments being superficially harvested. The surgical margins were invaded in two cases. In two cases, the HP of KA with moderately differentiated SCC corresponded clinically with diagnosis of nodular BCC and SCC, respectively, in other cases the clinical diagnosis was KA; 30% of the patients associated other cutaneous carcinomas. Lesions were noticed in both sexes, aged between 60 and 85 years, with no correlation between lesion size, the period of evolution and the malignancy occurrence.

KA with areas of acantholytic SCC represented 3.49% of the cases with malignant lesions. These lesions arose more frequently in men (80%) with chronic sun exposure, phototype II skin; the lesions evolved for about a year, were located on the back of the hand or frontal region, 1–1.5 cm in size, clinically characterized by infiltration and ulceration. Invasion was present in the deep dermis.

In 12.5% of KA with microcarcinoma and 20% of KA with moderately differentiated SCC, the invasion could not be determined precisely, because biopsy specimens were incomplete.

The margins of the surgically excised specimens were without tumor invasion in 100% of the cases with KA with microcarcinoma (in one case the limit of surgical resection corresponds to areas of pseudoepitheliomatous hyperplasia), and 80% of cases of KA with moderately differentiated SCC.

The immunohistochemical results in keratoacanthoma

Although several IHC stainings may be useful to differentiate KA from SCC, the utility of IHC markers for differentiation of KA from SCC is still controversial because there can be no certainty about a pattern to differentiate these tumors [15].

In the context of the need for criteria to differentiate between KA and SCC, the p53 immunoexpression in both lesions has been the subject of numerous studies. In both lesions, p53 immunoexpression was present, but the intensity and extent are higher in SCC compared with KA. The presence of p53 is an indicator of immaturity and proliferative capacity that disappears in cell differentiation. The difference of immunostaining between KA and SCC, according to some authors, is only in terms of intensity and extension of immunoexpression and this is due to the different proliferative activity and degree of differentiation between these two lesions. KA with nuclear atypia and intensely and extensively p53 immunostaining show a characteristic similar pattern to SCC [24].

Joshi *et al.* have reported a high frequency of tumor protein p53 (*TP53*) gene mutations (39.5% of cases) in studied KA that is associated with increased p53 levels, indicating a role for the p53 protein in KA development [25].

Lee *et al.*, assessing the p53 immunoexpression in KA, SCC and pseudoepitheliomatous hyperplasia have reported positive p53 immunostaining in 78.80% of cases (33 KA cases studied), 84.6% of cases having weak or moderate immunostaining, with 96.20% of cases involving more than 50% of the cells. The distribution of immunostaining was peripheral, particularly affecting the basal cells of papilloma's extensions that extend below the crater. In SCC cases, p53 was positive in 75.5% of cases [26].

In our study, all analyzed KA (10 cases) expressed nuclear p53. In cases without malignancy, 80% of the cases showed weak and moderate intensity of immunostaining, 40% of cases showed less than 5% positive cells, 60% of cases were positive in 5–50% of the cells. These results suggested that *p53* gene mutations in KA are low, so p53 protein maintains its role in apoptosis and even tumor regression. In malignant KA, in 60% of the cases showed moderate immunostaining, in 40% of the cases immunostaining was strongly positive, 40% of cases were more than 50% positive cells, 60% of the cases were positive in 5–50% of the study group. In malignant KA, the immunoexpression has a similar pattern with SCC, which reflects the presence of keratinocytes clones with increased proliferative characteristics and invasive, aggressive potential. The p53 immunoexpression cannot differentiate KA from SCC but is useful in highlighting a keratinocyte population that may have a potential for aggressive growth. The p53 immunoexpression is linked to malignant progression as an immunomarker of tumor aggressiveness and less than oncogenesis itself.

A particular importance in determining the apoptotic status of the cell has the relationship between Bcl-2 and Bcl-2-associated X (Bax), oncoproteins of Bcl-2 family. In the presence of Bcl-2, the apoptotic process is blocked, and the cell is pushed to proliferation. Bcl-2 oncoprotein is normally expressed in basal keratinocytes and shows expression changes in skin cancers. The study of Bcl-2 immunoexpression in KA showed the cytoplasmic positive immunostaining for Bcl-2 in seven (70%) of the 10 studied cases; all biopsies that expressed Bcl-2 positive cytoplasmic immunostaining had distribution in isolated cells, focal or diffuse. The immunostaining intensity was weak in 85.71% of the cases and moderate in one case, 57.14% of cases expressing Bcl-2 less than 5% of the cells.

