

Activation of Expression of Hedgehog Target Genes in Basal Cell Carcinomas

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Mutations in hedgehog signaling pathway genes, especially *PTC1* and *SMO*, are pivotal to the development of basal cell carcinomas. The study of basal cell carcinoma gene expression not only may elucidate mechanisms by which hedgehog signaling abnormalities produce aberrant tumor cell behavior but also can provide data on *in vivo* hedgehog target gene control in humans. We have found, in comparison with normal skin, that basal cell carcinomas have increased levels of mRNA for *PTC1*, *GLI1*, *HIP*,

***WNT2B*, and *WNT5a*; decreased levels of mRNA for *c-MYC*, *c-FOS*, and *WNT4*; and unchanged levels of mRNA for *PTC2*, *GLI2*, *WNT7B*, and *BMP2* and 4. These findings suggest that mutations in hedgehog signaling pathway genes may exert both cell autonomous and indirect effects and indicate that basal cell carcinoma tumor cells have a phenotype that at least in some aspects resembles that of epidermal stem cells. Key words: *GLI/PTC/signaling/skin carcinogenesis*. *J Invest Dermatol* 116:739–742, 2001**

Basal cell carcinoma (BCC), although the commonest human cancer, had been subjected to relatively little molecular analysis until several years ago, when heritable mutations in *PATCHED1* (*PTC1*) were found to underlie the basal cell nevus syndrome (MIM no. 109400) (Hahn *et al*, 1996; Johnson *et al*, 1996), a rare autosomal dominant affliction characterized by multiple phenotypic abnormalities, including the development of multiple BCC starting in youth. *PTC1* inhibits signaling by the membrane protein Smoothed (Smo), and this inhibition is relieved by binding Sonic Hedgehog (SHH) to PTC. Unrestricted Smo signaling can affect target gene transcription via the *GLI* family of transcription factors (*GLI1*, *GLI2*, and *GLI3*). *PTC1* is itself a target for HH signaling, thus forming a feedback loop in which decreased *PTC1* protein function leads to the production of more *PTC1* mRNA. In BCC, the feedback loop is disrupted because the increased mRNA encodes a nonfunctional *PTC1* protein, and indeed *PTC1* mRNA, as well as *SMO*, *GLI1*, and *GLI3* mRNA, have been reported to accumulate in BCC. Other genes whose expression has been described to be activated by HH signaling in various tissues include members of the *WNT/Wg* and *BMP/TGFβ* families of signaling molecules and the gene encoding the cell-surface HH-binding protein *HIP* (Dahmane *et al*, 1997; Ingham, 1998; Chuang and McMahon, 1999; Ghali *et al*, 1999).

So far, it remains unclear how mutations in *PTC1* and *SMO* and consequent changes in HH target gene expression produce the

abnormal proliferation, differentiation, and adhesion characteristic of BCC tumor cells. As part of an effort to investigate this question, we have surveyed BCC for *in vivo* quantitative abnormalities of mRNA encoding hedgehog target genes, hemidesmosomal proteins, and a selection of genes that encode proteins important in apoptosis and cell cycle regulation.

MATERIALS AND METHODS

Aliquots of nodular BCC removed from the skin by curettage and normal human skin (obtained as part of breast reduction surgery) or epidermis (the roof of suction blisters of the volar forearm) were frozen immediately in liquid nitrogen and were stored at -70°C until assayed or were fixed and processed for routine microscopic examination. We used a Qiagen RNeasy kit (Qiagen, Valencia, CA) to extract RNA from these tissues and from cultured normal cells [preconfluent epidermal keratinocytes (HEK) grown in 0.07 mM Ca^{2+} and fibroblasts] and cell lines (HaCaT, HeLa, and D259MG). mRNA for *PTC1*, *PTC2*, *GLI1*, *c-MYC*, *HIP*, and *GAPDH* was quantitated by TaqMan. Primer and probe sequences are available as follows: Patched 1: forward (5'-TCT TCA TGG CCG CGT TAA TC-3'), reverse (5'-TTG CAG GAA AAA TGA GCA GAA C-3'), probe (5'-FAM-AAT TCC CGC TCT GCG GGC G-TAMARA-3'); Patched 2: forward (5'-CCA GCA CCC CCT CAT CAG-3'), reverse (5'-GAC CCG AAG ACC AAT TCA GC-3'), probe (5'-FAM-CAC AAG GAG CGC CAC TGT CTG GAC-TAMARA-3'); *GLI1*: forward (5'-TGA GGC CCT TCA AAG CCC-3'), reverse (5'-GTA TGA CTT CCG GCA CCC TTC-3'), probe (5'-FAM-TGC TGG TGG TTC ACA TGC GCA GA-TAMARA-3'); *HIP*: forward (5'-TGC TAA GCC TCG CAT TCC A-3'), reverse (5'-ACA ACC CTA AGA ATG TGG TCA TGA-3'), probe (5'-FAM-TGT ATG TGT CCT ATA CCA CCA ACC AAG AAC GG-TA-3'); *c-Myc*: forward (5'-CAG CTG CTT AGA CGC TGG ATT-3'), reverse (5'-GAG GTC ATA GTT CCT GTT GGT GAA-3'), probe (5'-FAM-CTC CCG CGA CGA TGC CCC T-3'); *GADPH*: forward (5'-GAA GGT GAA GGT CGG AGT CA-3'), reverse (5'-GAA GAT GGT GAT GGG ATT TC 3'), probe (5'-FAM-CAA GCT TCC CGT TCT CAG CC-TAMARA-3'). Ribonuclease protection assays (RPA)

