

Peculiarities of epidermal proliferation and terminal differentiation in various histological types of seborrheic keratosis

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Aim of the study is to determine the peculiarities of epidermal proliferation and terminal differentiation in various histological types of seborrheic keratosis (SK).

Materials and methods. Pathomorphological and immunohistochemical analysis was performed on the skin biopsy material of 60 patients with SK, who constituted the study group, and on the material of 30 healthy skin samples, which were considered a control group.

Results. Level of expression of Ki-67 marker in papillomatous and acanthotic types of SK is significantly lower ($P < 0.005$) compared to healthy skin samples, which indicates a low proliferative potential of tumors. While the expression of the marker of terminal differentiation between these groups of SK and healthy skin is significantly higher ($P < 0.005$). Comparative characterization of the association of the expression degree of the immunohistochemical marker of proliferation Ki-67 depending on the activity of terminal differentiation processes in acanthotic and papillomatous types of SK indicates a statistically significant inverse correlation of these pathogenetically determined processes. Thus, activation of terminal differentiation with increase of caspase-14 expression level inhibits proliferation in basal epidermal layers that is accompanied by decrease of Ki-67 level.

Conclusions. Level of expression of Ki-67 as a marker of proliferative activity in patients with acanthotic and papillomatous types is significantly lower than in the control group, which may indicate no tendency to malignancy. The expression level of caspase-14 in acanthotic and papillomatous types is increased compared to healthy skin, which may indicate a violation of keratinization, which occurs during the terminal differentiation of epidermocytes. Statistical inverse conjugacy between caspase-14 and Ki-67 indicates inhibition of proliferation against the background of increased terminal differentiation activity and may justify the vectors of therapeutic action of topical agents.

Ключові слова:

себорейний кератоз, термінальне диференціювання, проліферація, Ki-67, каспаза-14.

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Особливості епідермальної проліферації та термінального диференціювання при різних гістологічних типах себорейного кератозу

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Мета роботи – визначити особливості епідермальної проліферації та термінального диференціювання при різних гістологічних типах себорейного кератозу (СК).

Матеріали та методи. Патоморфологічний та імуногістохімічний аналіз здійснили на біопсійному матеріалі шкіри 60 пацієнтів із СК – група дослідження; 30 зразків здорової шкіри вивчали як контроль.

Результати. Рівень експресії маркера Ki-67 у папіломатозному та акантотичному типах СК істотно нижчий ($p < 0,005$) порівняно зі зразками здорової шкіри. Це свідчить про низький проліферативний потенціал новоутворень. Показник експресії маркера термінального диференціювання вірогідно відрізнявся у біопсійних матеріалах груп СК і здорової шкіри ($p < 0,005$). Порівняльна характеристика асоціації ступеня експресії імуногістохімічного маркера проліферації Ki-67 залежно від виразності активності процесів термінального диференціювання при акантотичному та папіломатозному типах СК свідчить про статистично значущий зворотний зв'язок цих патогенетично зумовлених процесів саме у хворих порівняно з показниками практично здорових осіб. Отже, активація термінального диференціювання з підвищенням рівня експресії каспази-14 гальмує проліферацію в базальних шарах епідермісу, що супроводжується зниженням рівня Ki-67.

Висновки. Рівень експресії Ki-67 як маркера проліферативної активності в пацієнтів із акантотичним і папіломатозними типами істотно нижчий, ніж в осіб групи контролю. Це може свідчити про відсутність тенденції до злякисного перебігу дерматозу. Рівень експресії каспази-14 при акантотичному і папіломатозному типах підвищений порівняно з відповідними показниками здорової шкіри; це може вказувати на порушення кератинізації, що спостерігають під час термінального диференціювання епідермоцитів. Статистичний зворотний зв'язок між каспазою-14 та Ki-67 свідчить про пригнічення проліферації на тлі підвищення активності термінального диференціювання та може обґрунтовувати вектори терапевтичної дії топічних засобів.

Seborrheic keratosis (SK) belongs to the group of epithelial skin neoplasms with benign potential and absence of metastasis. The classic clinical picture of this dermatosis is represented by efflorescences in the form of papules with hyperkeratotic layering or verrucous surface

and color ranging from light to dark brown shades. It should be noted that the SK is characterized by a certain variability of clinical variants and histological types.

Thus, Noils K. et al. identified the following clinical variants of SK: dermatosis papulose nigra, stucco kera-

toxis, invert follicular keratosis, large cell acanthoma, lichenoid keratosis, flat and typical SK [1]. Morphologically, according to the WHO (2018), determine acanthotic, keratotic, reticulated, clonal, irritant, pigmented, and macular types [2].

