Lack of Evidence for Activation of the Hedgehog Pathway in Psoriasis

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Recent reports have suggested that the hedgehog (Hh) pathway is activated in lesional psoriatic skin, and that treatment with the Hh pathway antagonist cyclopamine may lead to rapid resolution of the disease. To assess Hh pathway activity in psoriasis, we isolated RNA from lesional and uninvolved skin of 58 psoriatic patients, and from 63 normal control subjects, and subjected these samples to global gene expression profiling on Affymetrix HU133 Plus 2.0 gene arrays. We were especially interested in Hh target genes (*PTCH1* and *GLI1*), whose expression is elevated in response to Hh signaling. The microarray data demonstrated downregulation of *PTCH1* expression in uninvolved and lesional skin (1.1-fold and 2-fold, respectively; $P<0.0001$). Additionally *GLI1* mRNA was downregulated in lesional skin (1.7 fold; $P<0.05$). No significant changes were observed between lesional and uninvolved skin for the Hh ligands or Smoothened. Quantitative PCR confirmed these findings. *In situ* hybridization for *GLI1* and *PTCH1* was positive in basal cell carcinoma tumor cells, but was negligible in uninvolved or lesional psoriatic skin. The absence of elevated Hh target gene expression in lesional psoriatic skin indicates that the Hh pathway is not activated in this disease, raising questions regarding the proposed use of Hh antagonists as antipsoriatic agents.

Journal of Investigative Dermatology advance online publication, 28 August 2008; doi:10.1038/jid.2008.266

INTRODUCTION

Psoriasis is a common chronic inflammatory skin disease characterized by complex alterations in epidermal growth, differentiation, and multiple, immunological, and vascular abnormalities. Psoriasis was historically considered to be a primary disorder of keratinocytes, but over the past two decades it has been firmly established that psoriasis is an immune disorder mediated by activated T cells (Gudjonsson *et al.*, 2004; Liu *et al.*, 2007). How activated T cells mediate the altered differentiation and hyperproliferation of keratinocytes and other changes observed in psoriasis is still unknown but is thought to involve a highly complex, yet incompletely understood, interaction of multiple cytokines and growth factors with multiple cellular effectors present within the psoriatic lesion. Additionally, the intercellular signaling pathways mediating these changes remain to be fully elucidated, although reports have indicated involvement of the signal transducer and activator of transcription 1 (Bowcock *et al.*, 2001), signal transducer and activator of transcription 3 (Sano *et al.*, 2005), mitogen-activated protein kinase (Johansen *et al.*, 2005b), activator protein 1 (Johansen *et al.*, 2004), and the NF-κB pathways (Lizzul *et al.*, 2005; Johansen *et al.*, 2005a).

Recent reports have suggested that the hedgehog (Hh) pathway is activated in lesional psoriatic skin (Kuenzli *et al.*, 2004; Tas and Avci, 2004; Endo *et al.*, 2006; Meth and Weinberg, 2006), and that pharmacological inhibition of this pathway using cyclopamine may lead to rapid resolution of the disease (Kuenzli *et al.*, 2004; Tas and Avci, 2004; Meth and Weinberg, 2006). The Hh signaling pathway is one of the major signaling pathways involved in embryonic development (Ingham and Placzek, 2006). During physiologic Hh signaling, Hh proteins bind to the cell surface receptor Patched (PTCH1), thereby releasing Smoothened (SMO) from PTCH-mediated inhibition. SMO activation then triggers a series of intracellular events, culminating in alterations in gene expression mediated by the Gli family of transcription factors (GLI1, GLI2, and GLI3; Ruiz i Altaba *et al.*, 2007; Figure 1). The transcripts for PTCH1 and GLI1 are reliable markers for both physiologic and pathologic Hh signaling activity as they are consistently induced when the Hh pathway is activated (McMahon *et al.*, 2003; Hutchin *et al.*, 2005). This pathway has been shown to be a crucial regulator of hair follicle growth and sebaceous gland biology (Allen *et al.*, 2003; Niemann *et al.*, 2003). On the other hand, sustained activation of the Hh pathway appears to be the driving force for basal cell carcinoma (BCC) development (Daya-Grosjean and Couve-Privat, 2005).