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Abbreviations: BCC, basal cell carcinoma; HEK, human epidermal keratinocyte; HH, hedgehog; RPA, ribonuclease protection assay.

were performed using the Ambion RPAII kit according to the manufacturer's recommendations (Ambion, Austin, TX). Probes used for RPA were produced by polymerase chain reaction (PCR) amplification of published sequences (*GLI2*, *INTEGRIN β 4*), were purchased from Pharmingen or Ambion, or were generous gifts (*PTC1*, *GLI1*, *INTEGRIN α 6*, and *BPAG1* and *BPAG2*). DNA was linearized to give anti-sense probes of 150–300 bp and was labeled with a Promega (Madison, WI) transcription kit. Gels were scanned for quantitative densitometry using a BioRad (Hercules, CA) Model GS-670 imaging densitometer. The relative levels of three controls ("housekeeping" genes – *GAPDH*, *ACTIN*, and *CYCLOPHILIN*) were consistent in each sample, suggesting that none of the three changed significantly in these samples. We identified *WNT* family members expressed in HEK and in BCC by reverse transcription–PCR amplification using consensus *WNT* family primers: forward (5'-GGG GAA TTC CAR GAR TGY AAR TGY CAY-3', reverse (5'-AAA ATC ATC TAG ARC ARC ACC ART GRA A-3') (Gavin *et al*, 1990).

RESULTS

PTC Levels of *PTC1* mRNA in all 27 BCC studied were elevated markedly compared with controls (epidermis, skin, or HEK) with a 15-fold variation among individual tumors. Levels were 10–500-fold higher by TaqMan and up to 1600-fold higher by RPA; however, *PTC1* expression in the controls was very low, and consequently precise quantitation by RPA was difficult. Like those of *PTC1*, levels of *PTC2* mRNA were high in BCC and were low in HEK; however, unlike those of *PTC1*, levels of *PTC2* mRNA also were high in normal epidermis. Cultured dermal fibroblasts and HaCaT cells had uniformly low levels of *PTC1* (albeit higher than the levels in HEK) and of *PTC2* mRNA. D259MG cells, the cell line from which amplified *GLI1* originally was isolated, had low levels of *PTC2* mRNA but, unlike other

cultured cells studied, had elevated levels of *PTC1* mRNA—as high as those in BCC (Table I).

GLI Levels of *GLI1* message were elevated (*vs* that in HEK or skin) in all 12 BCC studied by TaqMan, the average level being approximately 1/6 that in D259MG cells (Table I). In seven BCC studied by RPA, levels of *GLI2* varied over a 9-fold range, were absent in HEK and in D259MG cells, but were as high in skin as in BCC.

HIP Of five BCC tested by RPA three had mRNA levels 10-fold increased, and two had levels approximately 100-fold increased *vs* those in HEK. The levels in the two latter BCC were similar to that in D259MG cells. *HIP* expression was undetectable by RPA in extracts of normal skin (Fig 1). In five BCC tested by TaqMan, *HIP* expression averaged 40-fold higher than in normal skin and nearly 300-fold higher than in epidermis or HEK (Table I).