Significant prevalence of the disease among middle-aged and elderly patients, as well as their registration in younger people, contributes to a detailed consideration of this topic in the definition of triggers and pathogenetic mechanisms [3]. It is difficult to identify a single factor that triggers the process of SK on the skin. According to the literature, the etiological preconditions for the occurrence of foci may be excessive exposure to ultraviolet radiation, impaired transcription of 3 fibroblast growth factor receptor (FGFR3), or the presence of human papillomavirus of the genus β [4].

The pathogenetic basis of the SK also remains debatable. However, according to clinical and morphological signs of SK, the process of physiological keratinization suffers due to disruption of proliferative and apoptotic mechanisms in the skin. Changes in the activity levels of cyclin-dependent kinase inhibitors involved in the phases of the cell cycle have been reported. Wu Y. H. et al. by immunohistochemical study found that increase in the expression of p16 and p21 is more typical for Bowenoid transformation than for foci of SK [5]. According to A. K. Bruecks et al., in contrast to p16 and p21, p27 expression was strong and diffuse (78 % of keratinocytes) in all studied acanthotic SK [6]. This may indicate a contravention of the cell cycle of keratinocytes.

Ki-67 nuclear protein is an indicative marker of the proliferative activity of tumors in the epidermis, which is characteristic of SK. Histologically, the manifestations of SK are represented by acanthosis, hyperkeratosis, papillomatosis, respectively, impaired terminal differentiation of keratinocytes, a marker of which is caspase-14 [7].

That is why the comparison of the processes of proliferation and differential keratinization made us interested to research the links of SK formation and was the purpose of the study.

Aim

To determine peculiarities of epidermal proliferation and terminal differentiation in different histological types of seborrheic keratosis.

Materials and methods

A pathomorphological and immunohistochemical analysis of biopsy material was carried out in 60 patients with SK and 30 healthy individuals, which was chosen by specialists of the Zaporizhzhia State Medical University based on the Scientific Medical Center "University Clinic". The morphological examination has been provided by the Department of Pathologic Anatomy and Forensic Medicine of Zaporizhzhia State Medical University. 60 patients were examined for keratosis, the main study group consisted of 55 patients with foci of SK, which were pathomorphologically represented by acanthotic ($n = 33$) and papillomatous ($n = 22$) types. Reticular, irritated,

and stucco keratosis (total $n = 5$) were not significantly represented among the samples and thus did not provide reliability in the statistical processing of the data. Therefore, these 3 types were considered only for general descriptive changes in Ki-67 and caspase-14 expression. Biopsy material from 30 people represented by healthy skin, which was obtained during surgical dermatology intervention, was used as a control group.

Paraffin blocks were formed from the obtained tissue samples, serial standard sections of 4 μm thickness were made on a precision rotary microtome NM 3600 (MICROM Laborgerate GmbH, Germany), sections were placed on ordinary slides (for review histopathological staining) or on adhesive slides "SuperFrost Plus" (company DAKO, Denmark) – for immunohistochemical studies.

Determination of the histological pattern of SK was evaluated in serial paraffin sections stained with hematoxylin and eosin. IHC studies were performed in paraffin sections using monoclonal antibodies and polyclonal antibodies. Dewaxing and rehydration with simultaneous high-temperature unmasking of antigens was performed by heating in an autostainer with an RT module (Thermo Fisher Scientific, USA) in Dewax & HIER buffer H from Thermo Fisher Scientific, USA (pH = 9.0), suppressed endogenous peroxidase activity with 3 % H_2O_2 solution and applied the protein block. Incubation with primary antibodies was performed according to the instructions of the manufacturers, visualization of the IHC reaction was performed using the detection system UltraVision Quanto HRP + DAB System ("Thermo Scientific", USA). Sections were stained with Mayer's hematoxylin.

Monoclonal antibodies were used to determine the proliferative activity of epithelial cells: Mo a-Hu Ki-67 Antigen, Clone SP6 (NeoMarkers, USA); the process of differentiation of keratinocytes in different patterns of seborrheic keratosis was determined using antibodies Rb a-Hu Caspase 14 ("NeoMarkers", USA). Due to the recommendations of the St Gallen International Consensus of Experts the expression of proliferation index Ki-67 can be low (≤ 15 %), intermediate (16–30 %), and high (> 30 %). When > 50 % of cells were positively stained it showed a high level; intermediate – 25–50 % of the cells stained positive; weak – < 25 % or > 10 % of the cells were positively stained; and negative, if < 10 % of the cells were positively stained.

