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Increased Expression of TGF-β/Smad Proteins in Basal Cell Carcinoma

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Abstract
Background: Basal cell carcinoma (BCC) is the most common cancer in humans placing a significant burden on healthcare services worldwide. There is an increasing evidence that the development of cutaneous epithelial tumours is pathogenetically linked to dysregulations of the transforming growth factor β (TGF-β) and its signalling molecules, the Smads.
Objective: In the present study we aimed to investigate the mRNA as well as protein expression of TGF-β/Smad signalling proteins in patients with BCC and healthy controls.
Methods: In this prospective pilot study, 24 patients with BCC were recruited. Punch biopsies were harvested from the centre of the tumour (lesional) as well as an adjacent healthy skin site (non-lesional controls). In addition to the specimens of BCC patients, skin samples (healthy controls) were obtained from subjects who had no history of skin cancer (n = 25). Real-time RT-PCR as well as immunohistochemistry was performed.
Results: The mRNA levels of TGF-β/Smad transducers observed in healthy controls did not significantly differ from TGF-β/Smad levels observed in non-lesional skin of BCCs patients ($P > 0.05$). RT-PCR revealed significant mRNA overexpression of TGF-β1, Smad3, and Smad7 in BCCs as compared to non-lesional skin ($P < 0.05$). TGF-β1 mRNA expression significantly correlated with Smad3 ($r = 0.60$; $P < 0.05$) and Smad7 ($r = 0.76$; $P < 0.05$) levels. Immunohistochemistry demonstrated marked protein overexpression of Smad3 in tumour tissue as compared to non-lesional skin.
Conclusions: Our data suggest a possible role of TGF-β/Smad signalling in the pathogenesis of BCC.

Key words: Non-melanoma skin cancer; Epithelial neoplasia; Transforming growth factor

Abbreviations: Basal cell carcinoma, BCC; transforming growth factor β, TGF-β.

1. Introduction

Basal cell carcinoma (BCC) is the most common cancer in humans. It is classified, together with squamous cell carcinoma, as nonmelanoma skin cancer. The incidence of BCC is increasing worldwide by up to 10% a year. Although mortality is low as BCC rarely metastasises, this malignancy causes considerable morbidity and places a significant burden on healthcare services worldwide. Three main types of BCCs are generally distinguished with regard to the histopathological growth pattern: nodular, superficial, and morphoeic. BCCs are believed to derive from the epidermis, specifically the basal cell layer and the outer root sheath of the hair follicle. The development of BCC is clearly associated with mutated p53 tumour-suppressor gene and constitutive activation of the sonic hedgehog signalling pathway, including PATCHED (PTCH), sonic hedgehog (Shh) and smoothened (Smo), which regulate cell proliferation and cell fate determination [1, 2].

There is an increasing evidence that the development of cutaneous epithelial tumours such as squamous cell carcinoma (SSC) is pathogenetically linked to dysregulations of the transforming growth factor β (TGF-β) and its signalling molecules, the Smads [3-6]. Members of the TGF-β family, which includes three isoforms of TGF-β (TGF-β1/2/3) exert a wide spectrum of biological responses on a large variety cell types, e.g. regulation of cell growth, differentiation, matrix production, inflammation, host defense, and apoptosis [7, 8]. Typically, TGF-β signalling initiates with binding and activating specific dual cell-surface receptors (type I and II) that have intrinsic serine/threonine kinase activity. Smads are the initial responders to receptor activation of the TGF-β family and have been studied as transcriptional activators of cell differentiation. There are three types of Smads: receptor-regulated (R-Smads), common mediator (Co-Smads) and inhibitory (I-Smads) [7-9]. After ligand binding, the type II TGF-β receptor phosphorylates the type I TGF-β, which, in turn, phosphorylates and activates the R-Smads (e.g., Smad3). The R-Smads bind then to a Co-Smad (Smad4), and this complex translocates to the nucleus, where the complex regulates transcription of TGF-β-responsive genes. The inhibitory Smad7 associates with ligand-activated type I TGF-β receptor and interferes with phosphorylation of R-Smads (e.g., Smad3) by preventing their interaction with activated type I TGF-β receptor. Since the expression of Smad7 is induced by TGF-β1, Smad7 inhibits TGF-β signalling by a negative feedback system [8-9].

It has previously been shown that TGF-β1 plays a dual role in skin carcinogenesis, particularly in SCC.

