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Advances in Brief

Sporadic Medulloblastomas Contain *PTCH* Mutations[1]

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Abstract

Nevoid basal cell carcinoma syndrome (NBCCS), or Gorlin's syndrome, is an autosomal dominant disorder that predisposes to developmental defects and various forms of cancer. *PTCH* was recently proposed as a candidate gene for NBCCS due to its frequent mutation in basal cell carcinomas, the cancer most often associated with this syndrome. Another NBCCS-associated cancer is medulloblastoma, a common central nervous system tumor in children. Most medulloblastomas, however, occur without indication of an inherited predisposition. We have examined 24 sporadic medulloblastomas for loss of heterozygosity (LOH) at loci flanking as well as within *PTCH*. In cases with LOH, single-strand conformational polymorphism and sequencing analysis were performed to determine the status of the remaining *PTCH* allele. Microsatellite analysis indicated LOH of *PTCH* in 5 of 24 tumors, and in three of these cases a mutation of the remaining allele was identified. Two of the mutations were duplication insertions, and the third consisted of a single base deletion. It is interesting that all three mutations occur in exon 17 of the *PTCH* gene. These data suggest that inactivation of *PTCH* function is involved in the development of at least a subset of sporadic medulloblastomas.

Introduction

Primitive neuroectodermal tumors of the central nervous system account for 20% of pediatric brain tumors (1). These tumors occur most frequently in the posterior fossa, where they are called medulloblastoma, but may also occur in the cerebrum (cerebral neuroblastoma) or pineal gland (pineoblastoma). Medulloblastoma may occur in association with two familial cancer syndromes: NBCCS,[3] or Gorlin's syndrome, and Turcot's syndrome. NBCCS is an autosomal dominant disorder. Affected individuals develop multiple basal cell carcinomas, odontogenic keratocysts of the jaws, palmar and plantar dyskeratoses, and skeletal anomalies, especially rib malformations (2). In addition, at least 40 cases of medulloblastoma have been reported in patients with NBCCS, indicating that approximately 3% of individuals with this syndrome develop medulloblastoma (3, 4).

The gene for NBCCS has been mapped to chromosome 9q22.3 (5, 6) and has recently been identified as *PTCH*, the human homologue of the *Drosophila patched* gene (7, 8). In *Drosophila*, *patched* encodes a protein with 12 putative transmembrane domains that may function as a receptor or transporter (9, 10). *Patched* has an essential role in embryonic patterning in *Drosophila*; an analogous role in humans may explain the congenital anomalies associated with NBCCS.

Because most medulloblastomas occur in the absence of an inherited predisposition, examination of sporadic cases of this tumor for *PTCH* mutations should determine whether such alterations are associated with medulloblastoma development in patients without NBCCS. We have examined 24 sporadic medulloblastomas for genetic alterations in *PTCH*. Tumors were initially screened for LOH near or within the *PTCH* locus; five of the tumors had LOH. Somatic mutation of the remaining *PTCH* allele was identified in three of these cases, suggesting that *PTCH* inactivation is an important event in the development of some sporadic medulloblastomas.

