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Defensin *DEFB4A* transcript level in the differentiation of keratoacanthoma, squamous and basal cell carcinomas**

Poziom ekspresji defensyny *DEFB4A* w różnicowaniu rogowiaka kolczystokomórkowego, raka kolczystokomórkowego i raka podstawnokomórkowego

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Key words

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oligonucleotide microarrays (Affymetrix),
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Słowa kluczowe

defensyna *DEFB4A*, niemelanotyczne nowotwory skóry, mikromacierze oligonukleotydowe (Affymetrix), QRT-PCR w czasie rzeczywistym

Summary

Introduction. Defensins are peptide with antimicrobial, antiviral, antifungal activities and many other functions, such as induction of immunological response and antitumor activity. Changes in expression level of defensins was studied in many skin pathologies, including dermatological lesions such as psoriasis, atopic dermatitis and non-melanoma skin cancers (squamous cell carcinoma – SCC and basal cell carcinoma – BCC).

Aim. The objective of this study was to evaluate the mRNA profile of defensin-related genes' transcripts as an additional molecular marker of non-melonoma skin pathologies: SCC, BCC and keratoacanthoma (KA).

Material and methods. Tissue samples were obtained from the central part of tumours (KA, SCC and BCC) and healthy margins. mRNA profile of genes coding defensins and proteins involved in their activation was determined using oligonucleotide microarrays (Affymetrix). Validation of the microarray analysis was performed using real-time QRT-PCR.

Results. Microarray analysis revealed changes in defensin-related genes' profile. In all tumours *DEFB4A* (*defensin beta 2*) mRNA was up-regulated, compared with the healthy skin margins. Real-time QRT-PCR analysis showed increased *DEFB4A* transcript level both in KA and SCC comparing to BCC.

Conclusions. Defensin beta 2 mRNA level is a useful tool for the differentiation of KA and SCC from BCC. KA and SCC cannot be differentiated on the basis of the *DEFB4A* mRNA level.

Streszczenie

Wstęp. Defensyny stanowią grupę peptydów o aktywności antybakteryjnej, antywirusowej i antygrzybiczej. Uczestniczą także w indukcji odpowiedzi immunologicznej i przeciwnowotworowej. Zmiany w poziomie ekspresji defensyn były badane w wielu patologiach skórnych, m.in. w łuszczycy, atopowym zapaleniu skóry, a także w niemelanotycznych nowotworach skóry (raku kolczystokomórkowym – SCC i raku podstawnokomórkowym – BCC).

Cel pracy. Celem tej pracy była ocena profilu mRNA genów kodujących defensyny oraz białka zaangażowane w indukcję ich ekspresji jako dodatkowego markera niemelanotycznych patologii skórnych: SCC, BCC i rogowiaka kolczystokomórkowego (KA).

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Materiał i metody. Wycinki uzyskano z centralnej części guza (KA, SCC i BCC) i marginesów tkanki zdrowej. Profil mRNA genów kodujących defensyny oraz białka zaangażowane w indukcję ich ekspresji został wyznaczony techniką mikromacierzy oligonukleotydowych (Affymetrix). Walidację wyników przeprowadzono techniką QRT-PCR w czasie rzeczywistym.

Wyniki. Analiza techniką mikromacierzy wykazała zmiany profilu mRNA genów powiązanych z defensynami. We wszystkich guzach stwierdzono nadekspresję mRNA defensyny 2 (ang. *defensin beta 2 – DEFB4A*), w porównaniu do kontroli. Analiza techniką QRT-PCR w czasie rzeczywistym wykazała wzrost liczby kopii mRNA *DEFB4A* zarówno w KA, jak i SCC w porównaniu do BCC.

Wnioski. Poziom mRNA defensyny 2 jest przydatnym narzędziem w różnicowaniu KA i SCC od BCC. Rogowiak kolczystokomórkowy i rak kolczystokomórkowy nie mogą być różnicowane na podstawie liczby kopii mRNA *DEFB4A*.