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Abbreviations: BCC, basal cell carcinoma; CCN, cyclin; DHH, desert hedgehog; Hh, hedgehog; IHH, Indian hedgehog; PTCH, Patched; QT, quantitative; SHH, Sonic hedgehog; SMO, Smoothened

Received 27 April 2008; revised 26 June 2008; accepted 30 June 2008

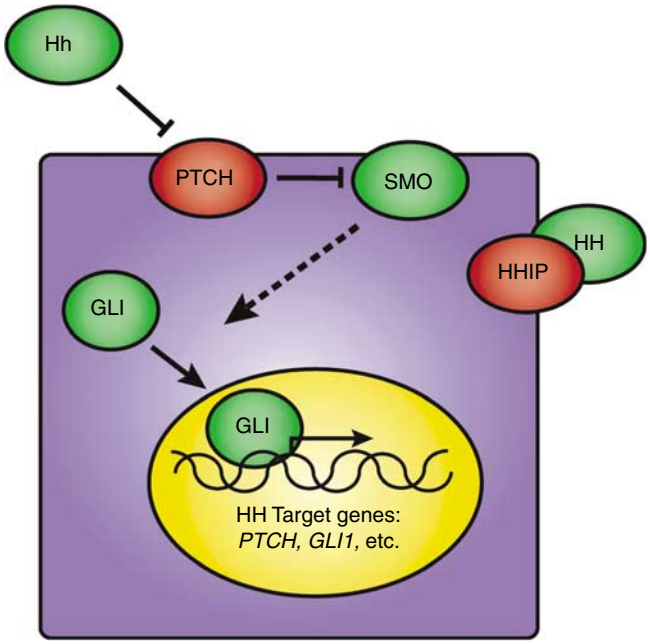

Figure 1. Highly simplified diagram illustrating key components of the Hh signaling pathway. Signaling activators are indicated in green, signaling inhibitors in red. In the absence of Hh ligands (SHH, IHH, or DHH), PTCH blocks the function of the key signaling effector, SMO. Hh ligands block the inhibitory effects of PTCH, leading to derepression of SMO and activation of intracellular signaling. HH pathway activation leads to reprogramming of gene expression via the GLI family of transcription factors (GLI1, GLI2, and GLI3). Hh target genes, in addition to PTCH and GLI1, include the Hh inhibitor protein HHIP, cyclins CCND1 and CCND2, and PTCH2.

The involvement of the Hh pathway in psoriasis was first suggested by the marked and rapid improvement observed in a clinical trial, in which 31 individual psoriatic lesions in seven patients, all with an established diagnosis of plaque or guttate psoriasis, were treated with topical cyclopamine (Tas and Avci, 2004). Furthermore, the authors asserted that topical cyclopamine was more effective than the potent topical steroid clobetasol-17 propionate, a typical first-line therapy (Tas and Avci, 2004). Cyclopamine is a steroid alkaloid that acts as an inhibitor of the Hh pathway by binding directly to SMO and thus blocking activation of the pathway. Cyclopamine is a potent teratogen that can lead to severe fetal malformations including cyclopia (holoprosencephaly; Belloni *et al.*, 1996; Roessler *et al.*, 1996). With application of cyclopamine to the lesional skin marked improvement was seen within 24 hours with near complete clinical and histological clearance observed in 96 hours (Tas and Avci, 2004). In another recent study it was demonstrated by immunohistochemistry that GLI1 was overexpressed in lesional skin whereas no expression was detected in either uninvolved or control skin (Endo *et al.*, 2006). Taken together, these data suggested that Hh pathway activation is proximal to other events in the pathogenesis of psoriasis and that therapeutic manipulation of this pathway may lead to rapid and possibly sustained improvement of psoriasis.

Despite these data, detailed evaluation of this pathway, and its activation status, in psoriasis has been lacking. We have collected large datasets on gene expression in psoriasis to evaluate and dissect the pathogenic basis of this enigmatic disease. Here, we investigate the principal components of the Hh pathway in lesional, uninvolved, and normal skin and expression of its downstream signature target genes.