Materials and Methods

Microsatellite Analysis. Normal and tumor DNAs were isolated and purified as described previously (11, 12). Serial dilutions of each DNA sample were prepared and subjected to PCR amplifications using the surrogate microsatellite primer *D10S224* to identify working concentrations that would yield similar autoradiographic intensities after amplification in the presence of [α-^{32}P]dCTP (Amersham, Arlington Heights, IL). The PCR reactions for these determinations, as well as those used in the assessment of LOH on 9q, consisted of 10–100 ng of DNA, 8–10 pM forward and reverse primers (Research Genetics, Huntsville, AL), 0.8 μCi [α-^{32}P]dCTP, and 0.2–0.35 units of *Taq* polymerase (Promega, Madison, WI) or AmpliTAQ Gold (Perkin-Elmer, Foster City, CA) in 10–15 μl of aqueous buffer containing 200 μM dGTP, dATP, and dTTP; 25–34 μM dCTP; 50 mM KCl, 10 mM Tris-HCl (pH 9.0); 0.1% Triton X-100; and 2.5 mM $MgCl_2$. Samples were placed in 96-well plates, overlayed with mineral oil, and amplified in a twin-block thermal cycler (Ericomp, San Diego, CA) at 95°C denaturation, 55°C annealing, and 72°C extension for 43 cycles, or by using a "touchdown" cycling profile: annealing temperature of 60°C once, decreasing by 1°C each cycle to the final annealing temperature of 55°C for 43 rounds of amplification. (Melting and extension temperatures were as indicated above.) At completion of PCR, an equal volume of denaturing buffer containing 95% formamide, 10 mM EDTA, and 1 mg/ml bromphenol blue and 0.03% xylene cyanol was added to each reaction, and samples were then heated to 95°C and quenched on ice. Two μl of each were applied to 4% or 6% acrylamide sequencing gels containing 8 M urea and 15% formamide and electrophoresed for 1.5–3 h at 75 W. Gels were dried and exposed to single-sided Kodak XAR film for 4–48 h. Autoradiographs were assessed for LOH by visual comparison of tumor and blood-band intensities. Losses were confirmed by repeated assays and by exposing dried gels to PhosphorImager screens and quantitating allele count intensities with Image-QuaNT version 3.2 software (Molecular Dynamics, Sunnyvale, CA). Loci investigated by microsatellite analysis for tumor DNA LOH on 9q are listed in Table 1.

Southern Analysis. Three to five μg of corresponding normal-tumor DNA pairs were digested to completion with *Pst*I. Samples were electrophoresed through 0.8% agarose gels, blot-transferred to a reinforced nitrocellulose membrane (Schleicher & Schuell, Keene, NH), and fixed to the membrane by baking under vacuum at 80°C for 2 h. Filters were prehybridized as described previously (13) and hybridized using 25 ng of [α-^{32}P]dCTP-labeled (14) *PTCH* exon 6 DNA. After membrane rinsing (13), hybridized filters were exposed to X-ray film (Kodak XAR) at −70°C for 2–5 days. After exposure, filters were stripped of *PTCH* exon 6 probe and rehybridized with a probe from the *D9S19* locus (15). Autoradiograms were examined by scanning densitometry, and resultant images were analyzed for *PTCH* signal response in tumor DNAs using the NIH Image 1.52 software program.

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[3] The abbreviations used are: NBCCS, nevoid basal cell carcinoma syndrome; LOH, loss of heterozygosity; TBE, Tris-borate EDTA; SSCP, single-strand conformational polymorphism.

Table 1 *Summary of LOH analysis*

	Microsatellite[a]							RFLP
Tumor	*D9S196*	*D9S280*	*D9S1809*	*AFM203*	*D9S287*	*D9S180*	*D9S176*	*PTCH*
M1	–	–	NI	NI	–	NI	NI	ND
M2	NI	+	+	NI	ND	+	NI	NI
M3	+	NI	+	NI	NI	+	+	+
M4	+	+	+	NI	+	NI	+	NI
M5	+	+	NI	+	+	NI	+	NI
M6	+	NI	NI	NI	+	ND	+	NI
M7	NI	+	NI	+	+	ND	+	+
M8	+	+	+	+	+	NI	+	NI
M9	NI	+	+	NI	+	ND	+	ND
M10	NI	+	+	NI	+	+	+	+
M11	NI	NI	–	NI	NI	–	–	NI[b]
M12	+	+	+	NI	NI	+	+	+
M13	+	+	+	+	+	+	NI	NI
M14	NI	NI	NI	NI	ND	+	NI	+
M15	–	–	NI	NI	–	NI	–	NI[b]
M16	+	+	+	NI	+	ND	+	NI
M17	NI	+	NI	NI	+	+	+	ND
M18	+	NI	NI	NI	+	+	+	NI
M19	+	+	+	NI	+	+	+	NI
M20	–	NI	–	NI	–	NI	–	ND
M21	NI	–	ND	–	–	NI	–	–
M22	+	+	+	NI	+	ND	ND	+
M23	NI	+	+	NI	+	+	+	+
M24	NI	+	ND	NI	+	ND	+	NI