When TGF-β1 transgene is targeted primarily in the suprabasal/differentiated layers of the epidermis in transgenic mice, TGF-β1 overexpression inhibits papilloma formation at early stages but promotes tumour progression, metastasis, and epithelial-mesenchymal transition at later stages of skin carcinogenesis [5, 10]. The earlier tumour-suppressive role of TGF-β1 is attributed to its growth-inhibitory effect on keratinocytes, whereas the later tumour promotion role is associated with its effects on the loss of epithelial cell adhesion, extracellular matrix remodelling, and enhanced angiogenesis [11]. Current data suggest, however, that TGF-β1 overexpression may have a tumour-promoting effect even at the early stages of skin carcinogenesis if overexpressed in proliferative cells of the epidermis/tumour epithelia [5]. Recently, the essential role of Smad3 and Smad4 in repressing as well as promoting skin tumour formation through TGF-β pathway has been documented in SCC [12, 13].

To our best knowledge, there have been published only four studies investigating the expression of TGF-β signal transducers in BCC [14-17]. Data of these trials using immunohistochemistry [14, 15, 17] and in situ hybridization [16], respectively, indicate that the expression of TGF-β1 and Smad proteins is altered in BCC. In the present study we aimed to investigate the mRNA as well as protein expression of TGF-β/Smad signalling proteins in patients with BCC and healthy controls.

2. Material and Methods

2.1. Subjects

In this prospective pilot study, 24 patients who were suspected of having BCC on the basis of clinical and dermoscopic features, underwent complete tumour excision in our dermatological surgery. Three millimetre punch biopsies were harvested from the centre of the tumour (lesional) as well as an adjacent healthy skin site approximately 5 mm in distance to the tumour border (non-lesional skin controls). In addition to the BCC patient group, skin samples (healthy skin controls) were obtained from subjects who had no history of skin cancer (n = 25). This study adhered to the Declaration of Helsinki and ethics approval for research was obtained from the local review board of the Ruhr-University Bochum (Bochum, Germany). All patients who participated in the investigation signed an informed consent form.

2.2. Quantitative Real-time RT-PCR

Quantitative analysis of real-time RT-PCR was performed using the method of Giulietti et al. [18]: total cellular RNA was isolated from skin tissue samples using RNeasy® Lipid Tissue Kit (QIAGEN, Chatsworth, CA) following the manufacturer´s protocol. Prior to cDNA synthesis. RNA was digested with RNase-free DNase I (Roche Diagnostics, North America). cDNA was synthesized by reverse transcription from DNase I treated RNA using MultiScribe™ reverse transcriptase enzyme and random hexamers primers (TagMan® Reverse transcription reagents, Applied Biosystems, Forster City, CA). Real-time PCR was performed using a Taqman SYBR Green PCR Master Mix and GeneAmp®Sequence Detection System (Applied Biosystems). PCR Primers for TGF-β1, Smad proteins, and the housekeeping gene (GAPDH) were designed using the computer program Primer Express (PE Biosystems) and produced by the custom oligonucleotide synthesis service TIB Molbiol (Germany). The primer sequences for are described in Table 1. PCR amplifications were performed in a total volume of 25 µl, containing 5 µl cDNA sample, 5 µM of each primer and 12.5 µl SYBR Green PCR Master Mix. PCR was started with 2 min at 50 °C and an initial 10 min denaturing temperature of 95 °C, followed by a total of 40 cycles of 15 sec of denaturing and 1 min of annealing and elongation at 60 °C. A melting curve and 2% agarose gel electrophoresis served as controls. Relative expression levels were calculated by the relative standard curve method as outlined in the manufacturer's technical bulletin. The comparative Δ-ΔC_t method was used as previously suggested by Livak and Schmittgen [19]. A standard curve was generated using the fluorescent data from 10-fold serial dilutions of total RNA of the highest expression sample. This was then used to calculate the relative amounts of target mRNA in test samples. Quantities of all targets in the test samples were normalized to the corresponding GAPDH mRNA transcript in the skin samples. In order to make the quantities of mRNA levels more illustrative, the ΔC_t values (logarithms) were re-transformed.