Microphotographs of histological and IHC skin samples of the study and control groups were recorded in an Axioplan 2 microscope ("Carl Zeiss", Germany) using a digital camera Camedia C5060WZ Olympus (Japan).

Statistical processing of the results was performed on a personal computer in the program Statistica® for Windows 13.0 (StatSoft Inc., license No. JPBZ804I382130ARCN10-J).

The hypothesis of the normality of the distribution of the studied indicators was tested using the Shapiro–Wilk test. Data with a different than normal distribution were presented using the median and interquartile range of Me (Q1; Q3). In the case of incorrect distribution of indicators, the Mann–Whitney U test was used to evaluate two independent groups. To assess the pathogenetic relationships and the degree of associative conjugation

between the level of the marker of terminal differentiation (caspase-14) and the severity of proliferative potential (Ki-67) in groups of acanthotic and papillomatous types of SK, regression analysis with scattering diagrams in different groups.

Results

Histological examination of the foci of SK in patients of the study group acanthotic type was recorded in 33 patients, which is almost half of all cases (55.0 %). Papillomatous SK was found in 22 (36.6 %) patients with foci of extensive fields of hyperkeratosis, uneven acanthosis. In the studied cohort of patients histologically 3 (5.0 %) cases of reticulated (adenoid) type were detected, irritated and stucco keratosis – 1 (1.7 %) SK, respectively.

Immunohistochemical study of the proliferative activity of epithelial cells in the acanthotic pattern of SK revealed that the expression area of the Ki-67 marker was 0.11 (0.09; 0.12) % and its intensity was a moderate nucleoplasmic expression, which was observed among certain groups basal and parabasal epitheliocytes, which were in the state of the most active division and are a feature of this histological type.

In more differentiated epitheliocytes of the intermediate layers of the epithelium, the number of proliferating cells decreased sharply, morphologically only single epitheliocytes with positive nuclear expression of Ki-67 were observed, in the centers of keratin cysts and apical keratinocytes negative was detected (Fig. 1).

When analyzing the expression profile in patients with a papillomatous type of SK, the area of immunopositive Ki-67 cells was 0.09 (0.08; 0.11) %, it was detected only in single basal epitheliocytes. According to its intensity, the expression had a moderate nucleoplasmic type, a sharp decrease in the intensity of the separation index was observed in intermediate epitheliocytes, in apical keratinocytes Ki-67 expression was negative (Fig. 2).

Apathomorphological study of the proliferative activity of the reticulated type of SK was observed in single cells and amounted to 0.33 (0.31; 0.35) %. Nucleoplasmic expression was significantly more intense compared to the expression in cells of the preserved layers of the integumentary epithelium. In the areas of preserved integumentary epithelium without violation of stratification properties, cells showed intensive expression of Ki-67 marker in basal and parabasal epitheliocytes with a gradual decrease in the number of positive cells as they differentiate in the intermediate and apical layer, which was morphologically healthy.

The most intense proliferative activity of cells was found in representatives of irritated SK. In the statistical processing of the obtained data, the number of positive cells was 0.36 (0.33; 0.40) %. Some groups of basal cells throughout the area had a pronounced nuclear intensity of expression of the nucleoplasmic type.

In the places of preserved stratification of the integumentary epithelium among the epitheliocytes of the intermediate and apical layers, the area of cells with proliferative activity decreased and had a focal character. Positive expression of Ki-67 in single lymphocytes and macrophages took place during the study of the zones of

acanthotic strands formation and in the loci of leukocyte-macrophage inflammatory infiltration.

The expression area of Ki-67 in the conditional control group was 0.21 (0.18; 0.24) %. According to its intensity, the expression had a nucleoplasmic variant and was linearly traced in most cells of the basal and parabasal layers of the epithelium.

In the areas of the pilosebaceous complex, positive expression was observed in single myoepithelial cells around the sebaceous glands and single cells of the mantle of hair follicles (Fig. 3).

Immunohistochemical study of keratinocyte differentiation in different types of SK showed that in the acanthotic pattern the total area of positive cells was 35.05 (32.82; 36.82) %, the most intense cytoplasmic expression of caspase-14 was observed around the formed keratin cysts (Fig. 4) and in the apical epithelial cells of the spinous layer, which are at the initial stage of completion of differentiation.

