

Identification of Mutations in the Human *PATCHED* Gene in Sporadic Basal Cell Carcinomas and in Patients with the Basal Cell Nevus Syndrome

Michelle Aszterbaum, Alana Rothman, Ronald L. Johnson,* Monte Fisher, Jingwu Xie, Jeannette M. Bonifas, Xioli Zhang, Mathew P. Scott,* and Ervin H. Epstein Jr

Department of Dermatology, University of California, San Francisco, California, U.S.A.; *Departments of Developmental Biology and Genetics, Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, California, U.S.A.

Mutations in *PATCHED* (*PTC*), the human homolog of the *Drosophila* *patched* gene, have been identified in most exons of the gene in patients with the basal cell nevus syndrome and in sporadic basal cell carcinomas. We have screened the 23 *PTC* exons for mutations using single strand conformation polymorphism analysis of DNA from 86 basal cell nevus syndrome probands, 26 sporadic basal cell carcinomas, and seven basal cell nevus

syndrome-associated basal cell carcinomas. This screen identified mutations located in eight exons in 13 of the basal cell nevus syndrome patients and in three of the tumors. The most common mutations were frameshifts resulting in premature chain termination. These results provide further evidence for the crucial role of *PTC* as a tumor suppressor in human keratinocytes. *Keyword:* *Hedgehog. J Invest Dermatol 110:885-888, 1998*

Basal cell carcinomas (BCC) are the commonest human cancer, with an estimated 750,000 cases per year in the U.S.A. alone (Miller and Weinstock, 1994). The great majority of these occur on sun-exposed skin, and their steadily rising incidence is commonly attributed to ultraviolet mutagenesis accompanying increased recreational sun exposure. BCC also occur in the context of the BCNS (nevoid basal cell carcinoma syndrome, Gorlin Syndrome, MIM #109400), a rare autosomal dominant disease in which patients have both developmental (e.g., skeletal) abnormalities and postnatal tumors (e.g., BCC, which occur in greater numbers and at an earlier age than in sporadic cases, and medulloblastomas) (Gorlin, 1987). Positional cloning efforts in many laboratories extending over a decade, culminated in 1996 in the identification of heritable mutations in *PTC* in patients with the BCNS and somatic mutations in *PTC* in sporadic BCC (Johnson *et al*, 1996; Hahn *et al*, 1996), and subsequently in the identification of *PTC* gene mutations in sporadic medulloblastomas and other cancers (Raffel *et al*, 1997; Xie *et al*, 1997; Wolter *et al*, 1997).

The *PTC* gene is the human homolog of the *Drosophila* segment polarity gene *ptc*. It encodes a 12 transmembrane domain protein that physically binds at least one of the three known vertebrate Hedgehog molecules – Sonic hedgehog (Shh) – with high affinity. Current models posit that Ptc protein inhibits the Hedgehog signaling pathway (HHSP) through its interaction with the Smoothed (Smo) protein, which is predicted to be a seven transmembrane protein with the configuration of a G protein-coupled receptor. According to this model, Ptc inhibition of Smo is relieved upon binding of Shh to Ptc (Stone *et al*, 1996;

Marigo *et al*, 1996) or following mutational inactivation of Ptc. Smo signaling activates transcription of Hh targets including *PTC*, and there is consistent upregulation of *PTC* transcription in BCC (Gailani *et al*, 1996b; Uden *et al*, 1997; Kallassy *et al*, 1997). BCC-like epidermal proliferation and BCNS-like skeletal abnormalities are produced by overexpression of Shh in murine and human epidermal keratinocytes, and identical (presumably activating) *SHH* somatic mutations have been identified in a sporadic basal cell carcinoma, a medulloblastoma, and a breast carcinoma (Oro *et al*, 1997; Fan *et al*, 1997).

Further, mutations in *SMO* also can be found in BCC (Xie *et al*, 1998). Thus, activation of transcription of Hh targets can be effected by mutations in any of the three components of the signaling pathway that interact at the cell membrane – activating mutations in the proto-oncogenes *SHH* and *SMO* or inactivating mutations in the tumor suppressor gene *PTC*.