2.3. Histology and Immunohistochemistry

The main portion of each tumour specimen was fixed in formalin, routinely processed, and embedded in paraffin. Sections were stained with haematoxylin and eosin. In addition, paraffin-embedded sections of five patients (lesional and non-lesional) were mounted on silanized slides and stored for one hour in a humid chamber at 60°C. Sections were deparaffinized in xylene and washed with 100%, 96%, and 70% ethanol for five minutes each and rinsed with demineralized water. After washing with Target Retrieval Solution, pH 9.0 (EDTA), DAKO, S2367 for 20 minutes, sections were stored for 20 minutes in a humid chamber at 25 °C. Sections were covered with 200 µl anti-rabbit Ig biotin TGF-β1 and Smad3/4/7 (Santa Cruz Biotechnology, California, USA) for 30 minutes at 25 °C in a DAKO Autostainer (DAKO Cytomation, Hamburg, Germany). After washing with Wash Buffer 10 times (DAKO Cytomation) for two minutes, streptavidin-AP, DAKO K5005 was used as enzyme for 15 minutes. Chromogen red (Red permanent), DAKO, K5005, was used for visualization before counterstaining with haematoxylin and mounting in Mowiol (Roche Molecular Biochemicals, Mannheim, Germany). All sections were separately evaluated for microscopic features of BCC. Staining sensitivity was verified using skin sections from healthy controls. The mean percentage of positively stained tumour and stroma cells was independently evaluated in three fields of view (0.0625 mm^2) by two investigators.

2.4. Statistics

Analysis of mRNA data was performed using the statistical package MedCalc Software (Mariakerke, Belgium). Non-normal distribution of data was confirmed by the D'Agostino-Pearson test. Hence, data were expressed in medians and range. The results were analysed using paired or independent non-parametric tests including the Wilcoxon-rank test, the Mann-Whitney test, and the Spearman rank correlation procedure, respectively. We constrained experiment-wise error rates due to multiple comparisons to the standard alpha (P) level of <0.05 by the Bonferroni method.

3. Results

We investigated 13 men and 11 women with a median age of 75.2 years and an age range of 66 to 93 years. Histological examination revealed 15 nodular BCCs and 9 superficial BCCs. The medians including the 95% confidence interval of the range of mRNA expression of TGF-β/Smad proteins in BCC patients (n = 24) and healthy controls (n = 25) are illustrated in Fig. 1. The values of the medians and range of TGF-β/Smad expression levels are detailed in Table 1. The mRNA levels of TGF-β/Smad proteins observed in healthy controls did not significantly ($P > 0.05$) differ from TGF-β/Smad levels observed in non-lesional of BCCs patients. BCCs showed significantly ($P < 0.05$) increased mRNA levels of TGF-β1, Smad3, and Smad7 as compared to non-lesional skin. By contrast, Smad4 mRNA expression did not significantly ($P > 0.05$) differ between lesional and non-lesional skin. TGF-β1 mRNA expression significantly correlated with Smad3 ($r = 0.60$; $P < 0.05$) and Smad7 ($r = 0.76$; $P < 0.05$) expression. The mRNA expression of TGF-β/Smad found in nodular and superficial BCCs did not significantly differ (data not shown). As shown in Table 1, immunohistochemistry revealed a markedly higher percentage of Smad3 positively stained cells in the tumour parenchym and stroma as compared to the epidermis and dermis of non-lesional skin (Fig. 2). However, staining for TGF-β1, Smad4, and Smad7 did not show substantial differences between BCC and non-lesional skin.

4. Discussion

This was the first systematic study investigating the expression of TGF-β1 and Smad proteins in human BCC using real-time RT-PCR as well as immunohistochemistry. Previously, Stamp et al. [17] immunohistologically studied 29 BCCs showing overexpression of extracellular TGF-β1 in the desmoplastic stroma as compared to normal skin. Fibroblasts and endothelial cells in the desmoplastic stroma also stained. Schmid et al. [16] analyzed and compared, by in situ hybridization, the mRNA expression patterns of all three mammalian TGF-β isoforms and of the TGF-β type II re-

Table 1. Data of quantitative real-time reverse transcritase polymerase chain reaction (RT-PCR; n = 24) and immunohistochemistry (IHC; n = 5) for TGF-β/Smad mRNA (# median, range) and protein (* mean ± SD) expression investigated in patients with basal cell carcinoma (lesional, non-lesional) and healthy controls (only RT-PCR data; n = 25).