Starting from the basal layer and as the epitheliocytes differentiated, the expression of caspase-14 was of the membrane type and was moderate in intensity.

In the morphological examination of the papillary layer of the dermis, local weak cytoplasmic expression of caspase-14 was observed only in single capillary endotheliocytes, collagen and elastic fibers had negative expression.

Processing of statistical data on the area of expression of caspase-14 in papillomatous type of SK was 41.47 (39.68; 42.64) %. Intense cytoplasmic expression was visualized among differentiated epitheliocytes, which were part of papillary structures, with weak membrane expression in basal epitheliocytes and negative expression in the formed fibrovascular stroma (Fig. 5).

The area of immunopositive cells in reticulated SK was 39.79 (36.28; 41.61) %, had a moderate type of cytoplasmic expression, while in fragments of the preserved integumentary epithelium, the intensification of expression was observed only in differentiated spinous epitheliocytes of the apical layers of the epithelium and in the areas of the most pronounced keratosis.

Statistical processing showed that the area of immunopositive cells in the study of the irritated type of seborrheic keratosis was 25.92 (21.22; 27.95) %. Local moderate expression of caspase 14 took place, which was most pronounced in the areas of acanthotic strands formation and among cells of the spinous layer.

Among cells of the basal and parabasal layers, there was a local, weak cytoplasmic expression, moderate membrane expression. Among collagen and elastic fibers and in the loci of lymph-macrophage inflammation – there was negative expression.

When comparing the expression of caspase-14 in the conditional control group, positive expression had the cytoplasmic variant over the entire epithelial area and among pilosebaceous complexes, mainly among sebocytes of sebaceous glands (Fig. 6), the area of immunopositive cells was 6.21 (5.51; 7.24) %.

The scatter diagram of the relationship between level of the terminal differentiation marker and expression of the Ki-67 proliferation marker is statistically significant only for acanthotic and papillomatous types. Because irritated, reticulated, and stucco types of keratosis are

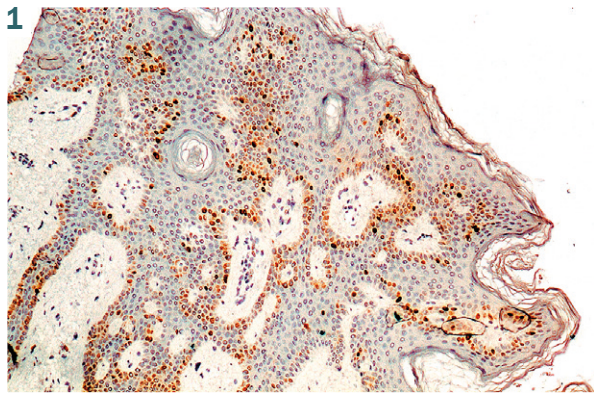


Fig. 1. Loci of basal epitheliocytes with Ki-67+ moderate nuclear expression. Mo a-Hu Ki-67 Antigen, clone SP6. Mag.: $\times 100$.

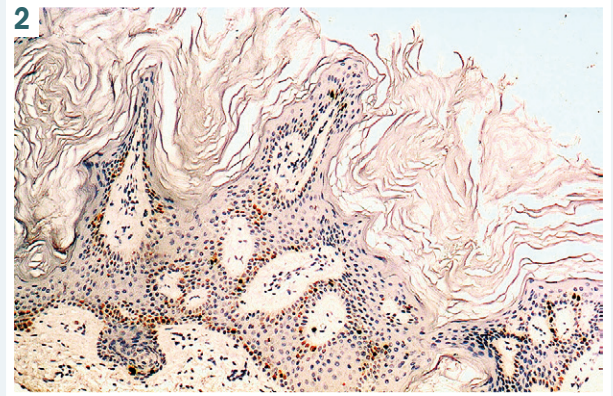


Fig. 2. Individual Ki-67 positive cells of the basal layer in the papillomatous variant of seborrheic keratosis. Mo a-Hu Ki-67 Antigen, clone SP6. Mag.: $\times 100$.