MATERIALS AND METHODS

Subjects One affected person from each of 86 unrelated BCNS kindreds was studied. All studied patients had multiple BCC with onset in the second decade of life in addition to at least one of the following: multiple keratocysts of the jaw, palmar pits, or skeletal abnormalities. No patients meeting the above criteria were excluded from the screen.

DNA isolation, amplification, and single strand conformation polymorphism (SSCP) analysis DNA was extracted from blood samples using standard phenol-chloroform extraction techniques. Paraffin-embedded BCC DNA was isolated as reported previously (Bonifas *et al*, 1994). All studied BCC occurred on sun-exposed sites. The following polymorphic microsatellite repeat loci for chromosome 9q were used to determine allelic deletions: *D9S152*, *D9S176*, *D9S180*, *D9S196*, *D9S197*, *D9S12*, *D9S119*, *D9S127*, *D9S280*, *D9S287* (Bonifas *et al*, 1994; Xie *et al*, 1997).

SSCP analysis was performed using intronic primers designed to flank each of the 23 exons of *PTC* (Xie *et al*, 1997). Forward primers were end-labeled with γ P³², and exons were amplified by PCR. PCR was carried out using 100 ng of genomic DNA with amplification conditions as follows: denaturation for 1 min at 95°C, annealing at 55°C for 1'30", extension at 72°C for 1'30" for 35 cycles. Amplification products were diluted 1:7 in stop solution, heated to 90°C for 5 min, and then chilled on ice prior to loading 3.5 μ l on a MDE

Manuscript received November 1, 1997; revised January 30, 1998; accepted for publication February 19, 1998.

Reprint requests to: Dr. Ervin H. Epstein, Jr., Bldg 100 Room 269, San Francisco General Hospital, 1001 Potrero Avenue, San Francisco, CA 94110.

Abbreviations: BCC, basal cell carcinoma; BCNS, basal cell nevus syndrome; HHSP, Hedgehog signaling pathway; Ptc, patched; Shh, Sonic hedgehog; Smo, smoothed; SSCP, single strand conformation polymorphism.

Table I. Mutations identified in BCNS probands and sporadic BCC^a

Source	Exon	Nucleotide change ^b	Consequence	Protein region ^c
BCNS	6	T886 del	frameshift	EL 1
BCNS	6	747-759 del	frameshift	EL 1
BCNS	7	T950 del	frameshift	EL 1
BCNS	8	G1144 ins	frameshift	EL 1
BCNS	8	T1127C	phe→pro	EL 1
BCNS	8	1208 5 bp deletion ATGTG	frameshift	EL 1
BCNS	10	G→C Intron/exon border	disrupted splice site	TM 2
BCNS	14	T2132 del	frameshift	CL 3
SP BCC ^d	14	1887 del	frameshift	CL 3
BCNS	17	C 2798 del	frameshift	EL 2
BCNS	17	G2778 A	premature stop	EL 2
S-BCC ^e	17	G2778 A	premature stop	EL 2
BCNS	18	3104T ins	frameshift	TM 8
SP BCC ^d	19	G3196T	premature stop	TM 9
BCNS	19	GTG 3246 ins	val insertion	TM 10
SP BCC ^d	20	A3340T	arg→trp	CL 4
BCNS	20	3364 10 bp del	frameshift	TM 11

^aMutation analysis via direct sequencing or automated sequencing of DNA extracted from blood of BCNS probands (BCNS) or sporadic BCC (SP BCC).

^bdel, deletion; ins, insertion.

^cRegion of Ptc protein predicted to correspond to mutation site. EL, extracellular loop; TM, transmembrane domain 2, 8, 9, 10, or 11; CL, cytosolic Loop 3.

^dAll sporadic BCC with identified *PTC* mutations also had loss of heterozygosity.

^eBCNS-associated BCC.

gel (BMC bioproducts) at room temperature or 4°C and run at 6 W for 10–12 h. After electrophoresis, the gels were transferred to Whatman chromatography paper and dried for 1 h at 94°C. Autoradiography with Kodak XAR film at room temperature allowed detection of PCR products after a 24 h exposure (Chen *et al*, 1995).

