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Advances in Brief

Missense Mutations in *SMOH* in Sporadic Basal Cell Carcinomas of the Skin and Primitive Neuroectodermal Tumors of the Central Nervous System[1]

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Abstract

About one-third of sporadic basal cell carcinomas (BCCs) of the skin and 10–15% of primitive neuroectodermal tumors (PNETs) of the central nervous system show mutations in the *PTCH* tumor suppressor gene. The *PTCH* gene product (Ptch) functions as a transmembrane receptor for the Sonic hedgehog protein (Shh) and interacts with another transmembrane protein called Smoh. To further elucidate the significance of alterations in the Shh signaling pathway, we investigated 31 sporadic BCCs and 15 PNETs for the mutation and/or expression of *SMOH*, *PTCH*, *SHH*, and *GLI1*. In addition, we fine-mapped the *SMOH* gene locus by fluorescence *in situ* hybridization to chromosomal band 7q32. Mutational analysis identified four BCCs with somatic missense mutations in *SMOH* affecting codon 535 (TGG⇒TTG: Trp⇒Leu) in three tumors and codon 199 (CGG⇒TGG: Arg⇒Trp) in one tumor. A missense mutation at codon 533 (AGC⇒AAC: Ser⇒Asn) was found in one PNET. *PTCH* mutations were detected in eight BCCs and one PNET. Two BCCs demonstrated mutations in both *SMOH* and *PTCH*. The majority of tumors showed an increased expression of *SMOH*, *PTCH*, and *GLI1* transcripts as compared with that of normal skin and nonneoplastic brain tissue, respectively. In contrast, only one BCC and one PNET expressed *SHH* mRNA at levels detectable by reverse transcription-PCR, and no *SHH* gene mutations were found. In summary, our results indicate that both *PTCH* and *SMOH* represent important targets for genetic alterations in sporadic BCCs and PNETs.

Introduction

The *PTCH* gene is the human homologue of the *Drosophila patched* gene and codes for a transmembrane protein (Ptch) that serves as a receptor for the secreted Sonic hedgehog (Shh) signaling protein (1, 2). Ptch forms a complex with another transmembrane protein, the human homologue of the *Drosophila* smoothened protein (Smoh; Refs. 1, 3, and 4). In the absence of Shh, Ptch inhibits the activity of Smoh (1, 4). The binding of Shh to Ptch can relieve this inhibition of Smoh, which results in the transduction of the Shh signal and transcriptional activation of several genes coding for members of the transforming growth factor β, Gli, and Wnt protein families, as well as Ptch itself (1, 4–6). Several recent studies have provided evidence that alterations in the Shh signaling cascade are involved in the pathogenesis of hereditary and sporadic human diseases. Germline mutations in the *SHH* gene were found to be responsible for holoprosencephaly type 3 (7). Germ-line mutations in the *PTCH* gene were identified in patients with nevoid BCC[4] syndrome, a rare autosomal dominant disorder that predisposes individuals to the development of multiple BCCs of the skin and a number of other tumor types including PNETs of the central nervous system as well as various developmental defects (8, 9). Somatic mutations in *PTCH* as well as in *SHH* were detected in sporadic BCCs, PNETs, and certain other types of sporadic tumors (10–15). The significance of these findings is supported by experimental studies showing that heterozygous *patched* mutant mice demonstrate an increased likelihood for the development of cerebellar PNETs (16), and that transgenic mice overexpressing Shh in the skin develop BCCs at a high frequency (15).

In contrast to *PTCH* and *SHH*, the involvement of *SMOH* gene alterations in the pathogenesis of human tumors is less established thus far. Here we report on the chromosomal fine-mapping of the *SMOH* gene locus and the molecular genetic analysis of *SMOH*, *PTCH*, *SHH*, and *GLI1* for mutation and/or expression in sporadic BCCs and PNETs. Our findings indicate that in addition to *PTCH* mutations, missense mutations in *SMOH* may contribute to the pathogenesis of some BCCs and PNETs, presumably by leading to constitutive activation of the protein.

Materials and Methods

Tumor Samples. The tumors were selected from the frozen tumor tissue collections at the Departments of Neuropathology and Dermatology, Heinrich-Heine-University (Düsseldorf, Germany). Frozen samples from four PNETs were provided by Dr. C. Sommer, Department of Neuropathology, Ruprecht-Karls-University (Heidelberg, Germany). The BCCs were from 19 male and 12 female patients (mean age at operation, 71 years; range, 33–89 years). None of the patients showed evidence of nevoid BCC syndrome. The PNETs were from 10 male and 5 female patients (mean age at operation, 16 years; range, 6 months to 46 years). Parts of the tumor tissue were frozen immediately after the operation and stored at −80°C. To assure that the tumor pieces taken for molecular genetic analysis contained a sufficient proportion of tumor cells, a histological evaluation of a representative part of each of these pieces was performed.