***WNT* genes** By cloning reverse transcription–PCR product prepared from RNA, we found five different *WNT* family members (2, 2b, 4, 5a, and 7b) to be expressed in cultured HEK and/or BCC, of which *WNT7b* was found the most commonly. By RPA *WNT2b* and *5a* were undetectable to very low in HEK and skin but clearly were expressed in BCC, albeit at varying levels. By contrast, *WNT4* was expressed in epidermis and skin but not in BCC (or in HEK). *WNT7b* was detected readily in all BCC, epidermis, and skin but was undetectable in HEK. *WNT2* expression was highly elevated in one of five BCCs studied but was undetectable by RPA in HEK.

c-MYC Of eight BCC tested by RPA all had near-complete loss of *c-MYC* mRNA as compared with that in HEK, epidermis, or whole skin. D259MG cells also have low *c-MYC* mRNA, whereas levels in HeLa and HaCaT cells are at least as high as those in HEK (Fig 2). With Taqman, *c-MYC* expression is very high in epidermis and at least 15-fold lower in BCC (Table I).

BMP mRNA for *BMP2* and *BMP4* were detectable at similar levels in BCC, skin, and epidermis. *BMP2* and *BMP4* expression levels were absent to very low in HEK.

Hemidesmosomal protein gene expression We assessed mRNA levels of the four major known components of hemidesmosomes in BCC. mRNA for *BPAG1* and *BPAG2* were readily detectable in BCC and were present at levels similar to those in HEK or whole skin. α 6 and β 4 integrin mRNA are visible

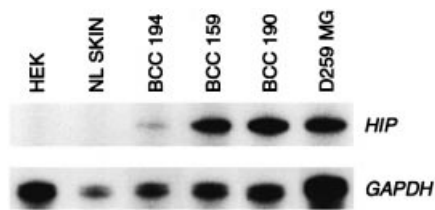


Figure 1. RNase protection assay of *HIP* expression in BCC and in control samples.

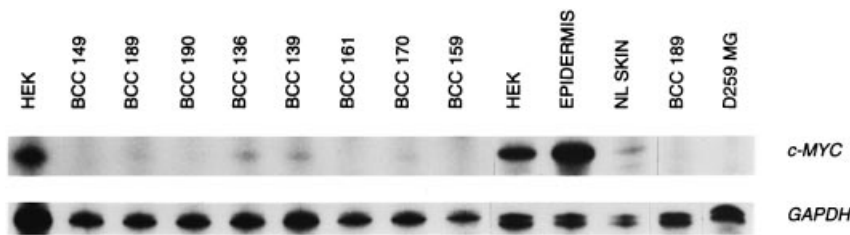


Figure 2. RNase protection assay of *c-MYC* expression in BCC and in control samples.

Table I. *PTC1*, *PTC2*, and *GLI1* expression in BCC mRNA levels were measured by TaqMan analysis and were normalized to the expression levels of *GAPDH*^a

	<i>PATCHED 1</i>	<i>PATCHED 2</i>	<i>GLI1</i>	<i>c-MYC</i>	<i>HIP</i>
HEK	0–0.14	0.1–0.5	0.007–0.2	17	0–0.05
Epidermis	0–0.59	11	0.1	94–99	0–0.01
Normal skin	0–2.2	1.6–62 (av. 18)	0.7–1.7	21	0.04
BCC	2–100 (av. 18)	2.3–54 (av. 17)	7–102 (av. 41)	0.8–14 (av. 6)	0.1–3.6 (av. 1.4)
D259MG	7–11	0.2	275	ND	ND

^aValues in the table are (2^ΔDelta CT) *100 and, for BCC, represent 12–20 individual tumors for *PATCHED 1*, *PATCHED 2*, and *GLI1* and five tumors for *c-MYC* and *HIP*.

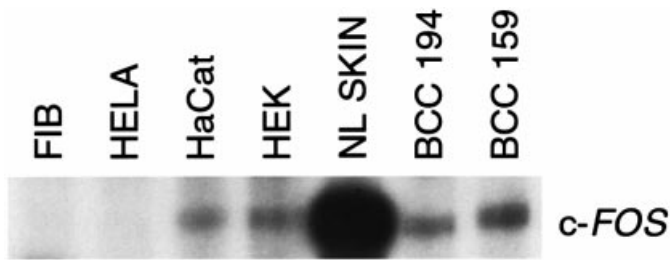


Figure 3. RNase protection assay of *c-FOS* expression in BCC and in control samples.

as faint bands in RPA of extracts of BCC, normal skin, or epidermis and are expressed at much higher levels in HEK. The single BCC with high $\alpha 6$ integrin expression did not differ clinically or histologically from those with lower expression.