RESULTS

Microarray data

Several of the genes involved in the Hh Pathway showed modest changes in expression among control, uninvolved, and lesional skin (Figure 2), but in no case did we detect evidence for activation of Hh signaling activity in psoriasis. Expression of the PTCH1 gene was only slightly downregulated in uninvolved psoriatic skin (89% of control, $P<0.0001$) with more pronounced downregulation in lesional skin (51% of uninvolved, $P<0.0001$; Table 1), compared to control skin. No changes were observed in the expression levels of the *PTCH2* gene. Both *GLI1* and *GLI2* were significantly downregulated in lesional psoriatic skin (60 and 68% of uninvolved, $P<0.05$ and $P<0.0001$, respectively; Table 1). Cyclin D1 (CCND1) was significantly downregulated compared to uninvolved (2.2-fold, $P<0.0001$) in lesional psoriatic skin whereas CCND2 was upregulated (1.5-fold, $P<0.0001$), consistent with previously published data (Belso *et al.*, 2008). Additionally, CDC2 and CCNB1 were also upregulated relative to uninvolved skin (1.5-fold, 3.3-fold, and 5.6-fold, respectively; $P<0.0001$). The Sonic hedgehog (*SHH*) and the Indian hedgehog (*IHH*) gene expressions were minimally upregulated in uninvolved psoriatic skin relative to control (1.06- and 1.05-fold, $P<0.05$) whereas no change was observed for the desert hedgehog (*DHH*) gene. No significant changes were observed between lesional and uninvolved skin for the Hh ligands or SMO (Table 1). Molecular network mapping performed by ingenuity pathway analysis demonstrated complete lack of activation of the Sonic hedgehog pathway (data not shown).

Real-time quantitative PCR data

For confirmation of the microarray data, quantitative (QT) real-time PCR was performed on RNA isolated from 10 control skin biopsies, 10 paired uninvolved and lesional psoriatic skin biopsies, and 12 BCC samples, which served as positive controls for Hh pathway activation. The expression of the principal Hh pathway factors, GLI1 and PTCH1, were significantly lower in lesional psoriatic skin than in BCC ($P<0.001$; Figure 3). Furthermore, expression of these two genes was lower in lesional skin compared to uninvolved skin ($P=0.10$ (GLI1), $P<0.05$ (PTCH1)). Expression of PTCH2 and HHIP were significantly lower in lesional, uninvolved, and control skin compared to BCC ($P<0.01$; not shown), whereas the expression of keratin 1b and CCND1 was decreased in both lesional skin and BCCs compared to normal and uninvolved skin ($P<0.001$, $P<0.01$, respectively; data not shown). CCND2 and myc myelocytomatosis viral related oncogene (neuroblastoma derived) expression levels were significantly lower in BCC