DNA and RNA Extraction. The extraction of high molecular weight DNA and RNA from frozen tumor tissue was carried out by ultracentrifugation as described by Ichimura *et al.* (17). The extraction of high molecular weight DNA from peripheral blood leukocytes was performed according to standard protocols.

Screening for Mutations by SSCP/Heteroduplex Analysis and Direct Sequencing. Mutational analysis of all 22 coding exons of *PTCH* was performed as described previously (13). Exons 1–3 of *SHH* were analyzed by using the primer sets and conditions as published (7). For the analysis of *SMOH*, 3 μg of total RNA from each tumor were reverse-transcribed into cDNA using random hexanucleotide primers and Superscript II reverse transcriptase (Life Technologies, Inc.). The *SMOH* coding sequence, except for the

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[4] The abbreviations used are: BCC, basal cell carcinoma; DAPI, 4′,6-diamidino-2-phenylindole; FISH, fluorescence *in situ* hybridization; PNET, primitive neuroectodermal tumor; SSCP, single-strand conformational polymorphism.

first 60 codons, was then amplified by PCR using a set of seven primer pairs. Six of the PCR products were cut with an appropriate restriction enzyme before SSCP/heteroduplex analysis (Table 1). The resulting fragments were screened for mutations by electrophoresis on 10% nondenaturating polyacrylamide gels at 120 V for 16 h. Each fragment was analyzed at room temperature and at 4°C. After electrophoresis, the SSCP/heteroduplex band patterns were visualized by silver staining of the gels. PCR products with aberrant SSCP/heteroduplex patterns were sequenced in both directions by manual sequencing using the USB Sequenase PCR product sequencing kit (Amersham).

Expression Analyses by Reverse Transcription-PCR. Analysis of *SMOH*, *PTCH*, and *GLI1* mRNA expression was performed by differential reverse transcription-PCR amplification using primers specific for each transcript (Table 1) together with primers for transcripts from either *GAPDH* (18) or *B2MG* (19). PCR was performed for 30 cycles (1 min at 94°C, 1 min at 56°C, and 1 min at 72°C) in PCR buffer containing 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate, 0.5 μM each primer, and 1 unit of Taq DNA polymerase (Eurogentec). Reference tissues included nonneoplastic adult cerebral and cerebellar tissue samples (cerebral cortex from the temporal lobe of a patient operated on for epilepsy, cerebellar cortex from a patient operated on for a cerebellar angioma, and normal cerebellar cortex obtained postmortem) and normal skin samples from two adult individuals. PCR products were separated on 2% agarose gels, and the ethidium bromide-stained bands were recorded by the Gel-Doc 1000 system (Bio-Rad). The expression of *SHH* mRNA was determined by reverse transcription-PCR using the primers listed in Table 1. PCR was performed for 30 cycles of 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C. To assess the cDNA quantity used as template, *β-actin* cDNA was amplified in a separate PCR reaction (13).

Southern Blot Analysis. For Southern blot analysis, 2.5 μg of DNA were digested with the restriction enzymes *Taq*I or *Hind*III, separated on 0.8% agarose gels, and alkali-blotted to Hybond-N^+ membranes (Amersham). The membranes were hybridized with [α-^{32}P]dCTP-labeled DNA probes. A 1.15-kb genomic probe for *SMOH* was generated by PCR using primers P6F (Table 1) and SR (5′-AGGGGCAGGGGGGTGAAG-3′). A PCR product covering exon 1 of the gene was used as a probe for *SHH*. As a reference for the assessment of gene copy number, the blots were hybridized with a probe for the *CCNA* gene locus at 4q31–q35. This probe was a 528-bp reverse transcription-PCR fragment corresponding to nucleotides 352–879 of the *CCNA* cDNA sequence (European Molecular Biology Laboratory accession number X51688). Densitometric evaluation of the target gene dose relative to control (leukocyte) DNA was performed with Molecular Analyst software after scanning the autoradiograms with a GS-700 imaging densitometer (Bio-Rad).