In KA without malignancy, the intensity of immunostaining was weak in 60% of cases and in 40% of the cases was negative. In 75% of the positive cases, the proportion of positive cells was less than 50%, and in 25% of the cases the proportion of positive cells was between 5–50%. We did not notice significant differences of immunostaining between KA with mild dysplasia and pseudoepitheliomatous hyperplasia, in all cases Bcl-2 immunostaining being weakly or moderately diffuse positive in basal keratinocytes and intensely positive in inflammatory infiltrate of the dermis. In malignant KAs, the immunostaining for Bcl-2 was positive in 80% of cases, 75% of the cases showed weak positive diffuse immunostaining in basal keratinocytes and isolated weak positivity in tumor cells, negative in carcinoma area and strongly positive in the inflammatory infiltrate in the dermis and intratumoral lymphocytes. We did not notice significant differences in terms of the intensity of immunostaining and the proportion of positive cells between malignant and nonmalignant cases.

The results are similar to those published by other authors, most references to Bcl-2 immunoexpression being contradictory. Some authors report positive immunostaining by different intensities in all studied KAs, others in a small percentage, while in SCC the positivity is noticed in all cases, little or not at all [27]. Sleater *et al.* reported positive Bcl-2 immunostaining in basal layer of proliferative KAs and in the rare basal cells in regressive KsA. These aspects would suggest that the degree and pattern of Bcl-2 immunoexpression indicates the loss of Bcl-2 immunoexpression with maturity of the tumor, with a possible role in their exposure to apoptosis and involution [28].

Amichai *et al.*, comparing the immunoexpression of Bcl-2 in 25 KAs, 15 well-differentiated SCC and 15 BCCs, reported positive cytoplasmic immunostaining for Bcl-2 in 8% of KAs, the immunoexpression being positive in rare cells in the portion of the lower tumor, in 80% of BCCs and no immunoexpression in SCC [29].

Infrequent presence or absence of Bcl-2 in KA and SCC may reflect the squamous differentiation in these tumors and the presence of Bcl-2 immunoexpression does not necessarily imply a low degree of terminal differentiation [30].

We noticed, in lesions with intense inflammatory infiltrate, the intensely positive immunoexpression of lymphocytes in the upper dermis and in some cases of intratumoral lymphocytes, issues described by other authors [31].

Bcl-2 homologous antagonist/killer (Bak) and Bcl-2 are proteins regulating the apoptosis, and increased expression of Bak protein in combination with low immunoexpression of Bcl-2 in regressive KA, compared to the SCC, suggest their role in tumor regression [15].

Based on IHC, HP, and clinical similarities some authors consider KA a particular form of SCC, which only rarely progresses into an invasive SCC, the only feature that could theoretically separate the two lesions being the

described involution of the KA. In KA, the immunoexpression of Bcl-2 is maintained, which allows cell apoptosis; Bcl-2 immunoexpression is decreased in the SCC. Tumor regression depends also on the host response mediated by cluster of differentiation (CD)8+ and CD4+ lymphocytes.

Recently, Ra *et al.* reported a molecular study sustaining that KA has a distinctive gene expression profile, considering KA a benign squamous neoplasia, different by SCC [32].

However, in our studied group, the large number of malignant KAs combined with no cases of spontaneous involution make us to follow the assumption that KA is more a "deficient" SCC, lacking a signal that would allow continued growth and protection against apoptosis and regression [33].

The Ki-67 immunoexpression was detected in all studied KAs as an important immunomarker that highlights nuclear atypia and atypical mitosis. In tumors, Ki-67 immunostaining provides a measure of the fraction of the tumor growth, which is an indicator of cell mitotic activity and cellular proliferation [26]. Ki-67 antigen is present in G1, S, G2 and M phases of the cell cycle, but it is absent in the G0 phase.