DNA cloning and sequencing Exons with detectable SSCP abnormalities were sequenced. PCR was performed under the same conditions described for SSCP. Direct sequencing was performed on samples using Sequenase PCR Product Sequencing Kit (Amersham Life Science). Further mutation detection was pursued in a subset of samples by cloning purified PCR product into plasmid vector pCR2.1 (Invitrogen) and sequencing cloned material either by the Sequenase Sequencing kit or by automated sequencing using the ABI Prism sequencer.

RESULTS

Of 26 sporadic BCC screened, 11 showed loss of heterozygosity at one or more of the polymorphic markers examined in the *PTC* gene region. All markers were at least 80% informative. Of these 11, three tumors were found to have *PTC* gene mutations, each in a different exon of the gene. One of these is predicted to result in an amino acid substitution, one in a premature stop codon, and one in a frameshift. The latter two of these identified BCC mutations cause premature chain termination. These three mutations are not those considered to be characteristic of UV-induced changes. Two of the mutations occurred in exons encoding Ptc protein cytosolic loop regions and one occurred in an exon encoding a transmembrane domain. We found no SSCP abnormalities in DNA from the 15 tumors that lacked loss of heterozygosity. Of the eight BCNS-associated BCC, two showed loss of heterozygosity and one of these had identical mutations identified in constitutional and tumor DNA. The results are summarized in **Table I**.

We identified 13 *PTC* mutations among the DNA samples screened from the 86 probands. These mutations were found in nine different exons. Nine of the 13 resulted in frameshifts (**Fig 1**), one resulted in an amino acid substitution, one in a premature stop codon, one in an amino acid insertion, and one in disruption of a splicing site. The results are summarized in **Table I**.

DISCUSSION

PTC gene mutations were identified in 13 of 86 BCNS probands, in three of 26 sporadic BCC, and in one of eight BCNS-associated BCC. Each of the BCC in which a mutation was identified had sustained deletion of the second allele. Our data are consistent with the conclusion that *PTC* acts as a classic tumor suppressor gene, requiring “two hits”

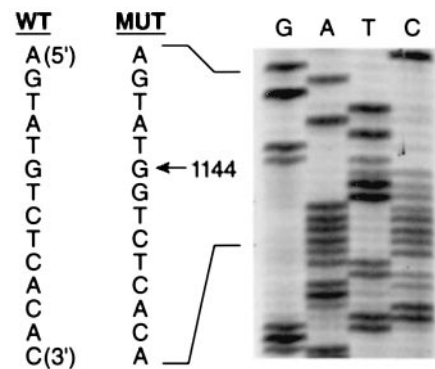


Figure 1. Identification of frameshift mutation (G1144 ins) in proband CO. Direct sequencing of PCR product of Exon 8 reveals a G insertion at position 1144, resulting in a frameshift. Wild-type sequence reads CGAGTAGTCTCACACATCAACTGG. Note that doublets occur distal to the base pair deletion.

for tumorigenesis (Knudson, 1971) in at least some BCC. Sporadic BCC require mutations of both alleles of *PTC* by postnatal insults such as UV or X-ray irradiation, chemical carcinogenesis, or random genetic alteration. In contrast, BCNS patients have greater susceptibility to tumorigenesis: they inherit a germline mutation of one allele of *PTC* and require only one postnatal genetic “hit” to inactivate the remaining wild-type allele.

The three *PTC* mutations in sporadic BCC from UV-exposed sites reported herein are not characteristic of UV-induced mutagenesis, although one (a deletion of base pair 1887) occurs at a dipyrimidine site. In contrast, 11 of 24 previously reported mutations in *PTC* in sporadic BCC have been of the UV signature type (Gailani *et al*, 1996b; Udden *et al*, 1996; Hahn *et al*, 1996; Johnson *et al*, 1996; Wolter *et al*, 1997). UVB-type signature mutations including C→T and CC→TT base substitutions comprise the majority of p53 gene mutations found in Caucasians in both BCC (65%) (Rady *et al*, 1992; Moles *et al*, 1993; Ziegler *et al*, 1993; van der Riet *et al*, 1994; Gailani *et al*, 1996a) and squamous cell carcinomas (SCC) (67%) (Brash *et al*, 1991; Pierceall *et al*, 1991; Moles *et al*, 1993). The lower incidence of UVB signature mutations in *PTC* (11 of 27, 41%) suggests that mutagenic events other than UV irradiation may also cause Ptc inactivation and trigger tumorigenesis. Furthermore, this relatively low fraction of BCC cells that have UVB induced mutations is consistent

with epidemiologic studies that have found poor correlation between UVB dose and incidence of BCC, unlike the better correlation between UVB dose and the incidence of SCC (Strickland *et al*, 1989; Vitasa *et al*, 1990).