Chromosomal Assignment and FISH Mapping of *SMOH*. The *SMOH* gene locus was assigned to human chromosome 7 by PCR analysis of a chromosome-specific hybrid panel (National Institute of General Medical Sciences Human/Rodent Somatic Cell Hybrid Mapping Panel 2; Coriell Cell Repositories, Camden, NJ) using primers P6F and SR. Primer SR corresponds to a sequence segment absent in the rat *smoothened* gene and therefore allows specific amplification of human *SMOH* from the hybrid panel. A human chromosome 7-specific cosmid library [No. 113 (L4/FS7) from the Resource Center/Primary Database of the German Human Genome Project, Berlin, Germany] was screened by hybridizing high-density filters with the genomic probe for *SMOH*. The initial screening identified seven positive cosmid clones from which five (ICRFc113 L2035Q4, N2035Q4, F1919Q4, O0637Q4, and I1950Q4) were selected and confirmed to contain *SMOH* sequences by PCR using primer pairs P2, P3, P6, and P7 (Table 1). Cosmids L2035Q4, N2035Q4, F1919Q4, and O0637Q4 were labeled with biotin-16-dUTP using standard nick translation protocols, and FISH was performed to normal human metaphase chromosomes separately for each cosmid as described previously (20). Hybridized probe was detected by avidin-FITC, and chromosomes were counterstained with DAPI. The experiments were analyzed by epifluorescence microscopy. Digitized images were obtained separately for DAPI and FITC with a cooled charge-coupled device camera (Photometrics, Tucson, AZ), electronically overlaid, and carefully aligned. From each experiment, at least 40 metaphase cells were analyzed microscopically, and 10 metaphase cells with particularly extended chromosomes were selected for digital image analysis.

Table 1 *Primers and restriction enzymes used for SSCP/heteroduplex analysis of SMOH (P1–P7) as well as expression analyses of SMOH (P8), PTCH (P9), SHH (P10), and GLI1 (P11)*

Primer sequences[a]	Restriction enzymes, fragment sizes, and nucleotides covered[b]
P1F 5′-CTG AGC CAC TGC GGC CGG-3′	204 bp (nt[c] 181–385)
P1R 5′-CGG CAC ACA GCA GGG GCT G-3′	
P2F 5′-GTC GGG CCT CCG GAA TGC-3′	*Cfo*I, 177 + 204 bp
P2R 5′-GCC GCG ATG TAG CTG TGC-3′	(nt 327–708)
P3F 5′-CTC TTC ACA GAG GCT GAG-3′	*Nde*II, 214 + 163 bp
P3R 5′-CAG GGC TTT GAA GGA AGT G-3′	(nt 661–1038)
P4F 5′-GGT TTG GTT TGT GGT CCT C-3′	*Msp*I, 315 + 98 bp
P4R 5′-AGG TAA TGA GCA CAA AGC C-3′	(nt 987–1399)
P5F 5′-ACC ATG CTG CGC CTG GGC-3′	*Hinf*I, 189 + 211 bp
P5R 5′-TGC AGG AGC TCG TGC CGC-3′	(nt 1342–1742)
P6F 5′-GCA AGA TGA TTG CCA AGG C-3′	*Pvu*II, 135 + 222 bp
P6R 5′-AGC GGG CAC ACC TCC TTC-3′	(nt 1697–2054)
P7F 5′-AGA GCT GCA GAA GCG CCT-3′	*Pvu*II, 129 + 246 bp
P7R 5′-TCA GAA GTC CGA GTC TGC-3′	(nt 1989–2364)
P8F 5′-CAC CTC CAA TGA GAC TCT GTC C-3′	519 bp (nt 918–1437)
P8R 5′-CTC AGC CTG GTT GAA GAA GTC G-3′	
P9F 5′-CGC CTA TGC CTG TCT AAC CAT GC-3′	450 bp (nt 1338–1788)
P9R 5′-TAA ATC CAT GCT GAG AAT TGC A-3′	
P10F 5′-CCA CTG CTC GGT GAA AGC AG-3′	181 bp (nt 694–875)
P10R 5′-GGA AAG TGA GGA AGT CGC TG-3′	
P11R 5′-CTC AAC AGG AGC TAC TGT GG-3′	396 bp (nt 2789–3185)
P11F 5′-GGG TTA CAT ACC TGT CCT TC-3′	

[a] F, forward; R, reverse.
[b] The numbering of nucleotides is according to GenBank accession numbers U84401 (*SMOH*), U43148 (*PTCH*), L38518 (SHH), and X03784 (*GLI1*).
[c] nt, nucleotide.

Results

The chromosomal localization of *SMOH* was determined by FISH of normal human metaphase chromosomes using four cosmids containing *SMOH* sequences as probes. All four cosmids mapped to the same chromosomal site, allowing the assignment of *SMOH* to 7q32 (Fig. 1*a*). No additional signals were found in other regions of the human genome for any of the cosmids tested.