Other Levels of expression of several genes involved in cell cycle regulation (*p53*, *Rb*, *p21*, and *cdk4*) and in control of apoptosis (*bclx*, *bax*, and *mcl*) varied among individual tumors and were not markedly different from those of skin. One consistent change seen was an at least 100-fold decrease in mRNA for *c-FOS* in BCC ($n = 8$) *vs* in normal skin (Fig 3), a finding that is consistent with previous reports of high levels of *c-FOS* expression in adult human skin, especially in suprabasal cells, and reduced levels in BCC (Basset-Seguín *et al*, 1990; Takahashi *et al*, 1994).

DISCUSSION

These data are consistent with the growing evidence that hedgehog target gene activation is the pivotal step in BCC carcinogenesis and is the first study of the levels of expression of *GLI2*, *WNT*, *BMP*, *c-MYC*, and *HIP* in BCC. Furthermore, we have generated quantitative data regarding the degree to which the message for three HH-regulated genes accumulate in these tumors: *PTC1*, *GLI1*, and *HIP* mRNA levels are at least 50–100-fold increased *in vivo* in BCC; levels that we have found to be matched *in vitro* only by the levels in D259MG cells, in which *GLI1* is highly amplified. The uniformity of this upregulation of *PTC1* mRNA in BCC indicates that loss of *PTC1* protein function rarely is due to deletions of both genomic copies, production of unstable mRNA, or prevention of transcription of *PTC1* message. The *PTC2* levels that we detected confirm those seen by *in situ* hybridization (Carpenter *et al*, 1998; Zaphiropoulos *et al*, 1999). The lack of upregulation of *PTC2* expression by amplified *GLI1* in D259MG cells, like the maintenance of *PTC2* expression in the lung and skin of *Shh*^{-/-} mouse embryos (Pepicelli *et al*, 1998; St-Jacques *et al*, 1998), is consistent with the conclusion that, at least in these tissues, *PTC2* expression is not an HH target gene. Overexpression of *GLI1* or *GLI2* in the epidermis of transgenic mice can drive the development of BCC (Grachtchouk *et al*, 2000; Nilsson *et al*, 2000). Our finding of levels of *GLI1* that were upregulated consistently, of levels of *GLI2* mRNA that were less consistent and on average were no higher than in normal skin, and of stronger RPA bands with *GLI1* than *GLI2* all suggest that *GLI1* may be more important *in vivo* in driving the aberrant cell behavior characteristic of BCC. We have no information, however, regarding possible post-translational activation of these proteins in epidermis, and so the primacy of *GLI1* must be considered a tentative conclusion.

HIP is a gene recently identified in a cell-free screen for murine genes encoding proteins that bind *Shh*. It is present at the cell surface where it binds *Shh* with an avidity similar to that of *PTC1*, is a transcriptional target of *Shh* signaling during mouse development, and appears to attenuate *Shh* signaling, most likely by binding *Shh* and thereby reducing its interaction with *Ptc1* (Ingham, 1998). Our finding of high levels of *HIP* mRNA in BCC indicates that its

expression is an *in vivo* target of HH signaling in humans as well as in mice.

The repertoire of *WNT* genes expressed in adult human epidermis has not previously been explored systematically. Saitoh *et al* (1998) analyzed *WNT* expression in adult murine epidermis using a strategy similar to ours: cloning of PCR product amplified from *WNT* consensus primers followed by RPA quantitation. They found clones of *Wnt4*, *7a*, and *10b*, expression only of *Wnt4* and *10b* in epidermis, and of *Wnt4* in newborn mouse epidermal keratinocytes in culture. We cloned sequences corresponding to *WNT2b*, *4*, *5a*, and *7b* from cDNA prepared from cultured HEK and to *WNT2*, *2b*, *4*, *5a*, and *7b* from cDNA prepared from BCC. Levels of *WNT2* expression measured by RPA were low to undetectable in HEK. Expression of *WNT2b*, *5a*, *7b* and *13* were readily detectable in BCC. Human (like mouse epidermis) does express *WNT4* but this *WNT* is undetectable in BCC. As the bulk of the cells in these samples were tumor cells, most likely the *WNT* messages detected in BCC were derived from the malignant cells rather than from “contaminating” normal stromal cells, but confirmation of this conclusion must await *in situ* studies. The expression of multiple *WNT* genes detectable at low levels by RPA is reminiscent of reports of similar studies in breast cancers (Huguet *et al*, 1994), and different *WNT* have been described as having varying transforming potencies (Wong *et al*, 1994).

