Medulloblastomas of the Desmoplastic Variant Carry Mutations of the Human Homologue of Drosophila patched

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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 (non-desmoplastic) 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

MBs3 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 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'-GACCATGTCCAGTGGCAGCTC-3' and 5'-CGTTCAAGATACACACAGCCC-3'; exon 12B (exon 12 turned out to consist of two exons), 5'-AGTTCCCTCTGATGGCCCGAG-3' and 5'-CACTTCTGACCCAACTTTATAGGCACC-3'; exon 20, 5'-TGGCTTCTGTTTCTTCTTGT-3' and 5'-GCCACCGGAAACACACGATTACC-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 MgCl2, 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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3The 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.

4Unpublished 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 β2-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 MgCl2 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'-ACATGTCACAGGCAGTG-3' and 5'-GCCAAAGGAGGCGAGCAGC-3'; product size, wild-type 192 bp, standard 182 bp; GAPDH, 5'-TGCCAAGGCTGTGGGCAAGG-3' and 5'-GCTTAGCTGCCTGACAC-3'; product size, wild-type 152 bp, standard 142 bp; β2-microglobulin, 5'-CTGAGACAAACCATGG-3' and 5'-CATGCTGGATTCCATGTC-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

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

<table>
<thead>
<tr>
<th>Tumor no.</th>
<th>MB variant</th>
<th>Age (yr)/Sex</th>
<th>LOH on 9q</th>
<th>Exon</th>
<th>Nucleotide change</th>
<th>Protein change</th>
</tr>
</thead>
<tbody>
<tr>
<td>D86</td>
<td>Desmoplastic</td>
<td>4, male</td>
<td>Yes</td>
<td>10</td>
<td>1444del6</td>
<td>del Gly-Leu</td>
</tr>
<tr>
<td>D92</td>
<td>Desmoplastic</td>
<td>1, female</td>
<td>No</td>
<td>10</td>
<td>1393insTGCC</td>
<td>Frameshift, truncation</td>
</tr>
<tr>
<td>D322</td>
<td>Desmoplastic</td>
<td>51, male</td>
<td>Yes</td>
<td>6</td>
<td>887delG</td>
<td>Frameshift, truncation</td>
</tr>
<tr>
<td>Polymorphisms</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D230 II</td>
<td>Classical</td>
<td>13, female</td>
<td>No</td>
<td>13</td>
<td>C2037T</td>
<td>No</td>
</tr>
<tr>
<td>D338</td>
<td>Classical</td>
<td>13, male</td>
<td>NA*</td>
<td>2</td>
<td>C306T</td>
<td>No</td>
</tr>
<tr>
<td>D358</td>
<td>Classical</td>
<td>10, female</td>
<td>NA</td>
<td>2</td>
<td>C306T</td>
<td>No</td>
</tr>
</tbody>
</table>

* NA, not analyzed.
PTCH MUTATIONS IN MEDULLOBLASTOMAS

of granule cell lineage-specific transcription factors (28) and PAX genes.

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.

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 desmoplasmatic type (16). In our panel of sporadic tumors, microsatellite analysis also revealed a higher rate of LOH at 9q in desmoplasmatic MBs, in agreement with Schofield et al. (16). LOH was present in 4 of 7 desmoplasmatic and in 3 of 33 classical MBs tested. Both the occurrence of LOH at 9q and mutations of PTCH were significantly associated with desmoplasmatic 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 desmoplasmatic and classical MBs and normal adult cerebellum. Interestingly, the cell line Daoy, which was derived from a desmoplasmatic 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 desmoplasmatic 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, \( β_2 \) 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 desmoplasmatic 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 desmoplasmatic 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 smoothed 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...
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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