Thirteen of the 16 mutations identified are expected to result in protein truncation, with the most proximal site being the first extracellular loop. Approximately 75% of previously reported constitutional mutations in BCNS patients and somatic mutations in BCC (whether in BCNS patients or in nonaffected persons) are predicted to cause premature protein truncation (Hahn *et al*, 1996; Johnson *et al*, 1996; Gailani *et al*, 1996b; Uden *et al*, 1996; Chidambaram *et al*, 1996; Wicking *et al*, 1997; Wolter *et al*, 1997; Lench *et al*, 1997). In addition, mutations in exons 6 and 8 appear to be a common site of *PTC* mutation (eight of 52 reported mutations in BCNS probands occur in these exons). The identification of two mutations in exons encoding the fourth cytosolic loop and the eleventh transmembrane domains in this study provides further evidence for the importance of these terminally located structures to Ptc protein tumor suppressor function. The mechanism by which these and other mutations alter the function of the human Ptc protein remains to be elucidated, but they are consistent with the idea that loss of Ptc protein function results in BCC tumorigenesis.

In *Drosophila*, Ptc functions as a critical component of the HHSP. Hedgehog, a diffusible protein, binds to and inhibits Ptc. In the absence of hedgehog inhibition, Ptc acts to inhibit Smo, an adjacent transmembrane protein. In *Drosophila*, activated Smo signaling activates Cubitus Interruptus, which encodes a transcription factor that is homologous to the human Gli family proteins (Von Ohlen *et al*, 1997). Cubitus Interruptus activates transcription of *wingless*, *ptc*, and *decapentaplegic* and represses transcription of *hedgehog* (Aza-Blanc *et al*, 1997; Von Ohlen *et al*, 1997).

The presence of a homologous HHSP that can result in tumorigenesis in mammals is suggested by the recent report that overexpression of wild-type Shh or mutant Smo in the epidermis of transgenic mice produces BCC-like proliferations in the skin (Oro *et al*, 1997; Xie *et al*, 1998). In addition, *in vitro* transformation of cell lines with an expression vector containing a mutant *SMO* gene in cooperation with an EIA-expressing construct resulted in an altered cell phenotype with loss of contact inhibition and formation of foci of transformation (Xie *et al*, 1998). Finally, *in situ* studies indicate that there is heightened transcription of *PTC* and *GLI* mRNA in BCC, whereas normal epidermis has no detectable *PTC* mRNA expression (Gailani *et al*, 1996b; Uden *et al*, 1997; Dahmane *et al*, 1997).

Although no kindred with mapping of the BCNS gene to any site other than chromosome 9q has been reported, we like others have identified *PTC* gene mutations in a rather low percentage of BCNS families. Possible explanations for this discrepancy may include insensitivity of SSCP, and indeed Hahn *et al* (1996) and Wicking *et al* (1997) found *PTC* gene mutations by sequencing samples without detectable SSCP abnormalities. In addition, SSCP temperature conditions were varied (22°C and 4°C) for only two exons due to limitations in the quantity of patient DNA, and this did in fact yield an additional aberrant band (due to a T2132 del) that had not been found at room temperature. Although only these two exons were additionally screened at this low temperature, it could be extrapolated that had there been sufficient patient DNA, further mutations might have been identified. Alternate explanations for our low mutation identification rate include possible promoter or enhancer region abnormalities, abnormal methylation, or the presence of another tumor suppressor gene in the same chromosomal region.

Future studies will delineate the effect of mutant Ptc, Shh, and Smo proteins on downstream HHSP components, including Gli, Wnt, and TGF- β , the mechanism by which HHSP target gene transcription triggers tumorigenesis, and therapeutic modes of suppressing pathway activation.