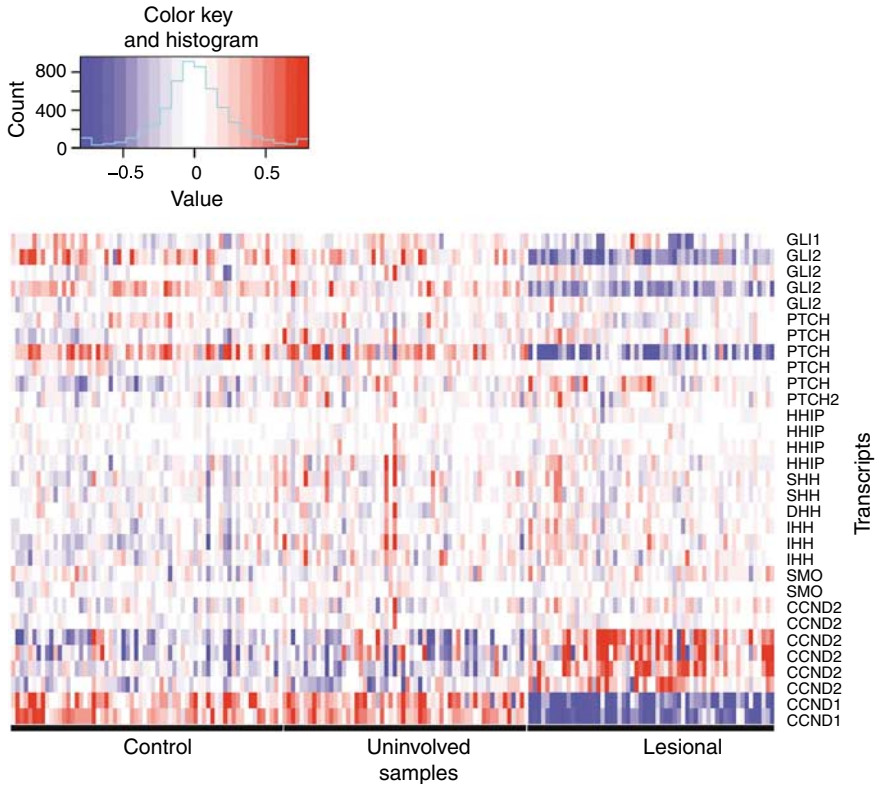

Figure 2. Heatmap of several Sonic hedgehog pathway genes. Several probes are present on the microarray for each gene. Different colors represent different expression values on the log scale after quantile normalization, and batch and gender adjustment. Specifically, red indicates high expression values whereas blue indicates low expression values on the log scale.

Table 1. Fold changes between uninvolved (U) and control skin (N), and lesional psoriatic (P) vs uninvolved

Genes	Fold changes U vs N (*P*-value)	Fold changes P vs U (*P*-value)
PTCH1	0.89 ($P<0.0001$)	0.51 ($P<0.0001$)
PTCH2	1.03 (NS)	1.00 (NS)
GLI1	1.03 (NS)	0.60 ($P<0.05$)
GLI2	0.92 (NS)	0.68 ($P<0.0001$)
HHIP	1.02 (NS)	1.00 (NS)
SHH	1.06 ($P<0.05$)	1.01 (NS)
DHH	1.00 (NS)	1.01 (NS)
IHH	1.05 ($P<0.05$)	1.06 (NS)
SMO	0.93 (NS)	1.06 (NS)
CCND1	0.96 (NS)	0.45 ($P<0.0001$)
CCND2	0.92 (NS)	1.47 ($P<0.0001$)
CDC2	1.09 (NS)	3.33 ($P<0.0001$)
CCNB1	1.07 (NS)	5.57 ($P<0.0001$)

NS, not significant.

compared to lesional, uninvolved, and control samples ($P<0.01$) whereas no difference was observed for GLI2, SMO or the ligands SHH, IHH, and DHH (data not shown).

In situ hybridization

In situ hybridization for *GLI1* and *PTCH1* was strongly positive in BCC tumor cells, which served as positive controls. In contrast, negligible expression of *GLI1* and *PTCH1* was detected in uninvolved or lesional psoriatic skin, whereas staining for the control transcript, *HPRT1*, was positive for all samples analyzed (Figure 4).

DISCUSSION

Psoriasis is characterized by marked hyperproliferation of keratinocytes and influx of T cells with activated CD4+ T cells predominating in the upper dermis and CD8+ T cells in the epidermis. The pathogenesis is highly complex with involvement and participation of multiple cell types (Bowcock and Krueger, 2005). However, despite the inherent complexity of the psoriatic process is has been shown to be critically dependent on T-cell activation, as medications inhibiting T-cell function can lead to remission of the disease (Liu *et al.*, 2007).