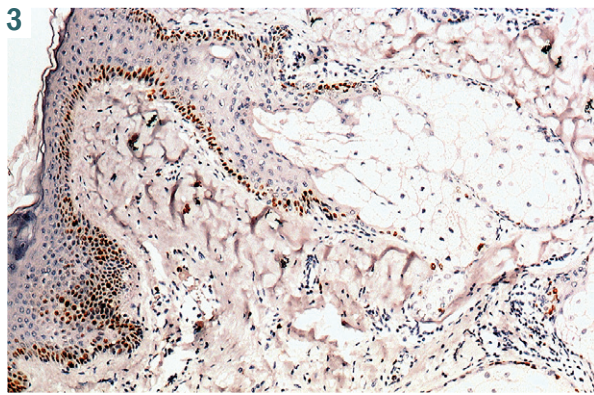


Fig. 3. Positive Ki-67 cells of the basal layer of the epithelium and single myoepithelial cells around the sebaceous glands. Mo a-Hu Ki-67 Antigen, clone SP6. Mag.: $\times 100$.

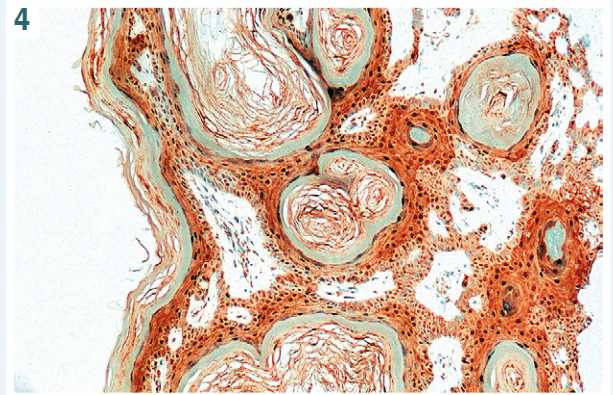


Fig. 4. Intensive expression of caspase-14 around keratin cysts in acanthotic variant of seborrheic keratosis. Rb a-Hu Caspase 14. Mag.: $\times 200$.

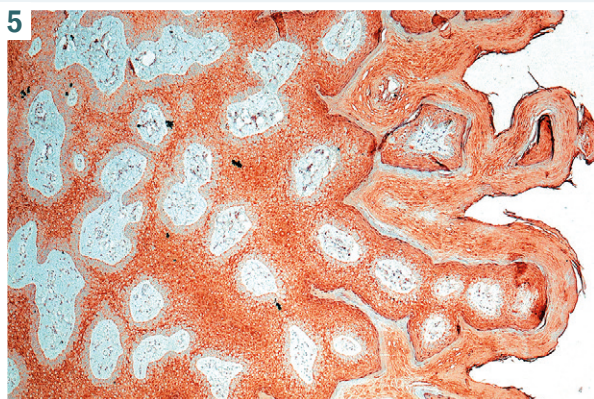


Fig. 5. Intense expression among differentiated epitheliocytes and negative stroma in papillomatous variant of seborrheic keratosis Rb a-Hu Caspase 14. Mag.: $\times 100$.

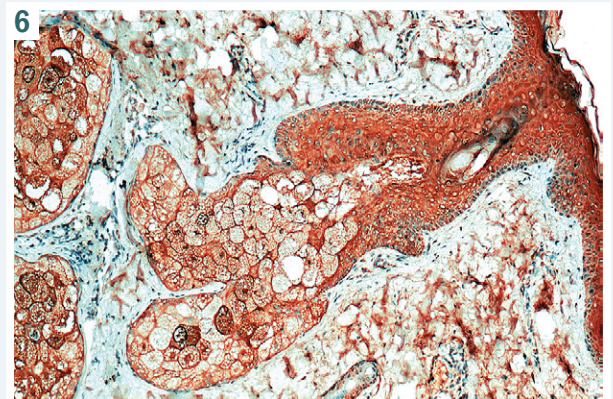


Fig. 6. Immunopositive cytoplasmic expression among spinous epithelial cells and sebocytes of sebaceous cells. Rb a-Hu Caspase 14. Mag.: $\times 100$.

nonrepresentable, which excludes them from a statistically significant sample.

According to the regression analysis procedure, it was shown that the relationship between the level of caspase-14 and the expression of the proliferative marker Ki-67 in patients with acanthotic type most adequately characterized the regression model of exponential type,

at $R = -0.81$, $R^2 = 0.65$, normalized $R^2 = 0.61$; $F = 14.81$, $t = -3.87$, $SE = 0.015$, $P = 0.0048$.

The results of the regression analysis show that the relationship between the level of caspase-14 and the expression of the proliferative marker Ki-67 in patients with papillomatous type most adequately characterized the regression model of polynomial (step), namely

quadratic type at $R = -0.87$, $R^2 = 0.76$, normalized $R^2 = 0.67$; $F = 19.21$, $t = -4.65$, $SE = 0.013$, $P = 0.0031$.