Diseases (AR39959 and AR43119) (EHE), and fellowships from the Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation (DRG 1218) (RLJ), Walter Berry Foundation (RLJ), the Dermatology Foundation through funding from the Neutrogena Corporation (JX), and the generous support of Patricia Hughes. MPS is an Investigator of the Howard Hughes Medical Institute. We also wish to thank Dr. James E. Cleaver for his critical review of our manuscript.

REFERENCES

- Aza-Blanc P, Ramirez-Weber FA, Laget MP, Schwartz C, Kornberg TB: Proteolysis that is inhibited by hedgehog targets cubitus interruptus protein to the nucleus and converts it to a repressor. *Cell* 89:1043-1053, 1997
- Bonifas JM, Bare JW, Kerschmann RL, Master SP, Epstein EH: Parental origin of chromosome 9q22.3-q31 lost in basal cell carcinomas from basal cell nevus syndrome patients. *Hum Mol Genet* 3:447-448, 1994
- Brash DE, Rudolph JA, Simon JA, *et al*: A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. *Proc Natl Acad Sci USA* 88:10124-10128, 1991
- Chen H, Bonifas JM, Matsumura K, Ikeda S, Leyden WA, Epstein EH Jr: Keratin 14 gene mutations in patients with epidermolysis bullosa simplex. *J Invest Dermatol* 105:629-632, 1995
- Chidambaram A, Goldstein AM, Gailani MR, *et al*: Mutations in the human homologue of the *Drosophila patched* gene in Caucasian and African-American nevoid basal cell carcinoma syndrome patients. *Cancer Res* 56:4599-4601, 1996
- Dahmane N, Lee J, Robins P, Heller P, Ruiz i Altaba A: Activation of the transcription factor Gli1 and the Sonic hedgehog signalling pathway in skin tumours. *Nature* 389:876-881, 1997
- Fan H, Oro AE, Scott MP, Khavari PA: Induction of basal cell carcinoma in transgenic human skin expressing sonic hedgehog. *Nature Med* 3:788-792, 1997
- Gailani MR, Lefell DJ, Ziegler AM, Gross EG, Brash DE, Bale AE: Relationship between sunlight exposure and a key genetic alteration in basal cell carcinoma. *J Natl Cancer Inst* 88:349-354, 1996a
- Gailani MR, Stahle-Backdahl M, Lefell DJ, *et al*: The role of the human homologue of *Drosophila patched* in sporadic basal cell carcinomas. *Nature Genet* 14:78-81, 1996b
- Gorlin RJ: Nevoid basal-cell carcinoma syndrome. *Medicine* 66:98-109, 1987
- Hahn H, Wicking C, Zaphiropoulos PG, *et al*: Mutations of the human homolog of *Drosophila patched* in the nevoid basal cell carcinoma syndrome. *Cell* 85:841-851, 1996
- Johnson RL, Rothman AL, Xie J, *et al*: Human homolog of *patched*, a candidate gene for the basal cell nevus syndrome. *Science* 272:1668-1671, 1996
- Kallassy M, Toftgård R, Ueda M, Nakazawa K, Vorechovsky I, Yamasaki H, Nakazawa H: Patched (*ptch*)-associated preferential expression of smoothened (*smo*) in human basal cell carcinoma of the skin. *Cancer Res* 57:4731-4735, 1997
- Knudson AG: Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci USA* 68:820-823, 1971
- Lench NJ, Telford EA, High AS, Markham AF, Wicking C, Wainwright BJ: Characterisation of human patched germ line mutations in naevoid basal cell carcinoma syndrome. *Hum Genet* 100:497-502, 1997
- Marigo V, Davey RA, Zuo Y, Cunningham JM, Tabin CJ: Biochemical evidence that patched is the hedgehog receptor. *Nature* 384:176-179, 1996
- Miller DL, Weinstock MA: Non-melanoma skin cancer in the United States: incidence. *J Am Acad Derm* 30:774-778, 1994
- Moles JP, Moyret C, Guillot B, Jeanteur P, Guilhou JJ, Theillet C, Basset-Segun N: p53 mutations in human epithelial skin cancers. *Oncogene* 8:583-588, 1993