None of the BCCs and PNETs demonstrated evidence of *SMOH* gene amplification by Southern blot analysis. Mutational screening of *SMOH* at the transcript level revealed point mutations in 4 of 31 (13%) BCCs and 1 of 15 (7%) PNETs. Three BCCs (BCC6, BCC7, and BCC20) carried an identical G to T exchange at nucleotide 1604 (numbering according to GenBank accession number U84401), which translates into an amino acid exchange from tryptophan to leucine at codon 535. The mutation was also present in genomic DNA from these tumors. However, the corresponding constitutional (leukocyte) DNA of these patients showed only the wild-type sequence at this position, indicating the somatic origin of the mutations (Fig. 1*b*). In all tumors, the mutant sequence was present together with the wild-type sequence, a finding suggesting that only one allele carried the mutation, whereas the second (wild-type) allele was retained. A heterozygous somatic C to T transition at nucleotide 595, which translates into an amino acid substitution from arginine to tryptophan at codon 199, was identified in tumor BCC33. This particular amino acid resides in the NH_2-terminal region of the protein between the signal sequence and the putative first transmembrane domain and is conserved in the rat protein but not in *Drosophila*. In one PNET (dpMB11), a heterozygous G to A transition was found at nucleotide 1598 that leads to the exchange from serine to asparagine at codon 533 (Fig. 1*c*). Unfortunately, a somatic origin of this alteration could not be confirmed, because no constitutional DNA was available from this patient. Nevertheless, like the mutations in BCC6, BCC7, and BCC20, the nucleotide sequence change in dpMB11 results in the substitution of an evolutionary conserved amino acid located in the putative seventh transmembrane domain. In addition, this sequence change was not found as a constitutional change in 110 different individuals. Thus, it is more likely that it represents a mutation rather than a

