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Advances in Brief

Medulloblastomas of the Desmoplastic Variant Carry Mutations of the Human Homologue of *Drosophila patched*[1]

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Abstract

Inactivating mutations in the *PTCH* gene, a human homologue of the *Drosophila* segment polarity gene *patched*, have been identified recently in patients with nevoid basal cell carcinoma syndrome. These patients are predisposed to various neoplasias including basal cell carcinomas and medulloblastomas (MBs). To determine the involvement of *PTCH* in sporadic MBs, which represent the most frequent malignant brain tumors in children, we screened for *PTCH* alterations in an unselected panel of 64 biopsy samples from 62 patients and four continuous MB cell lines, all derived from patients with sporadic MBs. Using single-strand conformational polymorphism analysis, we screened exons 2–22 and detected nonconservative *PTCH* mutations in 3 of 11 samples from sporadic cases of the desmoplastic variant of MB but none in 57 MBs with classical (nondesmoplastic) histology. In two of the tumors with mutations and in two additional desmoplastic cases, loss of heterozygosity was found at 9q22. These findings suggest that *PTCH* represents a tumor suppressor gene involved in the development of the desmoplastic variant of MB.

Introduction

MBs[3] are malignant primitive neuroectodermal tumors of the cerebellum. They occur predominantly in childhood with an incidence of approximately five per million children (1). Although most MBs are sporadic, the incidence is elevated to 3% in patients with NBCCS (2). NBCCS patients exhibit diverse developmental anomalies and are predisposed to several malignancies including BCCs and MBs (2–4). The *NBCCS* gene has been mapped to chromosome 9q22 (5) and was recently identified as a human homologue of the *Drosophila* segment polarity gene *patched*, which encodes a transmembrane receptor for the Hedgehog morphogen family (6–10). The human *PTCH* gene spans 34 kb and has at least 23 exons (8). Inactivating mutations of *PTCH* occur in NBCCS patients and their BCCs as well as in sporadic BCCs (7, 8, 11–14). LOH in the region of the *PTCH* locus has been observed in NBCCS-associated as well as in sporadic MBs (15, 16). Therefore, the *PTCH* gene represents a candidate gene for sporadic MBs. In this study, we searched for *PTCH* alterations by screening for mutations and analysis of the *PTCH* mRNA expression in sporadic MBs.

Materials and Methods

Patients, Tumors, and Cell Lines. A total of 68 MB samples were analyzed, including 64 biopsy samples and 4 from MB cell lines. The biopsy material was collected from 62 patients. In two of these patients, we were able to study both the primary and the recurrent tumors. The previously described MB cell lines D283Med, D341Med, and MHH-MED-1 have been derived from patients with sporadic MBs of the classical (nondesmoplastic) variant; the cell line Daoy has been generated from a desmoplastic MB (17). Constitutional DNA from peripheral blood was available in 40 patients. DNA samples from peripheral blood from healthy Caucasian volunteers were used as controls. A sample of normal cerebellum was analyzed. This biopsy specimen was from an adult patient with a cerebellar vascular malformation and was found to be normal upon histopathological review. The patients' age ranged from 1 month to 59 years; there were 46 males and 20 females. None of the patients had clinical signs of NBCCS or had first-degree relatives with NBCCS. All tumors were diagnosed according to the revised WHO classification of brain tumors using standard histological methods including H&E and reticulin stains and immunohistochemical reactions (18). Differentiation was assessed by immunostaining for embryonal neural cell adhesion molecule, neuron-specific enolase, synaptophysin, and glial acidic fibrillary protein. Frozen tumor samples were obtained at the time of surgical resection, snap frozen in liquid nitrogen, and stored at −80°C.

DNA Extraction and LOH Analysis. Tumor fragments were selected for extraction of DNA after careful examination of corresponding frozen sections to exclude contaminating necrotic debris or normal cerebellar tissue and to determine the histological characteristics of the tumors. DNA was extracted by standard proteinase K digestion and phenol/chloroform extraction (15). LOH was determined by microsatellite analysis with the markers *D9S287* and *D9S197*, which are tightly linked to the *PTCH* gene, and with two additional markers on 9q (*D9S302* and *D9S303*) essentially as described previously (15, 19).