INTRODUCTION

Defensins play a role as peptide antibiotics and constitute an important element of anti-infectious protection (1). Apart from a direct antimicrobial action, defensins also have many other functions, such as antifungal and antiviral activities, and chemotactic activity towards dendritic cells, T cells, basal cells and neutrophils. They may induce the production of certain chemokines and proinflammatory mediators; regulate complement activity; inhibit fibrinolysis and the production of certain glucocorticosteroids; intensify the proliferation of lymphocytes, endothelial cells and fibroblasts; bind and neutralize endotoxins; stimulate healing of sores; induce degranulation of basal cells and show antitumor activity (2). There are three groups of defensins – defensin α and β, and θ defensins – generated during the cyclisation of the two α defensins (1). In humans, six α (HNP 1-6 – human neutrophil peptide) and four β defensins (HBD 1-4) have been identified (3). Changed expression level of defensins was reported in many pathologies, including dermatological lesions such as psoriasis, acne vulgaris and atopic dermatitis (3-5). Examinations of cancer samples such as oesophageal squamous cell carcinoma, primary cutaneous squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) also showed changes in defensins profile, suggesting their potential participation in these pathologies (6-8).

Knowledge of molecular markers of tumour transformation is essential for malignant diseases because it enables a diagnosis to be made of a disease already at the level of molecular changes, and it aids cause-directed and efficient therapy. Non-melanoma skin cancers are still problematic in diagnosis because of clinical features resembling other pathologies i.e. keratoacanthoma. Keratoacanthoma (KA) is a relatively low-grade malignancy that histologically and clinically resembles well-differentiated SCC (9, 10). It develops on unchanged skin in the form of a dome shaped, well-limited, hard nodule, usually with the same colour as the surrounding skin. The central part of the tumour has a crater-like depression with embanked margins, filled up with a keratin mass (11, 12). KA is characterised by sometimes fast growth and a tendency to regression with scarring (11). BCC is the most common type of epithelial cancer; it is characterised by low clinical and histological malignancy and slow growth and rarely metastasises (13, 14). It is usually found on unchanged skin of the face and head and spreads in multiple directions with edgings. The multiple and multifocal presence of BCC is classified as nevoid BCC syndrome (Gorlin-Goltz syndrome) (13, 14). Clinically, the changes can be highly differentiated, superficial, corneous, nevus-like, scleroderma-like, nodular or sore-like (15). SCC is the second most frequent (after BCC) skin cancer. It originates from squamous epithelium and develops from premalignant states (leucoplakia). It is characterised by fast growth and metastases to regional lymph nodes. Clinical forms of SCC (sore and papular) morphologically resemble KA and BCC (9, 16). Therefore additional markers could facilitate diagnosis of non-melanoma skin pathologies.

Significant risk factors for BCC, SCC and KA are: UV radiation, ionized radiation, post-infectious and post-drug immunosuppression, chemical carcinogens (arsenic, wood tars), old age, male gender, light skin type and human papilloma virus infection (8, 9, 16-20). Most of these factors influence immunological response. Due to its multidirectional activity, defensins may be involved not only in the molecular pathogenesis of non-melanoma skin cancers, but also in the immunological reaction leading to self-regression of KA. Understanding of the molecular mechanisms underlying KA regression may contribute to the development of an efficient treatment for skin cancers. Better knowledge about defensins level in non-melanoma skin pathologies could improve their diagnosis and could affect better understanding of molecular mechanism underlying these pathologies. Till this time there is no research comparing defensins profile in these three non-melanoma pathologies (KA, SCC and BCC) in one study.

AIM

The aim of this work was to evaluate the mRNA profile of defensin family transcripts as an additional molecular marker of nonmelonoma skin pathologies.

MATERIAL AND METHODS

The study included a group of 39 patient (16 females and 23 males; mean age 71.8 ± 9.5 years) diagnosed and treated in the Dermatology Clinics and Department of Medical University of Silesia in Katowice. Based on clinical and pathomorphological

examination, 9 cases were kerathoacanthoma (KA), 11 were squamous cell carcinoma (SCC) and 19 were basal cell carcinoma (BCC). The changes were located on the skin of the face and head. The biopsies for the pathomorphological and molecular examination were obtained from the centre of the tumour and the margins of healthy tissues where no tumour cells were found. After surgical removal, the samples were immediately preserved in the RNA stabilisation reagent RNAlater (Qiagen GmbH, Hilden, Germany). For microarray analysis 19 samples (6 KA, 3 SCC, 7 BCC and 4 margins of healthy tissues) were selected. The study was approved by the Bioethical Commission of the Medical University of Silesia. All of the patients were informed about the research and signed an informed consent form.

RNA extraction

Total RNA was extracted from the tissue samples using TRIZOL® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA extracts were treated with DNase I (MBI Fermentas, Vilnius, Lithuania) and purified with a RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. The quality of RNA was estimated by electrophoresis on 1% agarose gel stained with ethidium bromide. The RNA concentration was determined on the basis of absorbance values at 260 nm using a Gene Quant Pro spectrophotometer (LKB Biochrom Ltd., Cambridge, UK).

