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Transforming Growth Factor beta in basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and keratoacanthoma (KA)

Transformujący czynnik wzrostu beta w raku podstawnokomórkowym, kolczystokomórkowym i rogowiaku kolczystokomórkowym

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Key words

TGFβ, non-melanoma skin cancers, oligonucleotide microarrays (Affymetrix)

Słowa kluczowe

TGFβ, niemelanotyczne nowotwory skóry, mikromacierze oligonukleotydowe (Affymetrix)

Summary

Introduction. Transforming Growth Factor β (TGFβ) activates signaling cascades which regulate cell proliferation, differentiation, apoptosis, inflammatory response and angiogenesis. In the early stages of malignant transformation this cytokine acts as an inhibitor of tumour growth. In the advanced stages of malignant transformation TGFβ acts as a promoter of metastasis. Changes in the expression of genes associated with TGFβ activity could provide a new strategy of molecularly targeted therapy.

Aim. The aim of this study was to compare the mRNA profile of genes associated with TGFβ signaling pathways in non-melanoma skin pathologies biopsy specimens of basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and keratoacanthoma (KA) in comparison to normal skin.

Material and methods. Tissue samples of KA, SCC and BCC were obtained from the central part of tumours. Healthy skin margins comprised the control group. mRNA profile of genes coding *TGFβ* and proteins involved in TGFβ-induced signaling pathways was determined using oligonucleotide microarrays (Affymetrix).

Results. Microarray analysis showed changes in profile of genes coding proteins involved in TGFβ-induced signaling pathways. In SCC *TGFβ-1* (*TGFB1*) was upregulated, comparing to controls. Both in KA and SCC, the most statistically significant change referred to *TGFBR3* (Transforming Growth Factor beta Receptor III) mRNA.

Conclusions. mRNA profile of genes coding proteins involved in TGFβ-induced signalization reveals strong molecular similarity of SCC and KA.

Streszczenie

Wstęp. Transformujący czynnik wzrostu β (TGFβ) aktywuje kaskady sygnałowe regulujące proliferację komórek, ich różnicowanie, apoptozę, odpowiedź immunologiczną i angiogenezę. W początkowych stadiach transformacji nowotworowej cytokina ta pełni funkcję inhibitora wzrostu guza. W zaawansowanych stadiach TGFβ działa jako promotor przerzutowania. Zmiany ekspresji genów powiązanych z aktywnością biologiczną TGFβ mogą przyczynić się do opracowania nowej strategii molekularnie ukierunkowanej terapii.

Cel pracy. Celem pracy było porównanie profilu ekspresji genów powiązanych z sygnalizacją indukowaną przez TGFβ w niemelanotycznych patologiach skóry: raku podstawnokomórkowym (BCC), raku kolczystokomórkowym (SCC) oraz rogowiaku kolczystokomórkowym (KA), w porównaniu do kontroli.

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Materiał i metody. Wycinki pobrano z centrum guza (BCC, SCC i KA) oraz marginesów tkanki histopatologicznie prawidłowej (kontrole). Profil mRNA genów kodujących *TGFβ* oraz białka zaangażowane w sygnalizację indukowaną przez TGFβ wyznaczono techniką mikromacierzy oligonukleotydowych (Affymetrix).

Wyniki. Analiza techniką mikromacierzy wykazała zmiany w profilu genów kodujących białka zaangażowanego w sygnalizację indukowaną przez TGFβ. W porównaniu do kontroli, w SCC stwierdzono nadekspresję *TGFβ-1* (*TGFB1*). Zarówno w SCC, jak i w KA największą zmianę wykazał gen kodujący receptor III dla TGFβ (*TGFBR3*).

Wnioski. Profil ekspresji genów kodujących *TGFβ* oraz białka zaangażowane w sygnalizację indukowaną przez TGFβ wykazuje silne molekularne podobieństwo pomiędzy SCC i KA.

INTRODUCTION

Molecular studies carried out at different levels of the flow of genetic information, allow precise characterization of what is really happening in normal cells or pathologically changed. Knowledge and understanding of the mechanisms responsible for the induction and progression of malignant transformation becomes more and more likely, what in the future may result in a modification of diagnostic algorithms and personalization of molecularly targeted therapy. Integrated analysis is made possible by the large-scale research platforms adapted to evaluate the integrity of the genome (whole-genome microarrays), analysis of transcriptome changes (expression microarrays), the analysis of the mechanisms responsible for transcription regulation (epigenetic control), metabolomics (phenotypic microarrays), the analysis of proteome (protein microarray) or kinomics (the state of proteins' phosphorylation).