SSCP Analysis and DNA Sequencing. SSCP analysis of exons 2–22 was performed using 22 published primer pairs (8, 12). In addition, we used the following primer sets: exon 12, 5′-GACCATGTCCAGTGCAGCTC-3′ and 5′-CGTTCAGGATCACCACAGCC-3′; exon 12B (exon 12 turned out to consist of two exons),[4] 5′-AGTCCTCTGATTGGGCGGAG-3′ and 5′-CCATTCTGCACCCAATCAAAAG-3′; and exon 20, 5′-TGTTCCCGTTTCCTCTTG-3′ and 5′-GCACAGGAAACACAGCATTC-3′. PCR reactions were performed in a volume of 10 μl with 20 ng of genomic DNA in a buffer containing 50 mM KCl, 1.0–2.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.5), 0.01% gelatin, 200 μM of each deoxynucleotide triphosphate, 20 pmol of the primers, and 0.25 unit of Taq polymerase (Life Technologies, Inc.) on a Uno Thermoblock cycler (Biometra). The products were analyzed on polyacrylamide gels with different acrylamide concentrations and acrylamide:bisacrylamide ratios. Gel composition and electrophoresis conditions were optimized for each individual primer pair. The single and double strands were visualized by silver staining, as described previously (15). PCR products that showed a gel mobility shift were excised from the wet gel, eluted (20), and

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[3] The abbreviations used are: MB, medulloblastoma; NBCCS, naevoid basal cell carcinoma syndrome; BCC, basal cell carcinoma; LOH, loss of heterozygosity; SSCP, single-strand conformational polymorphism; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR.

[4] Unpublished results.

reamplified by PCR with the same primers. The resulting products were purified using spin columns (Qiagen Quick Spin), and 20 ng were used for cycle sequencing with a fluorescent dideoxy terminator kit (ABI). The products were analyzed on an Applied Biosystems model 373A DNA sequencer.

Isolation of RNA and Quantitative RT-PCR for *PTCH* mRNA. Total cellular RNA was extracted by lysis in guanidinium isothiocyanate and ultracentrifugation through a cesium chloride cushion (20) or by extraction with the Trizol reagent (Life Technologies, Inc.) following the manufacturer's instructions. Again, individual samples were preexamined by frozen section histology to document the histopathological appearance of the specimen. Contaminating residual genomic DNA was removed by digestion with RNase-free DNase (Boehringer Mannheim). RNA standards with internal deletions for human *PTCH* and the housekeeping genes β_2-*microglobulin* and *GAPDH* were generated by *in vitro* mutagenesis and *in vitro* transcription (21). To achieve a semiquantitative assessment, preevaluated amounts of the specific standard RNAs covering the equimolar range of the corresponding mRNA transcripts were added to the MB sample RNAs, which were then reverse transcribed using the SuperScript Preamplification System (Life Technologies, Inc.) with random hexamers as primers in a final volume of 10 μl. cDNA (0.5 μl) was used as a template in RT-PCR reactions for amplification of *PTCH* and the housekeeping genes. The PCR was carried out on a Perkin-Elmer 9600 thermocycler in a final volume of 10 μl in the presence of 2 mM $MgCl_2$ and 0.25 unit Taq polymerase in PCR buffer (all from Life Technologies, Inc.) and 20 pmol of each primer. The primers used were: *PTCH*, 5′-ACATGTACAACAGGCAGTGG-3′ and 5′-GCAAAGGAGGTTTACCTAGG-3′, product size, wild-type 192 bp, standard 182 bp; *GAPDH*, 5′-TGCCAAGGCTGTGGGCAAGG-3′ and 5′-GCTTCACCACCTTCTTGATG-3′, product size, wild-type 152 bp, standard 142 bp; β_2-*microglobulin*, 5′-GCTGTGACAAAGTCACATGG-3′ and 5′-GATGCTGCTTACATGTCTCG-3′, product size, wild-type 148 bp, standard 130 bp. One of the primers for each gene was labeled with a fluorescent dye. All primers were chosen from adjacent exons spanning intronic sequences to avoid signals of the cDNA product size caused by residual genomic DNA. The PCR protocol consisted of an initial denaturation step of 94°C for 5 min, followed by 40 cycles of a three-step program of 94°C for 40 s, 58°C for 40 s, and 72°C for 50 s, and a final extension step of 72°C for 10 min. The PCR products were separated and analyzed on an Applied Biosystems model 373A DNA sequencer using the Genescan software (ABI). The expression levels of the individual genes were calculated from the signal ratios of the samples to the standards. The relative expression of *PTCH* mRNA to the housekeeping genes was defined as the ratio of the respective expression levels.