One intracellular pathway by which Wnt signaling proceeds is via stabilization of β -catenin, which interacts with the Tcf-Lef family of transcription factors to affect gene expression. Stabilization of β -catenin protein by mutations in the β -catenin gene in keratinocytes produce pilomatricomas, a skin tumor distinct from BCC (Chan *et al*, 1999). Stabilization of β -catenin protein in colon cancer cells, often by mutations in APC, is associated with upregulation of *c-MYC* expression (He *et al*, 1998). In contrast, despite their upregulation of *WNT2b* and *5a* and hence potential β -catenin stabilization, we have found that BCC have *c-MYC* downregulation as compared either to skin or to cultured normal HEK. Different *WNT* species clearly affect the phenotype of cells differently, as manifested both by their different patterns of expression in normal *vs* malignant cells and also by their differing abilities to stabilize β -catenin and to transform, e.g., mammary epithelial cells in which specific model *WNT4*, *5a*, and *7b* have no effect (Shimizu *et al*, 1997), and it is possible that different *WNT* produce different effects on *c-MYC* expression as well.

BCC have been suggested to arise from epidermal stem cells that are located in the bulge region of the hair follicles. That postulate is based not only on the continued proliferation and resistance to differentiation of BCC cells but also on their frequent expression of antigens similar to those expressed by the cells of the hair follicle bulge region, e.g., keratins 15 and 19 and $\alpha 2$, $\alpha 3$, and $\beta 1$ integrins. Also consistent with this view is the observation that *ptc*^{+/-} mice have upregulation of *ptc* promoter activity in the bulge cells as well as in BCC (Aszterbaum *et al*, 1999). Epidermal stem cells express low levels of *c-MYC* despite their relatively high levels of β -catenin protein and despite the ability of exogenous stabilized β -catenin to enhance stem cell numbers *in vitro* (Zhu and Watt, 1999). Upregulation of *c-MYC* expression can drive human epidermal stem cells into a transient amplifying phenotype (Gandarillas and Watt, 1997), and most but not all studies report that *c-MYC* expression then is downregulated coincident with the onset of keratinocyte terminal differentiation. Consistent with these findings, *c-MYC* expression in transgenic mice recently was found to drive epidermal keratinocyte proliferation and to inhibit normal complete differentiation *in vivo* (Pelengaris *et al*, 1999; Waikel *et al*, 1999). Hence, at least in their failure to switch from proliferation to differentiation and their downregulation of *c-MYC*, BCC resemble epidermal stem cells. Of note, however, the putative epidermal stem cells of the hair follicle bulge, unlike BCC, do express $\alpha 6$ and $\beta 4$ integrins and bullous pemphigoid proteins. Thus we speculate that upregulation of expression of *WNT2b* and/or *5a* (and/or downregulation of *WNT4* expression) and a possible resultant downregulation of *c-MYC* expression may be a crucial pathway by

which hedgehog target gene dysregulation produces at least some aspects of the BCC phenotype.

Our quantitation of hemidesmosomal protein mRNA levels suggests that the well-established loss of hemidesmosomal proteins in BCC may not be controlled by loss of mRNA for integrins $\alpha 6$ and $\beta 4$ or *BPAG1* or 2. However, in contrast to our results, others have found consistent loss of *BPAG1* and 2 mRNA by reverse transcription-PCR in BCC (Chopra *et al*, 1998).

The marked differences in gene expression between cultured and *in situ* keratinocytes complicate the choice of proper controls. Further, these differences are consistent with other data indicating that the response of cultured cells to the hedgehog ligand or to the loss of *PTC* is blunted (Fan *et al*, 1997). Our finding of upregulated *PTC1* and *HIP* in cells with amplified *GLI1* (D259MG cells) suggests that the block to the hedgehog response in cultured cells lies upstream of this transcription factor.

These findings further validate the concept of the primary role of hedgehog target gene activation in BCC tumorigenesis and are consistent with previously elucidated pathways of hedgehog signaling. Considerably more work, however, will be necessary to elucidate which of these changes described or which other changes yet to be described will be found to be the key to the mechanism by which aberrant hedgehog signaling leads to aberrant BCC cell behavior and which will be found to be epiphenomena.

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