Method	lesional	non-lesional	healthy controls
TGF-β1 (PS)	F 5´-GGTACCTGAACCCGTGTTGCT-3´, R 5´-TGTTGCTGTATTTCTGGTACAGCTC-3´		
RT-PCR	0.031 (0.003 – 0.144)	0.011 (0.000 – 0.107)	0.015 (0.005 – 0.390)
IHC	8.1 ± 13 / 12.3 ± 15	2.1 ± 19 / 8.7 ± 9.8	
Smad3 (PS)	F 5´-TGAGTTCGCCTTCAATATGAAGAA-3´, R 5´-CAGGAGGTAGAACTGGTGTCTCTACTCT-3´		
RT-PCR	0.466 (0.089 – 4.955)	0.235 (0.032 – 0.854)	0.259 (0.109 – 1.051)
IHC	51.5 ± 14.4 / 41.8 ± 14.6	30 ± 7.9 / 21.3 ± 15.9	
Smad4 (PS)	F 5´-ACTGCAGAGTAATGCTCCATCAAGT-3´, R 5´-GGATGGTTTGAATTGAATGTCCTT-3´		
RT-PCR	0.668 (0.043 – 1.661)	0.590 (0.211 – 1.119)	0.659 (0.356 – 2.88)
IHC	66.8 ± 9.7 / 67 ± 17.1	54.8 ± 14.3 / 75.2 ± 9.1	
Smad7 (PS)	F 5´-TAGCCGACTCTGCGAACTAGAGT-3´, R 5´-GGACAGTCTGCAGTTGGTTTGA-3´		
RT-PCR	0.025 (0.007 – 0.224)	0.013 (0.002 – 0.025)	0.017 (0.006 – 0.027)
IHC	20.2 ± 20 / 13.6 ± 14.7	19.9 ± 21 / 9.1 ± 7.4	
GAPDH (PS)	F 5`-CCTCAACTACATGGTTTACA-TGTTCC-3´, R 5`-ATGGGATTTCCATTGA-TGA-CAAG-3´		

PS, primer sequence; GAPDH, Glyseraldehyde-3-phosphate dehydrogenase ; # re-transformed ΔC_t values (logarithms); * mean percentage (%) of positively stained cells.