Results and Discussion

***PTCH* Mutation Analysis in MBs.** SSCP screening of DNA samples from 64 tumor biopsies and 4 cell lines derived from sporadic MBs revealed bandshifts in 6 samples (Table 1). Three of these were identified as silent polymorphisms. In three other tumors, the variants were not found in the corresponding germ-line DNA or in normal control DNA samples. Two mutations in exons 6 and 10, respectively, resulted in a frame shift with premature truncation of the protein (Table 1; Fig. 1). The third mutation (D86) was a 6-bp in-frame deletion in exon 10 leading to a deletion of two amino acids in transmembrane region 3 [according to the model proposed by Johnson *et al.* (7)]. This deletion may cause significant structural alterations of the PTCH protein and may result in a loss of function. According to Knudson's two-hit model, both alleles of a tumor suppressor gene need to be inactivated for

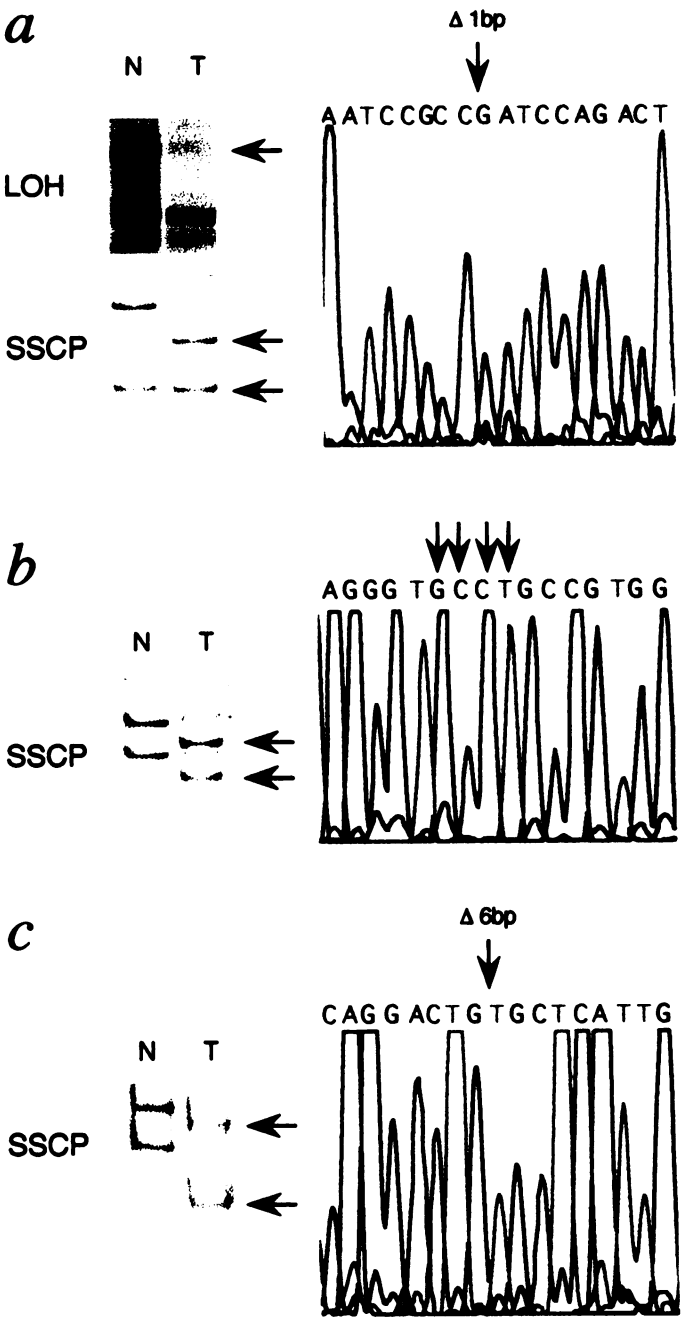

Fig. 1. *PTCH* mutations in desmoplastic MBs D322, D292, and D86. *a*, microsatellite *D9S302* shows loss of heterozygosity in tumor D322. In this tumor, the remaining allele of exon 6 exhibits an altered mobility. DNA sequencing shows a single base pair deletion in the tumor DNA that results in a frame shift and truncation of the protein. *b*, SSCP variants from exon 10 in tumor D292 without allelic loss for chromosome 9q. Sequencing of the altered allele demonstrates a 4-bp insertion at position 1393. *c*, sequencing of exon 10 in D86, a tumor with LOH, shows a 6-bp in-frame deletion at position 1444 (CTG GGC). This leads to a deletion of two amino acids (glycine and leucine) in transmembrane region 3.