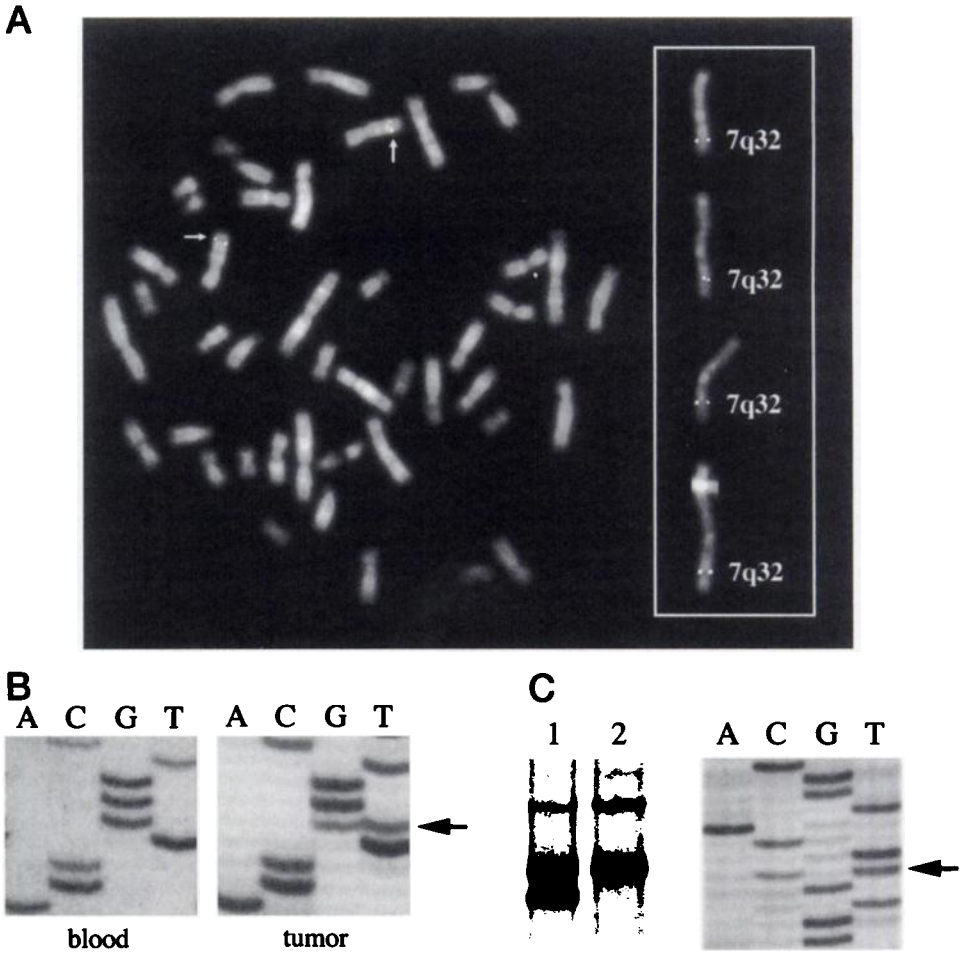

Fig. 1. *A*, chromosomal mapping of the *SMOH* gene to human metaphase chromosomes by FISH. The hybridized cosmids were detected via FITC, and chromosomes were counterstained with DAPI. A spread metaphase cell is shown on the *left*, illustrating the specificity of the hybridization experiment. *Right*, individual chromosome 7 homologues with probe hybridization signals are shown to demonstrate the position of the signal on the chromosome. Careful comparison of DAPI and FITC images revealed that *SMOH* maps to chromosomal band 7q32. *B*, demonstration of a somatic missense mutation in *SMOH* in BCC20. This tumor carried a heterozygous G to T transversion (*arrow*) at nucleotide 1604, which predicts an exchange of tryptophan to leucine at codon 535 (*right*). The mutation is absent in the constitutive (*blood*) DNA from this patient (*left*). *C*, SSCP analysis of *SMOH* (*left*) showing an aberrant pattern for the desmoplastic medulloblastoma dpMB11 (*Lane 1*) compared to a wild-type reference (*Lane 2*). DNA sequencing (*right*) revealed a heterozygous G to A transition (*arrow*) at nucleotide 1598 exchanging serine to asparagine at codon 533. The sequence shown in *C* is derived from the noncoding strand.

polymorphism. In contrast, another sequence alteration detected in dpMB11, *i.e.*, a G to A transition at nucleotide 808 leading to the exchange from valine to isoleucine at codon 270, probably represents a polymorphism, because it does not affect a conserved amino acid. In fact, the *Drosophila* sequence at codon 270 codes for isoleucine. Tumor BCC28 showed a nucleotide exchange in codon 647 (C1939T) that causes the substitution of proline by serine. This amino acid is conserved in rat but not in *Drosophila*. Because the same sequence change was present in the patient's constitutional DNA, it might represent a rare polymorphism. Additional polymorphisms that occurred in more than a single case and did not result in amino acid exchanges were identified in codon 245 (G735A), codon 284 (G852A), codon 379 (G1137A), and codon 388 (C1164G).

SSCP/heteroduplex analysis of the entire *PTCH* coding region revealed mutations in eight BCCs and one PNET. The *PTCH* mutations found in three BCCs (BCC3, BCC7, and BCC8) as well as in the PNET (stP3) had been reported previously (13). Here, *PTCH* mutations in five tumors (BCC10, BCC22, BCC27, BCC31, and BCC33) were newly identified by the screening of 22 additional BCCs. Four of these latter mutations predicted the generation of truncated proteins. BCC31 carried a 1-bp deletion (nt2861delA), and BCC33 carried a 1-bp insertion (nt278insT), both of which resulted in frameshifts and in the introduction of premature stop codons several bp downstream of the mutation site. Two nonsense mutations were found in BCC10 (G3042A) and BCC22 (C1237T) that both caused a premature stop of translation. One BCC (BCC27) carried a missense mutation, namely a G to A transition at nucleotide 3622, which predicts an amino acid exchange of glycine to serine in the intracellular COOH-terminal part of the protein. Two tumors (BCC7 and BCC33) showed mutations in both *PTCH* and *SMOH*. Mutational analysis of all coding exons of *SHH* did not identify any tumor with evidence of a mutation among the 31 BCCs and 15 PNETs investigated. In addition, no instance of *SHH* gene amplification was found by Southern blot analysis of these tumors.

Twenty-six BCCs and all 15 PNETs were investigated by reverse transcription-PCR for the expression of *SMOH*, *PTCH*, *SHH*, and *GLI1* (Table 2, Fig. 2). *SMOH* mRNA was expressed in the reference brain and skin tissue samples. Increased transcript levels relative to these reference tissues were found in 11 of 26 BCCs and 14 of 15 PNETs. The remaining tumors showed expression levels approximately equal to those of the respective reference tissues.

PTCH expression was increased relative to that of normal skin in 25 of 26 BCCs and relative to that of nonneoplastic brain tissue in all PNETs (Table 2, Fig. 2). None of the tumors lacked detectable *PTCH* transcripts. This finding corrects our previous study in which we reported three BCCs and three PNETs without detectable *PTCH* expression (13). When repeating the analysis of these tumors with new batches of cDNA and a modified detection method, *i.e.*, differential reverse transcription-PCR, *PTCH* transcripts were detected in all instances (Table 2).