Microarray analysis

The analysis of the expression profile of defensin-related genes was performed using commercially available oligonucleotide microarrays HG-U133A (Affymetrix, Santa Clara, CA) according to the manufacturer's protocol. For finding significant genes comparative analysis was performed with the use of GeneSpring 12.6.1 platform (Agilent Technologies, Inc., Santa Clara, CA, USA) and PL-Grid Infrastructure. The differences were analysed using the Oneway ANOVA test with Benjamini-Hochberg Multiple Testing Correction and TukeyHSD Post Hoc test. Genes were considered as potentially differentiating when FC $\geq$ 1.1 (fold change) and the significance level was set at $p < 0.05$.

Real-time QRT-PCR

The levels of the DEFB4A and β-actin transcripts were evaluated with the use of the real-time QRT-PCR TaqMan technique. The quantitative analysis was carried out using a Sequence Detector ABI PRISM™ 7000 (Applied Biosystems, Foster, CA, USA). Amplification was performed with the use of commercially available oligonucleotide primers specific for *DEFB4A* and *β-actin* genes (DEFB4A: TaqMan Gene Expression Assay defensin, beta 4; β-actin: TaqMan B-actin Detection Reagents Kit, Applied Biosystems, Inc., Foster, CA, USA) and QuantiTect Probe RT-PCR Kit (Qiagen GmbH, Hilden, Germany). For the assay, positive (*β-actin* mRNA) and negative (no template) controls were used. The thermal profile for one-step RT-PCR was as follows: 50°C for 30 min for reverse transcription, 95°C for 15 min, 45 cycles at 94°C for 15 s and at 60°C for 60 s. The standard curve was created for *β-actin* cDNA (TaqMan DNA Template Reagents Kit, Applied Biosystems, Inc., Foster, CA, USA). A standard curve was then generated by plotting the Ct values against the log of the known amount of the *β-actin* cDNA copy number. The mRNA copy numbers of the gene examined were recalculated per 1 μg of the total RNA. The RT-PCR products and the molecular weight marker pBR 322/Hae III (Fermentas International Inc., Ontario, Canada) were separated on 8% polyacrylamide gel and visualised with silver salts. The length of the amplified fragments was assessed by analysis with GelScan v.1.45 software (Kucharczyk TE, Warsaw, Poland). The statistical analysis of the real-time QRT-PCR results was performed with the use of Statistica version 9.0 software (StatSoft Inc., Oklahoma, USA). The one-way ANOVA followed by Tukey posthoc analysis of the logarithmic parameters were used to assess differences in the expression of the studied genes between KA, SCC and BCC. The differences between the tumour and the margin of the normal tissue were analysed using the t-test. All of results were expressed as means ± SD. The significance level was set at $p < 0.05$.

RESULTS

Microarray analysis

In the present study, changes in transcriptome of defensin family genes and genes coding proteins involved in their activation in non-melanoma skin pathologies were evaluated. Among 251 ID mRNA of defensin-related genes found in Affymetrix database (http://www.affymetrix.com) 11 transcripts were differentiating in non-melanoma skin pathologies (Oneway ANOVA test with Benjamini-Hochberg Multiple Testing Correction) (tab. 1A). Comparing to controls (C) 5 transcripts were differentiating for keratoacanthoma (KA), 4 for squamous cell carcinoma (SCC) and 2 for basal cell carcinoma (BCC) (TukeyHSD Post Hoc test) (tab. 1B).

Among 11 transcripts 6 were differentiating in comparison to controls (fig. 1). Some of them were characteristic only for particular group of transcriptomes, some were common for few groups. *DUSP7* was characteristic only for KA and *TRAF3* was characteristic only for SCC. *S100A8* and *S100A9* were common for KA and SCC. *MAPKAPK2* was common for KA and BCC. *DEFB4A* was common for all transcriptomes, comparing to controls (KA, SCC, BCC).

Keratoacanthoma transcriptomes comparing to BCC showed 3 upregulated genes (*S100A9*, *DUSP6*, *DUSP7*) and one gene down-regulated (*PELI2*). Comparing to SCC also *DUSP6* and *DUSP7* were up-regulated, while *TRAF3* was down-regulated in KA.