The TGFβ superfamily includes a large group of structurally related regulatory proteins with over 60 members, including at least 29-42 representatives encoded by the human genome (1). Until recently, the family TGFβ was divided into two basic subfamilies: TGF/Activins and BMP/GDF (bone morphogenetic protein/growth and differentiation factor) (2). Currently it is divided into 4 main groups: 1) TGFβ, 2) activins and inhibins, 3) bone morphogenetic proteins (BMPs) including at least 11 growth and differentiation factors – GDFs, and 4) MIF (also known as anti-Müllerian hormone – AMH) or MIS (Müllerian inhibitory substance) (3). TGFβ group comprise of five molecular isoforms not related to TGFα and each of them is encoded by separate gene. Three isoforms have been identified in mammals: TGFβ1, TGFβ2, TGFβ3. They are pleiotropic cytokines involved in cell cycle regulation (4), differentiation (5), apoptosis (6), cel migration (7) and in the formation and degradation of extracellular matrix components (8) including type I collagen (9). These factors are suppressors of proliferation of vascular endothelial cells and hematopoietic cells (10), significantly affecting the regulation of the immune response (4). In advanced stages of cancer of TGFβ acts as a promoter of metastasis by: modulating the microenvironment of the tumor cells and extracellular matrix synthesis, induction of chemokines secretion, silencing immunological response and participation in epithelial-mesenchymal transition (EMT) (11). Signaling pathways in tumors induced by TGFβ ligands may lead to inhibition of carcinogenesis or progression of cancer, depending on cancer staging (12). In the early stages of malignant transformation TGFβ activates signaling cascades which stimulate the expression of genes involved in inhibition of proliferation, cell differentiation stimulation, apoptosis or autophagy activation, suppression of angiogenesis and inflammatory response (13). In the advanced stages of the disease TGFβ acts as a promoter of metastasis through participation in epithelial-mesenchymal transition, remodeling of extracellular matrix and the microenvironment of tumor cells, inducing the synthesis of chemokines and immune response silencing (14).

TGFβ acts through two types of transmembrane serine-threonine kinase receptors: TβRI (TGFβR1) and TβRII (TGFβR2). In mammalian cells are present five kinds of receptor type II, seven kinds of type I and three kinds of type III receptor, which are involved in signal transduction activated by transforming growth factor as accessory/auxiliary receptors. These proteins have no functional intracellular domain, and therefore are not direct signal transmitters. Their involvement in the regulation of signaling pathways activity triggered by TGFβ involves presenting of cytokines to TGFβR1 and TGFβR2 receptors or limiting of their interaction with receptors. This type of receptors is specific only for TGFβ receptors group and is particularly important for TGFβ2 isoform, which has very low affinity for TGFβR2 and requires the presence of an auxiliary receptor TGFβR3 to facilitate formation of complexes with TGFβR2 (15). TGFβ type I receptors, known as ALK (Activin-like kinase) consist of the extracellular binding domain, transmembrane domain and a 30 amino acid regulatory region, rich in repeating glycine and serine residues (GS region) located above the catalytic domain of serine-threonine kinase (16). Type II receptors (TGFβR2), like TGFβR1, consist of the N-terminal extracellular ligand binding domain with characteristic cysteine CXCX4C pattern, transmembrane region and a C-terminal domain with serine-threonine kinase activity (3). Five receptors of TGFβ type II have been described: BMP receptor (BMP RII), activin type II receptor (Act RII), activin receptor β – Act RIIβ and Müllerian inhibitory substance type II receptor (MIS RII) (17). After binding with a ligand type II receptors phosphorylate

type I receptors, resulting in activation of SMAD family transcription factors – involved in the canonical TGFβ signaling pathway (16).

TGFβ and its antagonists have enormous potential in the treatment of diseases that are now resistant to conventional therapy. Analysis of gene expression associated with TGFβ activity and the design of additional analogs and antagonists of TGFβ is an object of many studies aimed at developing new molecularly targeted treatment strategies (18).

AIM

The aim of this study is to compare the concentration profile of 1050 mRNA associated with Transforming Growth Factor beta (TGFβ) signaling pathways in cancer biopsy specimens of basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and keratoacanthoma (KA) in comparison to normal skin and selecting mRNA significantly differentiating analyzed transcriptomes.