The Hh pathway is one of the main signaling pathways in the developing embryo (Ingham and Placzek, 2006) and has been shown to be crucial for the regulation and development of hair follicles and sebaceous glands, and maintenance of stem-cell populations in the skin (Levy *et al.*, 2005). Its role in human diseases, such as developmental disorders and cancer (Xie *et al.*, 1998; Mullor *et al.*, 2002; Rubin and de Sauvage, 2006), has been firmly established. Activation of the pathway

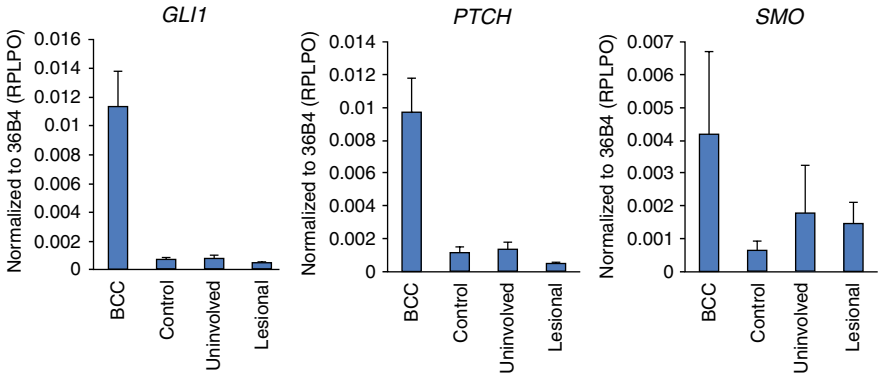

Figure 3. QRT-PCR results for *GLI1*, *PTCH1*, and *SMO* are shown. Error bars indicate standard error of the mean (SEM; BCC, $N=12$; control, $N=10$; uninvolved, $N=10$; lesional, $N=10$). Expression was significantly higher in basal cell carcinoma samples for GLI1 and PTCH1 genes ($P<0.001$), but was not significant for SMO. Lesional skin had significantly lower expression of PTCH1 compared to uninvolved and control ($P<0.05$). GLI1 showed a trend for being lower in lesional skin ($P=0.10$) whereas no significant changes were seen for SMO.