Table 1. The results of statistical analysis of 251 ID mRNA defensin-related genes in non-melanoma skin pathologies. A – The results of Oneway ANOVA test with Benjamini-Hochberg Multiple Testing Correction showing 11 differentiating entities at $p < 0.05$. B – TukeyHSD Post Hoc test results. Entities found to be differentially expressed between particular groups of samples are represented by grey colour.

A	P-value	P all	p < 0.05	p < 0.02	p < 0.01	p < 0.005	p < 0.001
	Number of ID *mRNA*	251	11	2	2	2	1
B	Transcriptomes	KA	SCC	BCC		C	
	KA	11	3	5		5	
	SCC	8	11	3		4	
	BCC	6	8	11		2	
	C	6	7	9		11	

KA – keratoacanthoma; SCC – squamous cell carcinoma; BCC – basal cell carcinoma; C – controls (healthy skin margins)

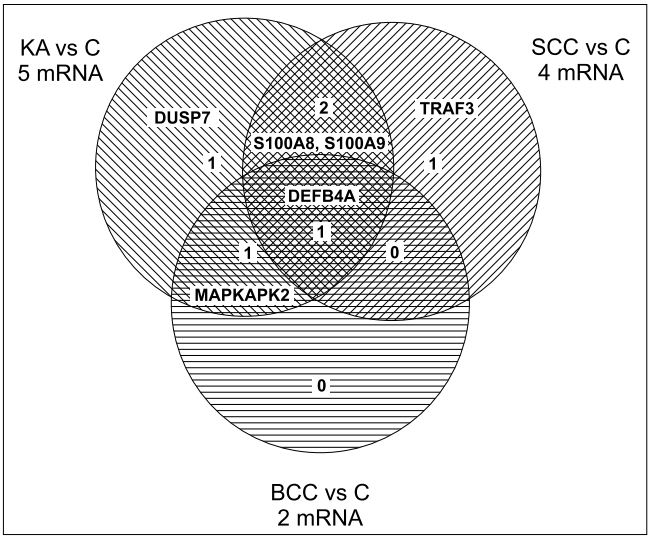

Fig. 1. Venn diagram showing number of transcripts differentiating in comparison to controls common for groups of transcriptomes: KA – keratoacanthoma, SCC – squamous cell carcinoma, BCC – basal cell carcinoma, C – controls (healthy skin margins).

In comparison of SCC to BCC *TRAF3* and *MAPK3* were upregulated, *LGR4* was down-regulated in SCC (fig. 2).

The most differentiating gene was *DEFB4A* which expression was upregulated in all skin non-melanoma pathologies comparing to controls. The highest fold change was for SCC (FC = 13.35), for KA FC = 9.86 and for BCC FC = 7.86.

Real-time QRT-PCR

In the next step the transcript level of the *DEFB4A* gene was validated using the QRT-PCR assay. The *DEFB4A* mRNA level was higher in non-melanoma skin tumours than in the margins of the healthy tissue (tab. 2). Analysis of the *DEFB4A* transcript level in the tumours revealed significant difference between BCC, KA and SCC. The *DEFB4A* mRNA level in BCC was lower than in the KA and SCC groups ($p = 0.0018$, $p = 0.0014$, respectively; Tukey's test). No statistically significant difference was found between KA and SCC ($p = 0.9902$) in the mRNA level of the *DEFB4A* gene. The *DEFB4A* transcript level in the margins of the healthy tissue was comparable ($p = 0.3101$).

Table 2. *DEFB4A* mRNA level in KA, SCC, BCC, C.

	KA	***SCC***	***BCC***	***p*[a]**
Tumour	5.32 ± 0.74	5.21 ± 0.55	4.39 ± 0.59	0.0002
Margin	4.03 ± 1.22	4.42 ± 0.96	3.83 ± 1.11	NS
p[b]	0.0051	0.0123	0.0315	

KA – keratoacanthoma; SCC – squamous cell carcinoma; BCC – basal cell carcinoma; C – controls (healthy skin margins); mean ± standard deviation; NS – not significant
[a]ANOVA
[b]t test

Additionally, the expression of the β-actin gene was assessed as an endogenous control. The transcript level of this gene was similar in tumours and healthy tissues. Statistical analysis revealed a marked difference in the *β-actin* mRNA level in the tumours. However, the number of mRNA copies in the BCC group was lower than that in the KA group ($p = 0.0012$; Tukey's test). In common with that found for the *DEFB4A* gene, the *β-actin* mRNA level in the margins of the healthy tissues was comparable ($p = 0.7467$).