MATERIAL AND METHODS

Material

The study included a group of 39 patients diagnosed and treated in the Dermatology Clinics and Department of Medical University of Silesia in Katowice. The tumours located on the skin of the face and head were pathomorphologically and clinically examined. Based on these results 19 samples were enrolled to transcriptome analysis: 6 cases of kerathoacanthoma (KA), 3 cases of squamous cell carcinoma (SCC), 7 of basal cell carcinoma (BCC) and 4 margins of healthy tissues. After surgical excision, tissue samples were immediately preserved in the RNA stabilisation reagent RNAlater (Qiagen GmbH, Hilden, Germany). All of the patients were informed about the research and signed an informed consent form. The study was approved by the Bioethical Commission of the Medical University of Silesia.

Extraction of total RNA

Total cellular RNA was isolated from tissue samples with the use of TRIZOL® reagent (Invitrogen Life Technologies, Kalifornia, USA), according to the manufacturer's protocol. Extracts of total RNA were purified with the use of RNeasy Mini Kit (Qiagen Gmbh, Hilden, Germany) and treated with DNAase I (Fermentas International Inc., Ontario, Kanada) according to the manufacturer's protocol. The RNA concentration was determined with the use of Gene Quant II spectrophotometer (Pharmacia LKB Biochrom Ltd., Cambridge, UK). The quality of RNA was estimated electrophoretically (1% agarose gel stained with ethidium bromide).

Oligonucleotide microarray

10 μg of purified RNA was reverse transcribed with the use of SuperScript Choice System (Invitrogen Life Technologies, California, USA). dsDNA was purified using Phase Lock Gel Light (Eppendorf, Germany). Synthesis of biotynylated cRNA was performed with the use of BioArray HighYield RNA Transcript Labeling Kit (Enzo Life Science, New York, USA). Biotynylated cRNA was purified using RNeasy Mini Kit (Qiagen Gmbh, Hilden, Germany). Fragmentation of 16 μg cRNA was performed with the use of Sample Cleanup Module (Qiagen Gmbh, Hilden, Germany). Hybridization with the oligonucleotide microarray HG U133A (Affymetrix, California, USA) was performed according to Affymetrix Gene Expression Analysis Technical Manual (Affymetrix, California, USA). Fluorescence intensity was measured with the use of Agilent GeneArray Scanner G2500A (Agilent Technologies, California, USA).

Statistical analysis

For finding significant genes between KA, SCC, BCC and control samples 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 $p < 0.05$.

RESULTS

mRNA concentration profiles of genes involved in TGFβ signalling pathways in KA, SCC, BCC and healthy skin margins were appointed with the use of oligonucleotide microarrays HG-U133A (Affymetrix). Comparative analysis of 10 ID mRNA for *TGFβ* and its receptors with the use of Oneway ANOVA test with Benjamini-Hochberg Multiple Testing Correction showed statistically significant differences of *TGFB1* (*TGFβ1*) and *TGFBR3* mRNA level ($p < 0.05$). *TGFB1* was upregulated in SCC, comparing to controls. *TGFBR3* mRNA level was down-regulated both in SCC and KA in comparison to healthy skin margins.

In the next step changes in transcriptome of 1050 mRNA of genes coding proteins involved in TGFβ biological activity were evaluated. The set of genes was created based on Affymetrix database searching results and literature data. Oneway ANOVA test showed 120 ID mRNA statistically significantly different in analysed groups (significance level set at $p < 0.05$) (tab. 1A). In SCC 47 ID mRNA were differentiating comparing to controls (TukeyHSD Post Hoc test) (tab. 1B), among them 29 ID mRNA were characteristic only for SCC, 17 were common for SCC and KA and only one ID mRNA was common for SCC and BCC (tab. 2; fig. 1). KA transcriptomes showed 40 differentially expressed ID mRNA comparing to controls, where 23 mRNA were characteristic only for KA and 4 were characteristic for BCC. Nonparametric T test with Benjamini-Hochberg Multiple Testing

Correction showed 13 ID mRNA in KA and 1 ID mRNA in SCC, comparing to controls ($p < 0.05$) (tab. 3). No differences were found for BCC in comparison to control samples.