Table 1 *Mutational analysis of the PTCH gene in MBs*

Tumor no.	MB variant	Age (yr)/Sex	LOH on 9q	Exon	Nucleotide change	Protein change
Mutations						
D86	Desmoplastic	4, male	Yes	10	1444del6	del Gly-Leu
D292	Desmoplastic	1, female	No	10	1393insTGCC	Frameshift, truncation
D322	Desmoplastic	51, male	Yes	6	887delG	Frameshift, truncation
Polymorphisms						
D230 II	Classical	13, female	No	13	C2037T	No
D338	Classical	13, male	NA[a]	2	C306T	No
D358	Classical	10, female	NA	2	C306T	No

[a] NA, not analyzed.

tumorigenesis to occur (22). This was the case in tumors D86 and D322, which showed LOH as well as a mutated *PTCH* allele. In case D292 without detectable LOH at 9q, only a single SSCP bandshift was found (in exon 10). Mutations of the other allele may be present but may not have been detected by SSCP screening because of the limited sensitivity of SSCP. It is possible that the true frequency of mutations is higher than revealed in this study.

Indeed, only 39% germ-line NBCCS mutations have been identified with this technique using the same primers in a previous study (13). Only the coding exons were screened so that mutations in other regions, such as regulatory domains, would not have been identified with our approach. A systematic sequencing analysis or alternative screening methods, such as protein truncation tests, may uncover additional *PTCH* mutations in MBs.

Association of *PTCH* Mutations with the Desmoplastic Variant of MB. In this study, mutations were only detected in a distinct histopathological variant of MB, the so-called nodular or "desmoplastic" MB. According to the WHO classification (18), this variant is characterized by islands of lower cellularity surrounded by densely packed, highly proliferative cells that produce a dense intercellular reticulin fiber network. The more frequent "classical" MB lacks this nodular appearance and reticulin pattern. Interestingly, the available data on NBCCS-associated MBs indicate that they are predominantly of the desmoplastic type (16). In our panel of sporadic tumors, microsatellite analysis also revealed a higher rate of LOH 9q in desmoplastic MBs, in agreement with Schofield *et al.* (16). LOH was present in 4 of 7 desmoplastic and in 3 of 33 classical MBs tested. Both the occurrence of LOH at 9q and mutations of *PTCH* were significantly associated with desmoplastic histology (Fisher's Exact test, $P < 0.05$). It remains to be shown if *PTCH* is the target tumor suppressor gene in all tumors with LOH on 9q.

***PTCH* mRNA Expression.** We have also studied the expression levels of *PTCH* mRNA in MB samples. In BCCs, high levels of *PTCH* mRNA were detected, suggesting that *PTCH* mutations may lead to an up-regulation of mRNA expression (14). Different histological subtypes of MBs with and without *PTCH* mutations or LOH at 9q22 were examined (Fig. 2; Table 2). A semiquantitative RT-PCR approach with specific RNA standards for *PTCH* and two housekeeping genes was used. *PTCH* was found to be expressed in both groups with a high variability in *PTCH* mRNA levels but without significant differences in the amounts of *PTCH* transcripts between desmoplastic and classical MBs and normal adult cerebellum. Interestingly, the cell line Daoy, which was derived from a desmoplastic MB, showed very low *PTCH* mRNA expression levels (Table 2). However, we have not uncovered any *PTCH* mutations in this cell line by SSCP analysis.

Role of PTCH in the Central Nervous System and in MBs. PTCH is expressed in the developing and adult central nervous system in the target tissues for its ligand, *Sonic hedgehog*. This points to an important role in the growth, migration, and differentiation of neural progenitors (23). Some NBCCS patients with haploinsufficiency of *PTCH* show dysgenesis of the corpus callosum, whereas mutations of the gene for its ligand *Sonic hedgehog* cause holoprosencephaly (24, 25), providing further evidence of an important role in neural development. MB cells display cytological and immunohistochemical characteristics of neural progenitors. The cellular origin of MB is controversial. One model postulates that MBs arise from neuroepithelial stem cells in the subependymal matrix layer (26). This hypothesis is supported by the fact that primitive neuroectodermal tumors with similar morphology may occur at extracerebellar sites of the brain. An alternative hypothesis proposes that MBs are derived from precursor cells in the external granular layer of the embryonic cerebellum (27), which differentiate into granule neurons. This view is supported by the finding of predominant neuronal differentiation in most MBs, especially of the desmoplastic type, and expression of granule cell lineage-specific transcription factors (28) and *PAX* genes