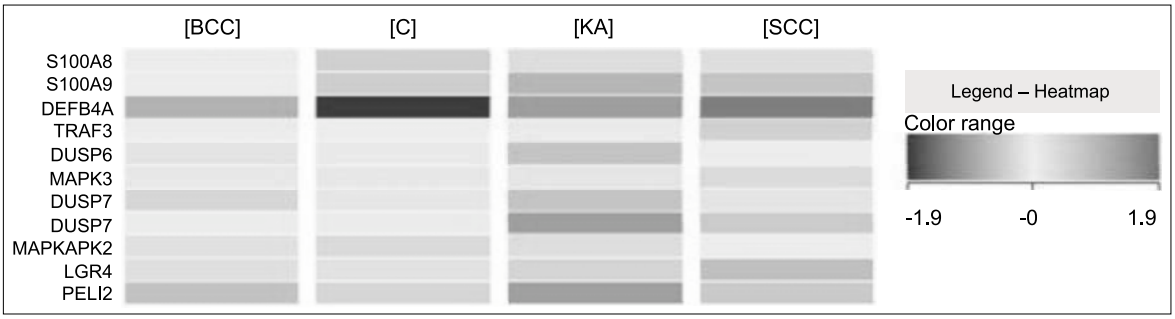

Fig. 2. Heat-map for differentiating ID mRNA of defensin-related genes in transcriptomes of KA – keratoacanthoma, SCC – squamous cell carcinoma, BCC – basal cell carcinoma, C – controls (healthy skin margins).

DISCUSSION

Defensins through multidirectional activity may influence many biological processes, including regulation of inflammatory response and wound healing (2). Many risk factors of NMSC (non-melanoma skin cancers) and KA can influence immunological reaction, thus potential involvement of defensins in mechanism underlying these pathologies cannot be excluded. Moreover, self-regression of KA is, *inter alia*, caused by changes in immunological reaction together with defensins' activity (21). There is much controversy whether KA should be described as a mild or intermediate form of SCC or whether it should be considered as a totally separate benign malignancy (12, 13). KA needs to be differentiated from both SCC and BCC. Some studies have been carried out to differentiate KA and SCC, other have compared molecular changes in SCC and BCC (16, 22-25). Comparing all three pathologies together could show changes helpful in differentiating KA from BCC and SCC, as well as SCC from BCC. Moreover, such approach could reveal molecular markers which could have impact on self-regression of KA. Therefore the aim of this study was to evaluate changes in mRNA profile of defensin family genes and genes coding proteins involved in their activation in three non-melanoma pathologies: KA, SCC and BCC in one study.

mRNA profiles of KA, BCC and SCC transcriptomes were appointed with the use of oligonucleotide microarrays (HGU133A, Affymetrix). Statistical analysis of transcriptomes assumed 251 ID mRNA of genes coding defensins and proteins involved in their activation, based on Affymetrix database results. Eleven ID mRNA were differentiating comparing to control samples. The only characteristic gene for KA was *DUSP7*, which expression was the highest in KA and it differentiated this lesion from controls, BCC and SCC. Additionally *DUSP6* was also upregulated in KA, but only in comparison to SCC and BCC. *DUSP7* and *DUSP6* encode phosphatases which inactivate the MAP kinases ERK1/2 (26, 27). ERK1/2 are responsible for proliferation of skin SCC and inhibition of this pathway is crucial in anti-tumour therapy (28). Our research showed upregulated *MAPK3* (*ERK1*) in SCC, comparing to BCC. Together with down-regulation of DUSP inactivators this result shows crucial role of ERK pathway in SCC. Moreover, the characteristic gene for SCC was *TRAF3*, which was upregulated in SCC comparing to controls, BCC and KA. TRAF3 is positive regulator of PI3K/AKT and JAK/STAT signalization and controls the growth of B and T cells (29). Both in KA and SCC, *S100A8* and *S100A9* were upregulated comparing to controls. *S100A9* was also upregulated in KA in comparison to BCC. S100A8 and S100A9 are calcium-binding proteins, highly expressed at sites of acute and chronic inflammation including psoriatic epidermis (30). These two proteins forms calprotectin, a heteridimeric complex which activate NF-kB signalization. Their high level was reported in inflammation, carcinogenesis and also during wound healing (30). Upregulated genes in BCC were *PELI2* (comparing to KA) and *LGR4* (comparing to SCC). PELI2 takes a part in TAK1 dependent activation of NF-kB, as well as in ERK phosphorylation (31). LGR4, G-protein-coupled receptor is crucial in the regulation of Wnt signalling (32). Common upregulated marker for KA and BCC was *MAPKAPK2* (*MK2*). This gene is coding serine/threonine kinase phosphorylated by p38 mitogen-activated protein kinase and plays a crucial role in inflammatory response (33). The participation of MK2 in immunological reaction along with protection of cells with DNA damage from apoptosis means that this kinase is crucial in skin tumours' development (33).