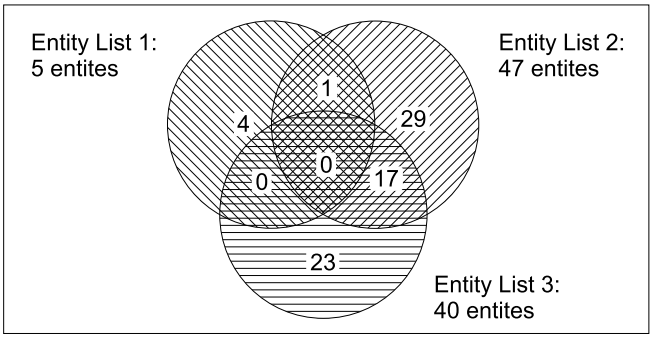

Fig. 1. Venn diagram showing number of differentiating transcripts in comparison to controls in groups of transcriptomes: KA – keratoacanthoma (Entity List 3), SCC – squamous cell carcinoma (Entity List 2), BCC – basal cell carcinoma (Entity List 1).

In the last stage of comparative analysis strength of mRNA in differentiation of SCC, KA and BCC from controls was estimated based on overrepresentation test results (19). Analysis revealed that among 120 ID mRNA indicated by Oneway ANOVA test 14 genes are significant for TGFβ signaling pathway and 7 genes are substantial for gonadotropin releasing hormone receptor pathway. Among 13 ID mRNA designated by nonparametric T test only 4 genes are significant for TGFβ signaling pathway (tab. 4). Diversity of fluorescence signals reflecting transcriptional activity of genes differentiating non-melanoma skin pathologies from controls is shown in the figure 2.

DISCUSSION

Malignant transformation is usually not a consequence of a single gene dysfunction but whole groups of genes involved in controlling of numerous metabolic and regulatory pathways in the cell. Analysis of expression of individual genes involved in these pathways is not sufficient to assess the physiological state of the cells. Expression microarray technology allows not only for analysis of transcriptional activity of over 20 thousand of genes in a single experiment, but also analysis of post-transcriptional modifications of mRNA. Because molecular changes precede phenotype features this technology can improve detection of lesions in the initial stage of their formation. It can also provide the basis for diagnostic strategy to facilitate the classification of histologically indistinguishable pathologies. In some cases the classical diagnostic methods are insufficient for the proper distinction of keratoacanthoma (KA) from well-differentiated squamous cell carcinoma (SCC) (20, 21). Moreover, clinical forms of

Table 1. The results of statistical analysis of 1050 IDmRNA of TGFβ responsive genes between the transcriptomes of KA – keratoacanthoma, SCC – squamous cell carcinoma, BCC – basal cell carcinoma, C – controls (healthy skin margins).
A – The results of Oneway ANOVA test with Benjamini-Hochberg Multiple Testing Correction showing 120 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	**All ID mRNA**	**p < 0.05**	**p < 0.02**	**p < 0.01**	**p < 0.005**	**p < 0.001**
	1050	120	42	27	19	4
B	**Group of transcriptomes**		**KA**	**SCC**	**BCC**	**C**
	KA		120	17	44	40
	SCC		103	120	55	47
	BCC		76	65	120	5
	C		80	73	115	120

Table 2. Differentiating transcripts illustrated on Venn diagram (fig. 1) characteristic for particular groups of transcriptomes of KA – keratoacanthoma, SCC – squamous cell carcinoma, BCC – basal cell carcinoma comparing to C – controls (healthy skin margins) appointed by TukeyHSD Post Hoc test.

Groups of transcriptomes	**Differentiating transcripts**	
	Number	**Symbol**
KA vs C – 40 ID mRNA		
KAvsC	23	SHC1; SH3BP5; NIT1; ITGB4; ITGB4; FOXD1; DPT; UBE2I; QRICH1; MFSD10; CITED2; PGF; PML; ITGB4; WIPF2; DPT; DPT; SHC1; ITGA6; ZFP106; RNF141; VGLL3; NCLN
SCC vs C – 47 ID mRNA		
KA vs C SCC vs C	17	PRDM4; ITGA6; TGFBR3; ACVR2A; PTHLH; SMAD1; PTHLH; NKX2-1; SMAD1; PTHLH; ACVR1B; WDR61; ARHGEF10; SMURF1; ZBTB3; C19orf54; AMIGO2
SCC vs C	29	TGFB1; PSG3; EML3; ARL4D; ICK; KRT15; INHBA; CDKN2B; IFNA14; CDKN1B; PSG9; KBP1A; NKX2-1; ESR2; GFR2; MGRN1; DHX30; ZC3H4; SMAD6; ROD1; PCDH1; RBM8A;NLK; RAB22A; TBX4; RUNX2; GDF15; IRGQ; TSR1
SCC vs C BCC vs C	1	EXPH5
BCC vs C – 4 ID mRNA		
BCC vs C	4	DYRK2; ACOX3; SMAD7; DUSP22

Table 3. Nonparametric T test with Benjamini-Hochberg Multiple Testing Correction results showing mRNA differentiating analysed trascriptomes of KA – keratoacanthoma, SCC – squamous cell carcinoma, BCC – basal cell carcinoma, comparing to C – controls (healthy skin margins).