- Oro AE, Higgins KM, Hu Z, Bonifas JM, Epstein EH, Scott MP: Basal cell carcinomas in mice overexpressing sonic hedgehog. *Science* 276:817-821, 1997
- Pierceall WE, Mukhopadhyay T, Goldberg LH, Ananthaswamy N: Mutations in the p53 tumor suppressor gene in human cutaneous squamous cell carcinomas. *Mol Carcinog* 4:445-449, 1991
- Rady P, Scinicariello Wagner RF, Tyring SK: p53 mutations in basal cell carcinomas. *Cancer Res* 52:3804-3806, 1992
- Raffel C, Jenkin RB, Frederick L, Hebrink D, Alderete B, Fults DW, James CD: Sporadic medulloblastomas contain *PTCH* mutations. *Cancer Res* 57:842-845, 1997
- van der Riet P, Karp D, Farmer E, *et al*: Progression of basal cell carcinoma through loss of chromosome 9q and inactivation of a single p53 allele. *Cancer Res* 54:25-27, 1994
- Stone DM, Hynes M, Armanini M, *et al*: The tumour-suppressor gene patched encodes a candidate receptor for sonic hedgehog. *Nature* 384:129-134, 1996
- Strickland PT, Vitasa BC, West SK, Rosenthal FS, Emmett EA, Taylor HR: Quantitative carcinogenesis in man: solar ultraviolet B dose dependence of skin cancer in Maryland watermen. *J Natl Cancer Inst* 81:1910-1913, 1989
- Uden AB, Holberg E, Lundh-Rozell B, Stahl-Backdahl M, Zaphiropoulos PG, Toftgård R, Vorechovsky I: Mutations in the human homologue of *Drosophila patched* (*PTCH*) in basal cell carcinomas and the Gorlin Syndrome: different in vivo mechanisms of *PTCH* inactivation. *Cancer Res* 56:4562-4565, 1996
- Uden AB, Zaphiropoulos PG, Bruce K, Toftgård R, Stahle-Blackdahl M: Human *patched* (*PTCH*) mRNA is overexpressed consistently in tumor cells of both familial and sporadic basal cell carcinoma. *Cancer Res* 57:2336-2340, 1997
- Vitasa BC, Taylor HR, Strickland PT, *et al*: Association of nonmelanoma skin cancer and actinic keratosis with cumulative solar ultraviolet exposure in Maryland watermen. *Cancer* 65:2811-2817, 1990
- Von Ohlen T, Lessing D, Nüsse Roel Hooper JE: Hedgehog signaling regulates transcription through cubitus interruptus, a sequence-specific DNA binding protein. *Proc Natl Acad Sci USA* 94:2404-2409, 1997
- Wicking C, Shanley S, Smyth I, *et al*: Most germ-line mutations in the nevoid basal cell

We thank the many patients and their families who participated in this study and the physicians who kindly referred them and aided in sample collection. This work was supported by grants from the National Institute of Arthritis, Musculoskeletal and Skin

- carcinoma syndrome lead to premature termination of the patched protein, and no genotype-phenotype correlations are evident. *Am J Hum Genet* 60:21-26, 1997
- Wolter M, Reifemberger J, Sommer C, Ruzicka T, Reifemberger G: Mutations in the human homologue of the *Drosophila* segment polarity gene patched (*PTCH*) in sporadic basal cell carcinomas of the skin and primitive neuroectodermal tumors of the central nervous system. *Cancer Res* 57:2581-2585, 1997
- Xie J, Johnson RL, Zhang X, et al: Mutations of the *Patched* gene in sporadic extracutaneous tumors. *Cancer Res* 57:2369-2372, 1997
- Xie J, Murone M, Luoh SM, et al: Activating *Smoothed* mutations in sporadic basal cell carcinomas. *Nature* 391:90-92, 1998
- Ziegler A, Leffell DJ, Kunala S, et al: Mutation hotspots due to sunlight in the p53 gene of nonmelanoma skin cancers. *Proc Natl Acad Sci USA* 90:4216-4220, 1993