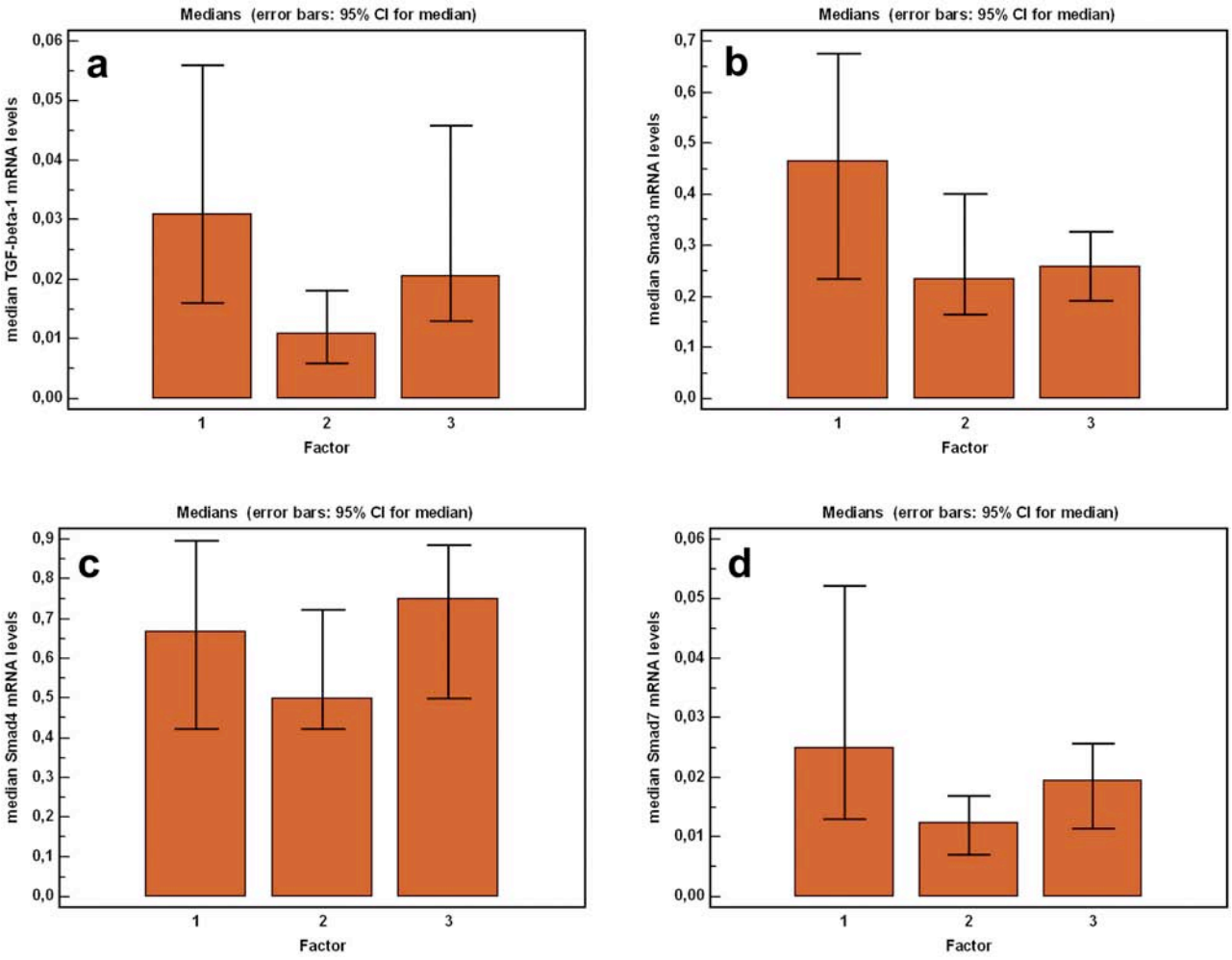

Fig. 1. Diagram illustrating a significant ($P < 0.05$) increase of median mRNA expression of TGF-β1 (a), Smad3 (b), and Smad7 (d) in basal cell carcinoma (n = 24), as compared to non-lesional skin. Smad4 (c) mRNA levels found in basal cell carcinoma did not significantly differ from non-lesional skin. For all TGF-β/Smad proteins investigated differences of mRNA expression between non-lesional skin and healthy controls (n = 25) were not significant ($P > 0.05$).

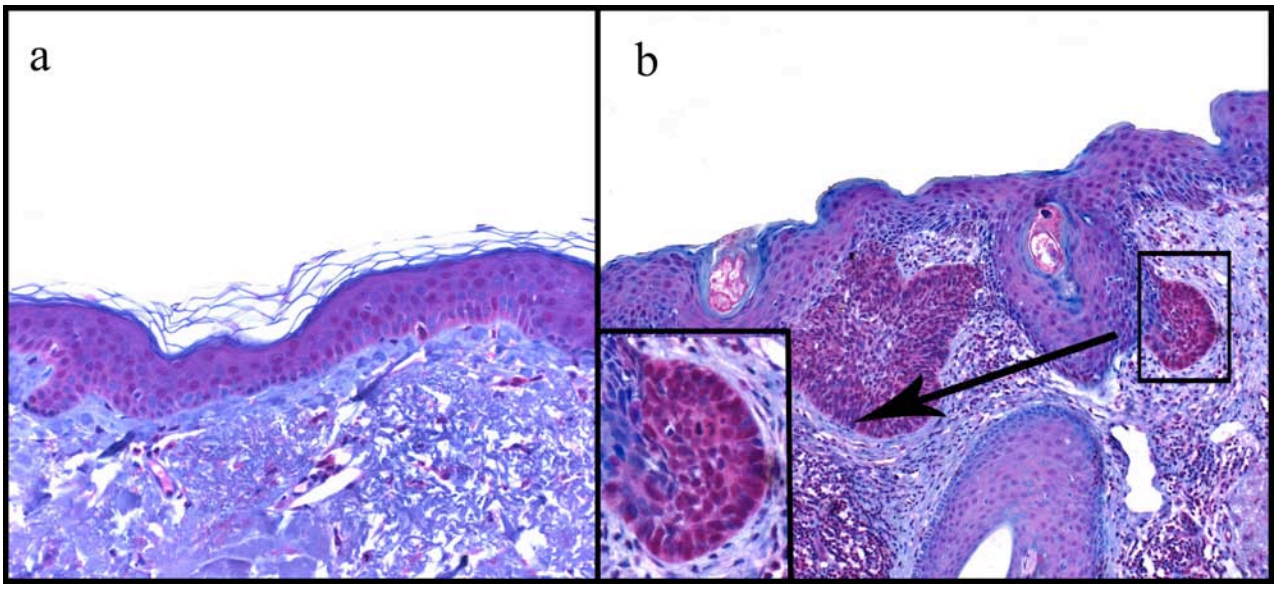

Fig. 2. Immunohistology for Smad3 of non-lesional skin (a) and BCC (b) demonstrating increased staining of the tumour parenchym (arrow) and stroma.