FC all	AK vs C				SCC vs C		BCC vs C	
	P all	P < 0.05	P < 0.01	P < 0.005	P all	P < 0.05	P all	P < 0.05
	1050	13	2	0	1050	1	1050	0
FC > 1.1	614	13	2	0	677	1	510	0
FC > 1.5	121	7	2	0	130	1	67	0
FC > 2.0	36	6	2	0	35	0	21	0
FC > 3.0	10	1	1	0	8	0	1	0
Differentiating mRNA		ITGA6; NIT1; ACVR2A; ZNF135; PTHLH; CITED2; PGF; PTHLH; PTHLH; WIPF2; ACVR1B; ZBTB3; SMURF1			ZC3H4		–	

Table 4. Overrepresentation test results showing strength of differentiating mRNA of TGFβ responsive genes (displaying only results with p < 0.05; p-value calculated by the Binomial statistic).

All		120 mRNA (Oneway ANOVA*)			13 mRNA (T Test unpaired**)		
PANTHER Pathways		Number	p-value	Gene symbol	Number	p-value	Gene symbol
Unclassified	19446	73	0.00E00		6	0.00E00	
TGF-beta signaling pathway	95	14	3.68E-15	SMAD7; SMAD6; SMAD3; SMAD1; TGFB1; GDF15; BMP6; INHBA; FKBP1A; SMURF1; CITED2; ACVR1B; CVR2A	4	1.79E-05	CITED2; SMURF1; ACVR1B; ACVR2A
Gonadotropin releasing hormone receptor pathway	228	7	1.35E-02	TGFBR3; TGFB1; ACVR2A; ACVR1B; SMAD3; SMAD1	–	–	–

*Multiple Testing Correction Benjamini-Hohberg
**Bonferoni correction

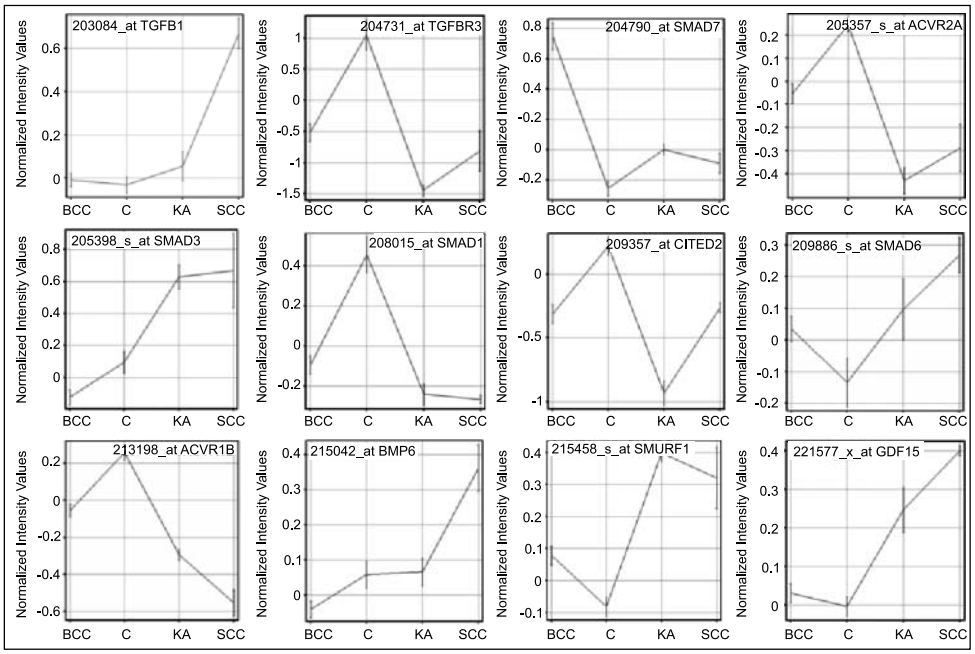

Fig. 2. Normalised intensity values of fluorescence signals (mean ± standard deviation) of differentiating mRNA in KA – keratoacanthoma, SCC – squamous cell carcinoma, BCC – basal cell carcinoma and C – controls (healthy skin margins).