Only two tumors (BCC14 and stP5) expressed *SHH* mRNA at levels detectable by reverse transcription-PCR (Table 2). Both tumors showed strong signals for *PTCH* and *SMOH* transcripts but no mutations in these genes. No *SHH* transcripts were detected in normal skin or in the reference brain tissue samples. Increased levels of *GLI1*

Table 2 *Summary of investigated tumors, mutational screening results, and expression analyses*

Tumor	Age (yr)	Sex	Location	*PTCH* mutation[a]	*SMOH* mutation[a]	*PTCH* mRNA expression[b]	*SMOH* mRNA expression[b]	*Gli1* mRNA expression[b]	*SHH* mRNA expression[c]
BCC1	74	M	Cheek			+++	+	++	−
BCC2	89	M	Upper lip			NA[d]	NA	NA	NA
BCC3	68	M	Lower leg	C1081T (premature stop)		+++	+++	+++	−
BCC4	68	M	Eye			NA	NA	NA	NA
BCC5	77	M	Shoulder			NA	NA	NA	NA
BCC6	84	M	Cheek		G1604T (Trp⇒Leu)	+++	+++	+++	−
BCC7	58	M	Nose	A3571T (Thr⇒Ser) T3932C (Leu⇒Pro)	G1604T (Trp⇒Leu)	+++	+	+++	−
BCC8	85	M	Chin	1422del4 (frameshift)		+++	+	+++	−
BCC9	61	M	Lower Eyelid			+++	+	++	−
BCC10	74	M	Forehead	G3042A (premature stop)		+++	+	++	−
BCC11	67	F	Upper lip			+++	+	+++	−
BCC12	75	F	Upper lip			+++	+++	+++	−
BCC13	79	M	Glabella			+++	+++	+++	−
BCC14	59	M	Axilla			+++	+++	+++	+
BCC15	88	M	Chin			++	+	++	−
BCC16	33	M	Cheek			++	+	++	−
BCC17	81	F	Nose			++	+++	+++	−
BCC18	65	M	Nose			+	+	++	−
BCC19	74	F	Chin			++	+++	+++	−
BCC20	33	M	Upper lip		G1604T (Trp⇒Leu)	+++	+++	+++	−
BCC21	84	F	Forehead			++	+++	+++	−
BCC22	68	F	Nose	C1237T (premature stop)		++	+++	+++	−
BCC23	75	M	Back			+++	+++	+++	−
BCC24	86	F	Cheek			+++	+	+++	−
BCC25	85	F	Neck			+++	+	++	−
BCC26	69	F	Head			++	+	−	−
BCC27	87	F	Forehead	G3622A (Gly⇒Ser)		++	+	+	−
BCC28	52	F	Head			+++	+	++	−
BCC31	74	M	Chin	2861delA (frameshift)		NA	NA	NA	NA
BCC32	59	M	Back			NA	NA	NA	NA
BCC33	76	F	Glabella	278insT (frameshift)	C595T (Arg⇒Trp)	+++	+	++	−
MB9[e]	8	M	Cerebellum			++	+++	−	−
MB11	6	F	Cerebellum			+++	+++	−	−
MB13	5	M	Cerebellum			++	+++	−	−
MB14	46	F	Cerebellum			+++	++	+++	−
MB15	37	M	Spinal cord			++	++	−	−
dpMB9[f]	23	F	Cerebellum			+++	++	+++	−
dpMB10	11	M	Cerebellum			++	+++	++	−
dpMB11	33	F	Cerebellum		G1598A (Ser⇒Asn)	+++	+++	+++	−
dpMB12	25	M	Cerebellum			++	+	++	−
dpMB13	12	M	Cerebellum			+++	+++	++	−
dpMB14	26	M	Cerebellum			+++	+++	+++	−
stP2[g]	0.5	M	Cerebrum			++	+++	−	−
stP3	7	M	Cerebrum	C2161T (Pro⇒Ser)		++	++	−	−
stP4	2	F	Cerebrum			+++	+++	++	−
stP5	5	M	Cerebrum			+++	+++	++	+

[a] The consequences at the protein level of the mutations detected are given in brackets.
[b] The expression levels were ranked according to the following scale: −, no detectable expression; +, expression level below or equal to that of the reference tissue (normal skin and nonneoplastic brain tissue, respectively); ++, moderately increased expression level relative to that of the reference tissue; +++, strongly increased expression level relative to that of the reference tissue.
[c] The absence (−) or presence (+) of detectable *SHH* transcripts is indicated.
[d] NA, not analyzed.
[e] MB, medulloblastoma.
[f] dpMB, desmoplastic MB.
[g] stP, supratentorial PNET.

mRNA relative to the respective reference tissues were found in 24 of 26 BCCs and 9 of 15 PNETs (Table 2, Fig. 2).