ceptor in normal skin and 11 BCCs. The stroma of most BCCs revealed enhanced TGF-β1 and TGF-β type II receptor mRNA expression when compared with normal skin. The expression of TGF-β1 mRNA was comparably weak in tumour tissues and normal skin epithelia. Immunohistological studies performed by Furue et al. [14] confirmed the accumulation of stromal cells possessing TGF-β1 and TGF-β type II receptor in the fibrotic stroma of some BCCs, suggesting that the secretion of collagens from the stromal cells may be enhanced by TGF-β1 in an autocrine fashion, at least in certain stages of development of BCC [14]. Lange et al. [15] immunohistologically investigated the expression of TGF-β related Smad proteins in 11 BCCs. In contrast to the data of the aforementioned studies and the present work, they found a markedly decreased protein expression of TGF-β1. Furthermore, they observed a significant reduction of Smad2/3/4/6/7 expression in BCCs when compared with normal skin.

In the present study, we demonstrated that mRNA levels of TGF-β1 and Smad transducers do not significantly differ between non-lesional skin of BCC patients and healthy controls. We observed a significant overexpression of TGF-β1 mRNA in BCC which is also seen in human SCC and its precursors [4]. However, our immunohistological studies revealed only a slight increase of TGF-β1 protein in the tumour tissue. This discrepancy in the pattern of TGF-β1 expression at mRNA and protein levels can be explained by the fact that real-time RT-PCR is a much more sensitive technique able to document the slightest evidence of gene expression. However, immunohistochemical analysis is important since it shows whether the transcript is translated and is probably functionally available. Moreover, the stability of the protein, in particular a nuclear protein, is also an issue. The protein may be translated at normal levels but degraded at an abnormal level.

Interestingly, we observed a strong positive correlation between mRNA overexpression of TGF-β1 and Smad3/7. The significant overexpression of Smad7 mRNA that was observed in tumour tissue could however not be confirmed by immunohistochemistry. Since Smad7 is induced by TGF-β1 and its expression level is regulated by negative feed-back loops, the significant increase of Smad7 mRNA that was observed in BCCs could be directly linked to the overexpression of TGF-β1 [6]. Notably, overexpression of Smad7 has been found in pancreatic carcinoma as well [20].

We observed significant overexpression of Smad3 mRNA in BCC which could also be confirmed by our immunohistological studies. Recently, Tannehill-Gregg et al. [13] demonstrated that transgenic mice heterozygous for Smad3 exhibit greater resistance to chemically induced squamous cell carcinomas than wild-type mice. This would suggest that Smad3 can function in an oncogenic fashion because heterozygous loss is protective against chemically induced cutaneous neoplasms. Paradoxically, complete loss of Smad3 protein expression has been observed in chemically induced skin tumours in mice as well [21]. Furthermore, analysis of head and neck squamous cell carcinomas for Smad3 protein by immunohistochemistry demonstrated comparable nuclear staining in both normal and neoplastic cells [22]. Although the role of Smad3 in the development of BCC has not been established, Smads are generally thought of as tumour suppressors. However, our data indicate that Smad3 may act as an oncogene in BCC which is supported by the results of Tannehill-Gregg et al. [13]. By contrast, Qiao et al. [12], showed in a knockout mouse model that skin-specific disruption of Smad4 impairs differentiation of hair follicles, and increased proliferation of keratinocytes of the epidermis, consequently leading to malignant tumour formation. The majority of tumours were SCCs, whereas BCCs only infrequently occurred [12]. In the present study, mRNA as well as protein expression of Smad4 in lesional skin did not significantly differ from non-lesional skin possibly indicating that Smad4 does not play a predominant role in the pathogenesis of BCC in human skin [23]. Epithelia-mesenchymal transitions in embryonic cells are induced by TGF-β/Smad signalling. The finding that recombinant TGF-β1 can induce an epithelia-mesenchymal transition in vivo contributes to our understanding of the molecular mechanisms responsible for changes in cell plasticity essential to both embryogenesis and oncogenesis.

In conclusion, TGF-β/Smad expression appears to be comparable in non-lesional skin of BCC patients and healthy controls. Significantly correlating overexpression of TGF-β1 and Smad3/7 mRNA is observed in tissue of BCC when compared to non-lesional skin. Our data suggest a possible role of TGF-β/Smad signalling in the pathogenesis of BCC. However, the validity of our data is limited, since first, it is not possible to ascribe the differences in TGF-β/Smad mRNA expression to a certain cell type, and second, we did not perform functional studies. Hence, further studies are needed to fully explore the significance of the TGF-β/Smad pathway in BCC.

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