SCC (sore and papular) morphologically resemble not only KA, but also basal cell carcinoma (BCC) (20, 22). Therefore finding additional markers is necessary for improvement of diagnosis of non-melanoma skin pathologies. Another interesting feature of keratoacanthoma is its tendency to self-regression with scarring (23). Molecular mechanism of this phenomenon is still unclear. Great expectations are associated with research aimed at understanding the complex function of TGFβ, both in tumour suppression and promotion. It is suggested that this cytokine may constitute one of the main factors responsible for modulation of cancer stem cells. TGFβ1 pathway is involved in transformation of human mesenchymal stem cells (MSCs) to cancer-associated fibroblasts (CAF), promoting tumor growth and metastasis (24).

In the present study transcriptomes of genes associated with the TGFβ activity in BCC, SCC, KA and

healthy skin margins (controls) were analyzed using oligonucleotide microarray (Affymetrix). In the first stage of comparative analysis 10 ID mRNA for TGFβ and its receptors were evaluated. Comparing to healthy skin margins *TGFB1* (*TGFβ1*) was upregulated in SCC, while *TGFBR3* (TβRIII) was down-regulated both in SCC and KA. Soluble form of TβRIII binds to TGFβ neutralizing its tumor promoting activity in late stage of tumors (25). The down-regulation or the loss of TβRIII expression was reported in many types of cancer at stage of tumour progression and metastasis. The loss of TβRIII expression was reported in breast cancer, ovarian cancer, renal cell carcinoma, and prostate cancer (25). The down-regulation at the mRNA and protein level was observed in non-small cell lung cancer, pancreatic adenocarcinomas, and well-differentiated endometrial carcinoma, as well as total inhibition of TβRIII expression in poorly differentiated endometrial carcinoma (25). In our study down-regulation of TβRIII expression, both in SCC and KA, supports previous observations that KA is a benign variant of squamous cell carcinoma (SCC) (26). However, the *TGFB1* mRNA was increased only in SCC, what suggests that intracellular signalization induced by TGFβ1 could not be identical in these two skin lesions. Consequently, comparative analysis of mRNA profile of genes coding proteins involved in TGFβ-induced signalization was performed. This profile was strongly changed in SCC, where 47 ID mRNA were differentiating comparing to controls. In keratoacanthoma 40 ID mRNA were differentially expressed, while in BCC only 5 ID mRNA were differentiating. Moreover, 17 mRNA were common for both, SCC and KA, and only one mRNA was common for SCC and BCC. KA and BCC had no mutual mRNA. These results show that, both in SCC and KA, TGFβ1-induced signalization could play a crucial role, and reveal molecular similarity of these skin lesions.

To assess the strength of mRNA in differentiation of SCC, KA and BCC from controls overrepresentation test was carried out. The binomial statistics tool of the PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System revealed that in the group of 13 differentiating mRNA (nonparametric T test), 4 mRNA are significant in TGF-beta signaling pathway: *CITED2* (*Cbp/p300-interacting transactivator*, with *Glu/Asp-rich carboxy-terminal domain*, *2*), *SMURF1* (*SMAD specific E3 ubiquitin protein ligase 1*), *ACVR1B* (*activin a receptor*, *type IB*; *ALK4*), and *ACVR2A* (*activin A receptor*, *type IIA*; *ActRIIA*). CITED2 is transcriptional modulator inhibited by TGFβ at the posttranscriptional level (27). Mice lacking CITED2 expression show abnormal fetal lung development with aberrant differentiation of alveolar epithelial cells (27). In our research *CITED2* was significantly down-regulated in KA, with the highest expression in controls. *SMURF1* was overexpressed, both in KA and SCC. High expression of SMURF1 has been shown in human colorectal cancer, pancreatic cancer, and in drug resistant HNSCC (head and neck squamous cell carcinoma) (28). It promotes tumor invasiveness and correlate with cancer progression and prognosis. Both activin receptors – *ACVR1B* and *ACVR2A* were down-regulated in SCC and KA. A homozygous deletion of the activin A receptor, type IB was reported in pancreatic cancer, resulting in an aggressive cancer phenotype (29). ActRIIA, together with BMPRII (Bone Morphogenetic Protein Type II Receptor), is necessary for endoglin-mediated suppression of prostate cancer invasion (30).

CONCLUSIONS

In conclusion, mRNA profile of genes coding proteins involved in TGFβ-induced signalization reveals strong molecular similarity of SCC and KA. This result indicate that TGFβ biological activity could play a crucial role in the molecular mechanism of these skin lesions development.

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