Discussion

We report on the detection of missense mutations in the *SMOH* gene in 4 of 31 BCCs and 1 of 15 PNETs. The mutations found in three BCCs and in the PNET predict the exchange of single amino acids in the hydrophobic region of the putative seventh transmembrane domain of Smoh. This domain exhibits a high degree of homology to the *Drosophila* and rat smoothened proteins as well as to the *Drosophila* frizzled family of transmembrane glycoproteins (3). Point mutations in this conserved domain could possibly result in conformational changes that lead to constitutional activation of the Smoh protein. The finding of an increased expression of two genes known to be transcriptionally up-regulated by Shh, *i.e.*, *PTCH* and *GLI1* (4–6), in the tumors with *SMOH* mutations supports this hypothesis. One tumor (BCC33) carried a somatic point mutation altering codon 199 of the Smoh protein. The expression of *PTCH* and *GLI1* transcripts was elevated in BCC33, however, this tumor also had a mutation in *PTCH*. Therefore, the significance of the codon 199 mutation in Smoh remains to be further investigated.

Experimental evidence for the activating nature of the mutation found in BCC6, BCC7, and BCC20 has been provided by a recent study that was published during preparation of our manuscript (21). These authors identified missense mutations in *SMOH* in 3 of 47 sporadic BCCs. Two tumors carried the identical mutation (G1604T) found in BCC6, BCC7, and BCC20. Xie *et al.* (21) also showed that Smoh containing either this particular mutation or another missense mutation (Arg to Gln at codon 562) can cooperate with adenovirus E1A to transform rat embryonic fibroblasts. In addition, BCC-like lesions were found to develop in transgenic murine skin overexpressing mutant Smoh (21). Thus, there is evidence that *SMOH* represents

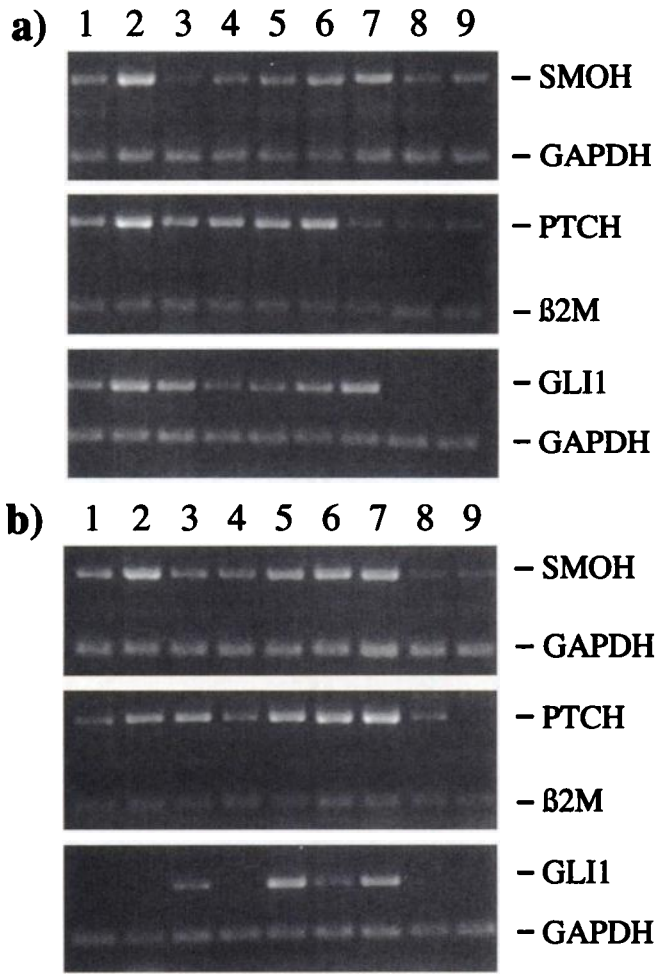

Fig. 2. Analysis of *SMOH*, *PTCH*, and *GLI1* mRNA expression by differential reverse transcription-PCR in BCCs (*a*) and PNETs (*b*). *a*, *Lanes 1–7*: BCC3, BCC6, BCC7, BCC8, BCC13, BCC20, and BCC22, respectively; *Lanes 8* and *9*, two normal skin samples. *b*, *Lanes 1–7*: MB9, MB11, MB14, MB15, dpMB11, dpMB13, and dpMB14, respectively; *Lanes 8* and *9*, two nonneoplastic cerebellar tissue samples. The majority of tumors show increased signals compared to the respective nonneoplastic tissues. No *GLI1* transcripts were detected in tumors MB9 (*b*, *Lane 1*), MB11 (*b*, *Lane 2*), and MB15 (*b*, *Lane 4*).

a cellular oncogene that can be activated by missense mutations. The precise mechanisms by which mutations result in Smoh activation and neoplastic transformation remain to be elucidated. One possibility is that *SMOH* mutations alter the interaction between Smoh and Ptch and thereby prevent Smoh from Ptch inhibition. However, other mechanisms leading to constitutional Smoh activity may also be possible.