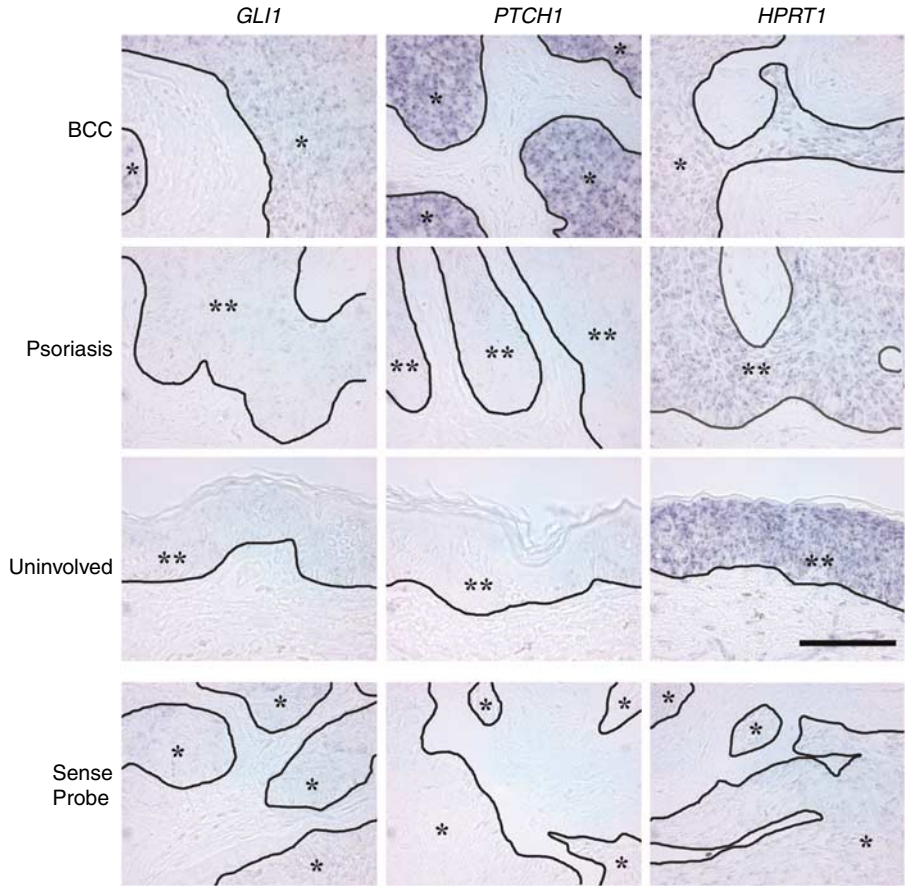

Figure 4. *In situ* hybridization reveals upregulation of Hh target genes, *GLI1*, and *PTCH1* in BCC, but not psoriasis. Expression of the housekeeping transcript *HPRT1* confirmed RNA integrity in all samples. Staining with sense probe was done on BCC samples. Asterisks indicate regions of epithelium (**) and basal cell carcinoma (*) (bar = 50 μm).

in keratinocytes has been shown to induce hyperproliferation and resistance to exhaustion of replicative growth capacity (Fan and Khavari, 1999). Additionally, the Hh pathway has also been implicated in modulation of immune function of peripheral CD4+ T-cells in a pro-inflammatory manner (Lowrey *et al.*, 2002; Stewart *et al.*, 2002; Chan *et al.*, 2006), suggesting that the involvement of this pathway in psoriasis pathogenesis might be plausible.

In this study, which is the largest and most detailed, yet on the function and activity of this pathway in psoriasis we failed to find any evidence of activation of the Hh pathway. Importantly, no expression of GLI1 or PTCH1, the established

target genes of this pathway, could be observed in either the inflammatory infiltrate or in the hyperproliferative epidermis. In contrast to the expected upregulation of this pathway, our results suggest that the activity of this pathway is in fact slightly suppressed in lesional psoriatic skin. However, if the Hh pathway is suppressed in lesional psoriatic skin, how can the clinical efficacy of topical applied cyclopamine (Tas and Avci, 2004) be explained? Cyclopamine is a steroidal alkaloid derived from the false hellebore, or corn lily (*Veratrum californicum*; reviewed in Kuenzli *et al.*, 2004; McFerren, 2006). It is a potent teratogen that on ingestion can lead to severe fetal malformations including cyclopia and holoprosencephaly (Cooper *et al.*, 1998; Incardona *et al.*, 2000). It has been demonstrated that cyclopamine inhibits Hh pathway activation by binding directly to SMO and this inhibition is the underlying mechanisms whereby this compound mediates its teratogenic effect (Chen *et al.*, 2002) as well as its inhibitory effect on BCCs (Taipale *et al.*, 2000). Whether the molecular effects of cyclopamine on BCCs and psoriatic lesions share a common mechanism is not known. Supporting such a common mechanism is a recently published study where evidence of increased Hh pathway activation in lesional psoriatic skin was provided using immunohistochemistry to show upregulation of GLI1 expression in lesional psoriatic skin (Endo *et al.*, 2006). However, the staining was weak and only found diffusely in the upper spinous layer, and Hh pathway activation was not confirmed using other markers (Endo *et al.*, 2006). As immunohistochemistry is prone to a variety of artifacts, we believe that it cannot be considered a reliable marker for Hh pathway activity unless accompanied by other supportive data. We could not find evidence for increased activity of the Hh pathway in psoriasis, as determined by gene microarray analysis, molecular pathway analyses, QT-PCR, and *in situ* hybridization. Thus, our data indicate that the effect of this compound, if truly effective in psoriasis, is likely to be mediated through some other mechanism than blockade of Hh signaling.

In conclusion, we could not confirm previously published studies on the activation of the Hh pathway in lesional psoriasis; in fact our results indicate that this pathway may be modestly suppressed. Given the potential for harmful effects of Hh antagonists, and the lack of evidence for Hh pathway activation in psoriasis, our study raises questions regarding the proposed use of these compounds as antipsoriatic agents.