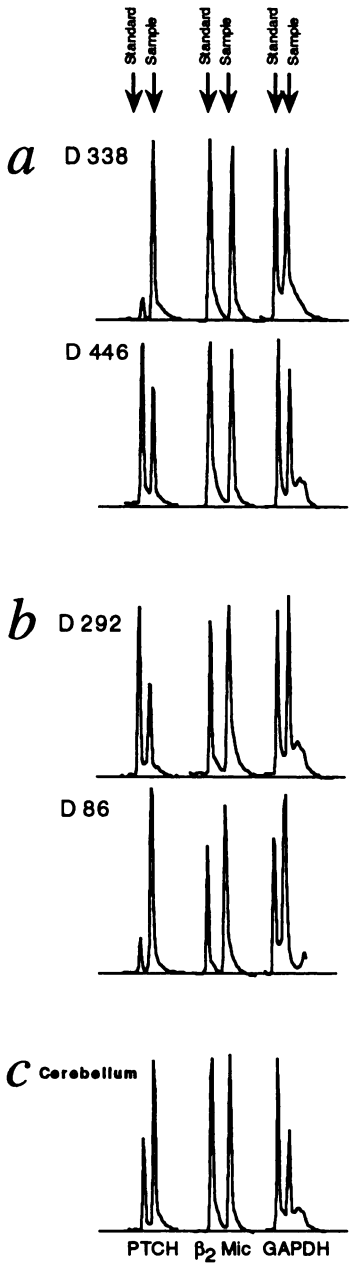

Fig. 2. Expression of *PTCH* mRNA in MBs. mRNA levels were determined using a semiquantitative RT-PCR approach. Reverse transcription of 250 ng of RNA in 10 μl was carried out in the presence of 40 pg of each of the standard RNAs with internal deletions for *PTCH*, *β2-microglobulin*, and *GAPDH*. The cDNAs were then amplified with primers for *PTCH* and the housekeeping genes. The products were separated and quantitated on an ABI 373A sequencer. The expression levels of the three genes were determined as the ratio of signals of the sample (*right peaks*) to the specific standards (*left peaks*). *a*, *PTCH* expression in two representative tumors of the classical variant of MB; *b*, expression in desmoplastic MBs; *c*, expression in adult human cerebellum.

(29) in MBs. The molecular pathogenesis of MBs is poorly understood. The detection of inactivating mutations of the *PTCH* gene in desmoplastic MBs is compatible with a model that these tumors arise from neural progenitors that lack the repression signal normally provided by intact Patched protein at a critical stage of neural differentiation. Some of the downstream components in the *Hedgehog/patched* signaling pathway have been identified, including the *patched*-regulated receptor *smoothened* and putative tumor suppressor genes and oncogenes such as WNTs and members of the transforming growth factor β family of proteins (9, 30). Further studies will focus on the function of these components in neural progenitors and MBs. Additional studies will be necessary to

Table 2 *Expression of the PTCH gene in MBs*

Sample no.	MB subtype	LOH on 9q	*PTCH* mutation detected by SSCP	mRNA expression ratio PTCH:GAPDH	mRNA expression ratio PTCH:β-microglobulin
D 338	Classical	NA[a]	No	5.8 (3.9–7.4)[b]	10.3 (8.2–14.2)
D 230 II	Classical	No	No	1.7	NA
D 286	Classical	NA	No	1.2	NA
D 245 II	Classical	No	No	0.7	NA
D 446	Classical	No	No	1.8 (0.8–2.8)	1.5 (1.0–1.8)
D 447	Classical	No	No	0.6	NA
D 86	Desmoplastic	Yes	Yes, exon 10	3.6 (1.4–5.4)	2.6 (1.7–3.9)
D 292	Desmoplastic	No	Yes, exon 10	0.6 (0.6–0.6)	0.3 (0.3–0.4)
D 322	Desmoplastic	Yes	Yes, exon 6	1.1	NA
D 448	Desmoplastic	Yes	No	4.3 (3.3–5.5)	2.9 (2.5–3.6)
D 398	Desmoplastic	No	No	26.5 (22.4–30.0)	18.4 (12.0–24.4)
D 444	Desmoplastic	Yes	No	4.1 (3.7–4.7)	4.5 (3.3–5.5)
D 365[c]	Desmoplastic	NA	No	0.3 (0.2–0.4)	0.1 (0.1–0.2)
Cerebellum	–	NA	NA	2.5 (1.55–3.64)	1.76 (1.42–2.09)

[a] NA, not analyzed.
[b] Mean and range of relative expression (analyzed by semiquantitative RT-PCR).
[c] D365, cell line Daoy.

determine whether other MB-associated loci that show LOH, such as 1q31–32.1 (19) or 17p13.3 (20), harbor genes that are components of or interact with the *patched* signal transduction pathway. Cell lines derived from desmoplastic MBs with homozygously mutated *PTCH* would constitute useful tools for a functional analysis of *patched.*

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Note Added in Proof

After submission of the manuscript, Raffel *et al.* (Cancer Res., *57:* 842–845) reported on *PTCH* mutations in three cases of medulloblastoma.

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