[a] Microsatellite markers are ordered from left (centromeric) to right (telomeric) based on information obtained from the Genetic Location data base at the University of Southampton (Southampton, United Kingdom). Primers used for each marker (forward primer listed first for each pair): GGGATTACACCTCAAAACCA, ACCACACTGCGGGACTT (*D9S196*) TTTCGCTTCCCACCCA, CACGCCACTGATCTAGGCT (*D9S280*) AGCTCCTGTTCAACATCTCCTC, AAGGTTTGTGCTTCCTGGG (*D9S1809*) AGACAGTTTGAGGCTGCG, AATTTTGCCCAGGAGACTTG (*AFM203WH8*) AGGATGCTCCTCACGC, ACCACTACATTGTTCAAGGG (*D9S287*) CAGTGGTTTGGAATCGAACC, AGCTATTTTTGGGGGCTGAG (*D9S180*) AGCTGGCTGTTGGAGAAA, TGACCAATGGCAGGGTAT (*D9S176*). +, tumor maintenance of heterozygosity −, tumor loss of heterozygosity NI, noninformative ND, not determined.

[b] Densitometric analysis of RFLP autoradiograms indicated allelic loss (Fig. 2).

SSCP and Sequence Analysis. Template for SSCP and sequence analysis was generated using primers described previously (8, 16). For SSCP, 10–100 ng of genomic DNA was amplified in a 15-μl reaction volume containing: 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 1.5 mM $MgCl_2$; 200 μM each dATP, dGTP, and dTTP; 25 μM dCTP; 15 pmol of forward and reverse primers; 0.5 units of *Taq* polymerase (Boehringer Mannheim); and 0.06 μCi [α-^{32}P]dCTP. Reaction programs consisted of 35 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C; final extension was at 72°C for 10 min. Reaction products were diluted 1:2 in stop solution (50% formamide, 7 M urea, 25 mM EDTA, 0.03% bromphenol blue, and 0.03% xylene cyanol), denatured at 95°C for 2 min, chilled, and loaded onto 0.5× mutation detection enhancement (ATGC Corp., Malvern, PA), 1× TBE nondenaturing gels. After electrophoresis at 35 W and 4°C for 5–8 h, gels were dried and exposed to X-ray film (Kodak XAR) overnight at room temperature.

Templates for DNA sequencing were generated in 50-μl reaction volumes containing 20 pmol of forward and reverse primers, 200 μM dNTPs (Perkin-Elmer, Foster City, CA), 1.25 units of *Taq* polymerase (Boehringer Mannheim), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% gelatin. PCR amplifications were for 43 cycles: 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min (final extension at 72°C for 10 min) after sample denaturation at 95°C for 9 min. One μl, containing approximately 20 ng of each amplified reaction, was added to 8 μl of 50 mM Tris-HCl (pH 8.3); 50 mM KCl; 2.5 mM $MgCl_2$; 10% DMSO; 400 μM ddATP; 600 μM ddTTP; 60 μM ddGTP; 200 μM ddCTP; 10 μM each dATP, dTTP, and dCTP; 20 μM 7-deaza-dGTP (Boehringer Mannheim); 0.05 μM [γ-^{32}P]ATP end-labeled sequencing primer; and 0.5 units of *Taq* polymerase (Boehringer Mannheim). Sequencing reactions were carried out in a twin-block thermocycler (Ericomp) for 30 cycles at 95°C for 20 s, 58°C for 30 s, and 72°C for 1 min using a 1-min ramp time between annealing and elongation phases. Reactions were terminated by the addition of 6 μl of stop buffer (same as listed above). Samples were then denatured at 95°C for 2 min, chilled on ice, and loaded onto a 6% sequencing gel (19:1 acrylamide, 7 M urea, 0.5× TBE, 15% formamide). Electrophoresis was at 75 W and room temperature for 1–3 h, after which the gels were dried and exposed to X-ray film overnight at room temperature.

Results

Medulloblastomas from 24 patients were used for this study; two of these, M1 and M16, were of the desmoplastic subtype. Seven microsatellite markers closely linked to the *PTCH* locus, as well as a single RFLP marker within *PTCH*, were used to examine the tumors for LOH. The microsatellite analysis revealed five tumors with allelic loss in the *PTCH* region (Fig. 1, Table 1). All five demonstrated loss of all informative markers in the region examined; no tumor exhibited partial loss. Because all patients were heterozygous at one or more loci, the data suggest that no patient had germ-line loss of the *PTCH* region.