The data obtained during the regression analysis indicate a lack of relationship between the level of caspase-14 and the severity of expression by immunohistochemical evaluation of Ki-67 in patients of the control group ($R = 0.30$, $R^2 = 0.095$, normalized $R^2 = 0.018$; $F = 0.838$, $t = 0.92$, $SE = 0.069$, $P = 0.387$).

Discussion

In this study, the expression level of the Ki-67 marker in individuals with acanthotic and papillomatous types of SK was significantly lower compared to healthy skin samples, indicating a low proliferative potential of tumors. Thus, when comparing the expression of this marker for acanthotic and papillomatous types of SK, statistically significant difference was determined ($P < 0.005$). We also recorded increase in proliferative potential in irritated SK, but given the limited prevalence of this type in our sampling of the patients, statistical evaluation is not relevant. In addition, it should be noted that a low level of Ki-67 expression may also indicate the absence of risk of malignant transformation of tumors or collisions with more aggressive neoplasm. According to E. Bahrani et al. high expression of Ki-67 is more typical for the Pagetoid form of Bowen's disease than the microclonal variant of SK [8].

Caspases are key enzymes of apoptosis. In addition, they can split and activate each other, thereby regulating and amplifying apoptotic signals [9]. Such tissue-specific programmed cell death leads to the formation of the stratum corneum as the foundation of the skin's protective function.

In humans, caspases-1, -4 and -5 are involved in the intracellular activation of proinflammatory cytokines IL1 and IL18, while caspases-2, -3, -6, -7, -8, -9 and -10 specialize in activation, enhancement and implementing a cell destruction program [10,11]. While caspase-14 is a representative of another generation belonging to the evolutionary group of cysteinyl-aspartate specific proteases, which are mostly involved in inflammation and apoptosis. However, recent experimental data indicate their direct involvement in proliferation and differentiation, which correlates with keratinization processes [12].

As confirmed by the studies of L. Eckhart et al., human caspase-14 has a limited distribution in tissues and is expressed mainly in the epidermis. Its accumulation occurs in the granular layer, activation during the formation of the stratum corneum in vitro, and the cleavage products of caspase-14 are present in the normal epidermis and in the stratum corneum in situ. All these data may indicate that these representatives of caspases are involved in the final differentiation of keratinocytes [13].

In a study by G. Denecker et al., it is shown that the skin of mice with caspase-14 deficiency was shiny and lichenified, which indicates a changed composition of the stratum corneum. Epidermis with deficiency of the marker of terminal differentiation contained significantly more alveolar F-granules of keratohyalin, prophyllagrin [14].

In our study, there was a significant increase in the expression of caspase-14 in patients with foci of SK.

Thus, when comparing the expression level of the marker of terminal differentiation between the groups of acanthotic and papillomatous types of SK versus healthy skin, there is a statistically significant difference ($P < 0.005$).

Determining the relationship between proliferation and terminal differentiation for skin neoplasms has significant prognostic potential. After all, it is necessary to take into account the dynamics of changes in the tumor, the risk of malignant transformation, which affects the further choice of pathogenetic and morphological justification of treatment tactics. Thus, for squamous cell carcinoma, overexpression of caspase-14 provoked a decrease in cell proliferation activity [15].

Comparative characteristics of the association of the degree of expression of the prognostic immunohistochemical marker of the proliferation of Ki-67 nuclear antigen depending on the severity of the activity of terminal differentiation in acanthotic and papillomatous types of SK indicate a statistically significant inverse conjugation of these pathogenetically determined patients.

Thus, the activation of terminal differentiation with increasing expression of caspase-14 inhibits proliferation in the basal layers of the epidermis, accompanied by a decrease in the level of Ki-67. The results of this study indicate pathogenetic links to explain morpho-functional changes in patients with SK.

Conclusions

1. The level of Ki-67 expression as a marker of proliferative activity in patients with acanthotic and papillomatous types is significantly lower than in the control group, which may indicate no tendency to malignancy.
2. The level of caspase-14 expression in acanthotic and papillomatous types is increased compared to healthy skin, which may indicate a violation of keratinization, which occurs during the terminal differentiation of epidermocytes.
3. Statistical inverse conjugation between caspase-14 and Ki-67 indicates inhibition of proliferation against the background of increased terminal differentiation activity and may justify the vectors of therapeutic action of topical agents.

Future research perspectives. The obtained results can serve as a prerequisite for the development of methods of therapy taking into account the peculiarities of the processes of epidermal proliferation and terminal differentiation in patients with SK.

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