MATERIALS AND METHODS

Biopsies, reagent, and gene chips

A total of 58 patients with chronic plaque psoriasis and 63 controls were included in the study. The study was conducted according to the Declaration of Helsinki Principles and was approved by the University of Michigan Institutional Review Board and all patients signed an informed consent before inclusion into the study. The patients had not received any topical or systemic treatment for 2 weeks before the study. After local anesthesia with 1% lidocaine HCl and 1:100,000 epinephrine (Hospira Inc. Lake Forest, IL), two 6 mm punch biopsies were obtained from each study subject. From patients, one biopsy was obtained from lesional skin and the other from uninvolved skin, taken at least 10 cm away from any active plaque. The nonlesional biopsies were taken from the buttocks or upper thighs of patients and controls. BCC samples were obtained from Mohs micrographic surgery. The biopsies were snap frozen in liquid nitrogen and stored at −80 °C. At processing the biopsy samples were homogenized while still frozen and total RNA extraction was performed using the RNeasy kit protocol (Qiagen, Chatsworth, CA). RNA quantity and quality was measured on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). RNA stabilization, isolation, and microarray sample labeling were performed using standard methods for reverse transcription and one round of *in vitro* transcription on 5 μg of total RNA. Samples were run on HU133 Plus 2.0 and processed per the manufacturer's protocol (Affymetrix, Foster City, CA). The raw data from the microarrays were processed using the Robust Multichip Average method (Irizarry *et al.*, 2003) and then normalized to account for gender and batch effects. Specifically, we used a linear model to estimate gender and batch effect and then subtracted them from the raw expression data to get the normalized data.

In situ hybridization

In situ hybridization was performed using 5 μm sections cut from NBF-fixed, paraffin-embedded tissue as previously described (Grachtchouk *et al.*, 2003). Plasmid DNA, containing full-length cDNA, to make riboprobe against human HPRT1 was purchased from Invitrogen (clone ID 3163726) (Invitrogen, Carlsbad, CA). *Bgl*II or *Eco*RI digestions followed by *in vitro* transcription using Sp6 or T7 promoters were used to generate sense or senti-sense probes, respectively.

Real-time PCR

Quantitative (QT) real-time PCR was performed on paired lesional and uninvolved samples from 10 psoriatic patients, 10 normal controls, and 11 BCC samples. The RNA used was from the same samples used for the gene microarrays. Primers for the genes, *GLI1*, *GLI2*, *PTCH1*, *PTCH2*, *SMO*, *CCND1*, *CCND2*, *IHH*, *SHH*, *DHH*, *HHIP*, *RPLPO* were obtained from Superarray Biosciences (Frederick, MD). Results were normalized to the expression of the housekeeping gene; Ribosomal protein, large, P0. The reverse transcription reaction was performed on 0.5 μg of RNA template and cDNA was synthesized using anchored-oligo$(dT)_{18}$ primers as instructed by the manufacturer (Roche Diagnostics, Mannheim, Germany). QT real-time PCR was carried out on the LightCycler 2.0 system (Roche Diagnostics). LightCycler FastStart DNA Master^{PLUS} SYBR Green I was used for all PCR reactions as instructed by the manufacturer (Roche Diagnostics). The reaction profile consisted of an initial denaturation at 95 °C for 15 minutes followed by 40 cycles of PCR at 95 °C for 10 seconds (denaturation), 58 °C for 10 seconds (annealing), and 72 °C for 10 seconds (extension). The fluorescence emitted was captured at the end of the extension step of each cycle at 530 nm.

Statistical analysis

Student's *T*-test was used to analyze differences among the three groups. Bonferroni correction was used and *P*-values were generated from the microarray data. Paired *T*-test was used when uninvolved and lesional psoriatic datasets were compared. The Sonic hedgehog pathway network of genes and network analysis of microarray data was performed using ingenuity pathway analysis (Ingenuity Systems: www.analysis.ingenuity.com).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by the Dermatology Foundation (JEG), the American Skin Association (JEG), the National Institute of Arthritis, Musculoskeletal, and Skin Diseases (AR052889; JTE). We acknowledge Dr D Thomas for generously contributing reagents. We thank the volunteers who provided blood and skin samples for this study, and acknowledge the skilled technical assistance of Linda Hodges, Kathleen McCarthy, and Suzan Rehbine.

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