In KA, we noticed positive nuclear immunostaining for Ki-67 in all studied cases. 60% of the KAs expressed Ki-67 weak and moderate, 75% of the cases having less than 50% positive cells. In malignant KA, 75% of cases had a low intensity of immunostaining, noticing the same proportion of positive cells as in non-malignant cases.

In KA without pseudoepitheliomatous hyperplasia, we noticed positive immunostaining only in keratinocytes from periphery of the tumor, while in KA with pseudoepitheliomatous hyperplasia positive cells increase up to 1/3 of the epidermis, with diffuse distribution. In malignant KA, immunostaining was diffuse in the carcinoma area, in about 30% of the tumoral cells. These results are consistent with those published by other authors.

Immunostaining for Ki-67 cell proliferation marker shows a peripheral pattern in KA, unlike SCC, in which the pattern is more diffuse; also, a reduced Ki-67 positive tumoral cells in KA with increases immunoexpression in SCC was noticed.

Many studies have reported a reduced expression of p53, Ki-67 antigen and cyclooxygenase-2 (COX-2) in KA compared to SCC, while the immunoexpression of p16 protein appears to be similar in both tumors [30, 33, 34]. We found a strong correlation between p53 and Ki-67 in all analyzed KAs.

The PCNA study showed that PCNA antigen in classical KA was located peripherally around the basal layer, while in well-differentiated SCC, the pattern was relatively diffuse [24].

In our studied KAs, PCNA was expressed in all cases, the intensity of the immunostaining being moderate in 70% of the cases and low in 30% of cases; the percentage of positive cells is less than 50% in 90% of cases. We have seen a peripheral pattern of immunoexpression in forms with mild dysplasia, while in forms with moderate dysplasia and pseudoepitheliomatous hyperplasia, the proportion of affected cells increased, affecting diffusely between 1/2 and 2/3 of the epidermis. In the presence of malignancy, we have noticed a diffuse distribution of the immunostaining in the carcinoma area. PCNA was focally positive in parabasal and basal areas of KA, the percentage of positive cells increasing with the dysplasia degree, being positive in over 80% of tumor cells in the area of acantholytic carcinoma. The results are consistent with those published by other authors, the same peripheral distribution of PCNA in KA being already reported by Benedek *et al.* [11].

The IHC study of KA contribute to the understanding of skin carcinogenesis and the complex mechanisms involved in tumor regression and progression of the keratinocyte precancers into SCC.

Using a panel of immunoperoxidase staining for proteins associated with apoptosis, cell cycle, cell growth and proliferation, providing different patterns of immunoexpression, seems to be sufficient to distinguish between classical KAs and SCC, in most cases.

Currently, based on molecular, IHC and HP studies, KA is considered by some authors an evolving malignant neoplasia, due to the existence of some features of malignancy, but other authors consider it a keratinocyte benign neoplasia, distinct from SCC [12, 16, 32].

Despite numerous existing controversies regarding the nosological framing of KA, the large number of malignant KAs in our studied group support the idea that KA has a high malignant potential, which requires prompt treatment and strengthening measures of photoprotection.

Conclusions

KA is an epithelial tumor of the skin with closely clinically and histopathologically resemblances to SCC. Although classically it is considered as having a low malignancy risk, the age over 70 years, women from rural area and chronic sun exposure increased the malignant transformation up to 75.77% in our study. There are a low accuracy and specificity of the clinical diagnosis, both for benign and malignant KA, therefore histopathology should be used to confirm the clinical diagnosis; history of the disease and tumor size have no influence on clinical diagnosis if malignancy is present in the KA lesion. IHC markers may be useful for identifying potentially invasive KAs. KA tends to persist and progress into invasive SCC; in this regard, we have noticed the progression of neoplastic process from KA to KA with dysplasia, KA with areas of carcinoma *in situ*, KA with microcarcinoma, KA with invasive SCC. Along with the important number of malignant KAs with benign clinical appearance in our study, this progression confirms the new framing of KA as a well-differentiated SCC–KA type. This study emphasizes that KA requires an active therapeutic attitude (surgical removal), not expectation.

Conflict of interests

The authors declare that they have no conflict of interests.

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