Xie *et al.* (21) mapped the *SMOH* gene locus to the chromosomal region 7q31–q32. Our FISH results refine this localization to 7q32. This chromosomal segment was found to be amplified in certain types of human tumors including malignant gliomas (22). However, none of the BCCs and PNETs investigated here showed evidence of *SMOH* gene amplification. Thus, gene amplification can be excluded as a common mechanism of *SMOH* activation in these tumors. Furthermore, the finding that wild-type Smoh, in contrast to mutant Smoh, did not transform rat fibroblasts in cooperation with adenovirus E1A (21) suggests that increased expression of Smoh on its own is not sufficient to induce the growth of BCCs and PNETs.

The current data (the present study and Ref. 21) indicate that only small fractions of BCCs and PNETs have *SMOH* mutations. The actual frequency of *SMOH* alterations in these tumors may be somewhat higher, because the sensitivity of the SSCP screening is limited. The sensitivity of SSCP analysis in detecting single-base substitutions has been shown to range from 97% for fragments of 150 bp to about 70% for fragments of 250 bp, respectively (23). Because one fragment analyzed in our study was even larger than 250 bp, and the first 60 codons were not screened, it is possible that we have missed some cases with *SMOH* mutations.

Similarly, the actual incidence of *PTCH* mutations in BCCs is likely to be higher than that suggested by the values of 26% (the present study) and 32% (10) determined by SSCP screening of two independent tumor series. This assumption is supported by the finding of allelic loss spanning the *PTCH* gene locus on 9q22 in more than 60% of BCCs (24, 25). In addition, direct sequencing of *PTCH* in two BCCs without SSCP abnormalities revealed inactivating mutations in both tumors (10). In line with studies from other laboratories (8–12), the majority of *PTCH* mutations identified in our series of BCCs and PNETs (the present study and Ref. 13) were frameshift or nonsense mutations that result in truncated proteins, which are likely to be functionally impaired or inactivated.

All tumors with *SMOH* mutations and the majority of tumors with *PTCH* mutations showed elevated *PTCH* and *GLI1* transcript levels compared to those of normal skin and nonneoplastic brain tissue. However, an increased expression of *PTCH* and *GLI1* as well as *SMOH* was also detected in the majority of BCCs and PNETs without a demonstrated mutation in *PTCH* or *SMOH*. These findings corroborate earlier reports showing consistent overexpression of *PTCH* mRNA in sporadic and familial BCCs (26), concordant overexpression of *SMOH* and *PTCH* transcripts in the majority of sporadic BCCs (27), and increased expression of *GLI1* mRNA in sporadic BCCs with and without a demonstrated *PTCH* mutation (28). Thus, it is possible that some tumors carry alterations in other genes that may result in activation of the Shh signal transduction pathway. A recent study reported on a somatic missense mutation exchanging histidine to tyrosine at codon 133 of the Shh protein in 1 of 43 BCCs, 1 of 14 medulloblastomas, and 1 of 6 breast carcinomas (15). However, we failed to identify this particular mutation or any other *SHH* alteration in our series of 31 BCCs and 15 PNETs. Therefore, it seems that *SHH* is only rarely altered in these tumors. In addition, the vast majority of BCCs and PNETs investigated here lacked expression of *SHH* mRNA at levels detectable by reverse transcription-PCR. Similar to our results, other authors have also found *SHH* expression restricted to single cases of BCC (16, 28). Both tumors of our series that expressed *SHH* transcripts showed no detectable *PTCH* and *SMOH* mutations but overexpressed *GLI1* and *PTCH*. Thus, it is possible that *SHH* mutation and/or increased expression contribute to tumorigenesis in a small fraction of BCCs and PNETs. An additional candidate for genetic alterations in these tumors is the proto-oncogene *GLI1*. Like mutant Smoh, Gli1 can transform rat fibroblasts in cooperation with adenovirus E1A (29). Ectopic expression of Gli1 in embryonic frog epidermis resulted in BCC-like tumors (28). However, it remains to be investigated whether *GLI1* is directly targeted by genetic alterations in BCCs and PNETs. Finally, mutations in yet other genes, *e.g.*, *TP53* or *CDKN1A* (27), could possibly up-regulate the expression of proteins involved in the Shh pathway.

In summary, we have provided evidence that not only *PTCH* but also *SMOH* represents an important target for mutations in human sporadic BCCs and PNETs. Genetic alteration of *SHH* seems to be less frequent in these tumors. The fact that increased expression of *PTCH*, *SMOH*, and *GLI1* is not restricted to BCCs and PNETs with a demonstrated mutation in *PTCH* or *SMOH* suggests the possibility that additional genes coding for proteins involved in the Shh signaling cascade are altered in these tumors.

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