In all skin non-melanoma pathologies upregulated gene was *DEFB4A* (the official symbol of *defensin beta 2* – *HBD2*, according to HUGO Gene Nomenclature Committee). DEFB4A belongs to the defensin family of small cationic peptides. It is produced by keratinocytes during inflammatory reactions, and it is present in lamellar bodies of the stimulated keratinocytes in the granular epidermal layer, in the extracellular matrix and in the stratum corneum (34, 35). DEFB4A is a protein closely associated with the development and maintenance of inflammation in the skin. It activates many mechanisms of adaptive and innate immune responses, which eventually lead to development of a local inflammatory response. Earlier research evaluated defensin beta 2 in relation to numerous pathologies: psoriasis and atopic dermatitis, irritable bowel syndrome, lung cancer, bacterial infections, BCC and SCC (3, 6, 8, 36-40). In our study oligonucleotide microarray analysis showed that the greater difference in *DEFB4A* mRNA level in comparison to controls was in SCC (FC = 13.35), in KA this difference was lower (FC = 9.86) and the lowest was in BCC (FC = 7.86). Significant fact is that all these non-melanoma skin pathologies showed up-regulation of this gene. Until now, there has been no study concerning *DEFB4A* mRNA expression in KA, SCC and BCC simultaneously.

In the present study, the level of defensin beta 2 mRNA in KA, SCC and BCC was validated by the use of real-time QRT-PCR. Due to a marked difference between the analysed tumours, the expression of *β-actin* mRNA could not be used for normalisation of the results obtained. Thus, the number of mRNA copies was recalculated per microgram of total RNA. All of the tumours analysed showed higher expression of *DEFB4A* mRNA compared with the margins of the healthy tissues. Our results are in agreement with other published data. Gambichler et al. demonstrated up-regulation of *DEFB4A* mRNA in BCC compared with healthy controls (8). In oral SCC, Yoshimoto et al. observed the expression of *HBD-2* both in inflamed lesions with bacterial infection and non-inflamed carcinomas using the *in situ* hybridization technique (41). Moreover, they demonstrated the expression of *HBD-2* in normal oral epithelia only in areas adjacent to the SCCs. Shi et al. reported overexpression of HBD-2 in rat

oesophageal squamous cell carcinoma (SCC) and in oesophageal SCC tissues in patients (6). We compared the mRNA level of the *DEFB4A* gene between KA, SCC and BCC. The expression was significantly higher both in KA and SCC compared with BCC. Our results showed that the expression of *DEFB4A* mRNA is similar in KA and SCC. However, such similarity does not indicate the same outcome in these two tumours. Defensin beta 2 is one of the most important factors involved in the immunological reaction and keratinisation. In KA, induction of the immune response, which inhibits uncontrolled growth and limits the proliferation potential of KA cells, may lead to its regression (21). Furthermore, the regression of KA may be related to the cessation of the differentiation of the cells, which become keratinised and are not able to proliferate any more, and, therefore, die. The cessation of the tumour cells' differentiation is then manifested by keratinisation. Yoshimoto et al. suggested that HBD-2 may lead to the death of normal keratinocytes adjacent to the SCCs, which might, in turn, indirectly assist in the multiplication of tumour cells (41). The molecular mechanism underlying KA transformation into SCC is still unclear. Probably, transformed cells (SCC) present in the KA tumour proliferate during the keratinisation of KA. Along with changes at mRNA level of genes coding proteins involved in intracellular signalization of KA common for BCC and SCC, changes in *DEFB4A* expression suggest complex interactions at protein level of unknown results. Therefore, planning therapeutic approaches to KA based on stimulation of the *DEFB4A* gene expression should be considered carefully.

The defensin beta 2 mRNA level may constitute a useful tool for the differentiation of BCC from KA and from SCC. KA and SCC cannot be differentiated on the basis of the *DEFB4A* mRNA level. To definitely confirm the usefulness of the expression of *defensin beta 2* in differentiation of KA from SCC and BCC, more detailed studies of larger populations are needed.

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