The noncoding sequence that flanks exon 6 of *PTCH* contains a polymorphic *Pst*I site that can be used for RFLP analysis. Of the tumors that showed loss on 9q22.3, one was informative with this RFLP marker and revealed LOH (Fig. 2, *M21*). Two of the remaining four patients displaying microsatellite LOH, and for whom there was sufficient DNA to permit Southern analysis, were uninformative for the exon 6 marker, but revealed a decreased band intensity in the tumor DNA consistent with allelic loss (Fig. 2, *M11 and M15*). Five tumors without LOH by microsatellite analysis were informative in the RFLP analysis; none showed LOH by RFLP. (Results for three of these, M7, M10, and M12, are shown in Fig. 2).

Using a combination of SSCP and direct sequencing analysis, 21 exons were examined from the retained *PTCH* allele in the five tumors with 9q22.3 LOH. *PTCH* mutations were identified in three instances; it is interesting that all of these were in exon 17. Two of the mutations were duplication insertions (Fig. 3*A*): 27 bp in tumor M15 (3146ins27) and 19 bp in tumor M21 (3112ins19; Fig. 3*B*). The latter results in a reading frame shift and the introduction of a stop codon at position 1005, rather than the normal position of 1444. The third mutation was a single-base deletion in a run of five adenosine residues (del2967; Fig. 3*C*) that results in a frame shift with multiple resultant downstream stop codons. None of these alterations was observed in germ-line DNA. SSCP and sequence analysis of exon 17 in the

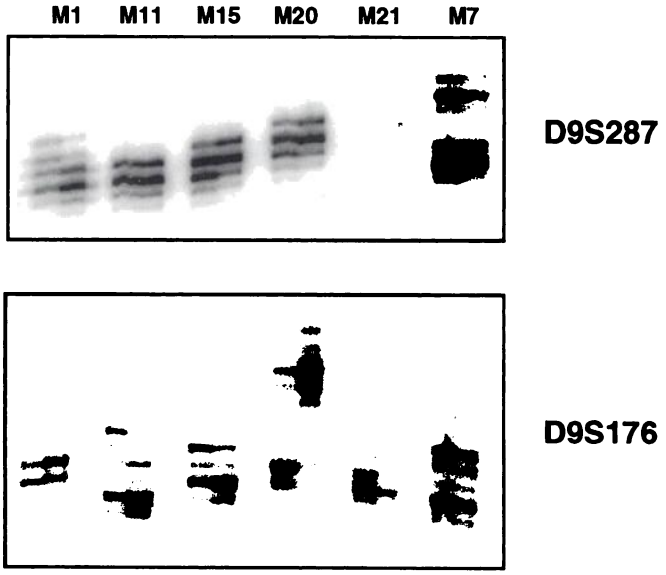

Fig. 1. Medulloblastoma LOH in the *PTCH* region as indicated by microsatellite analysis. Ten-ng to 100-ng quantities of paired normal (*left*) and tumor (*right*) DNAs were subjected to 48 cycles of amplification in the presence of ^{32}P[dCTP] and primers for the indicated loci. Reaction products were resolved by electrophoresis through a 6% sequencing gel that was subsequently dried and exposed to X-ray film. Results indicate LOH at both loci for specimens *M1* (densitometric analysis for the D9S287 result indicated a 50% reduction in relative intensity of the higher mobility allele for the M1 tumor specimen), *M15*, *M20*, and *M21*, and at D9S176 only for *M11* (patient was uninformative for D9S287). Specimen M7 demonstrates tumor maintenance of heterozygosity at both loci.

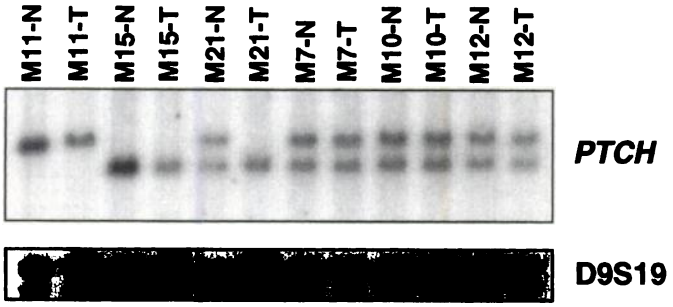

Fig. 2. LOH within *PTCH* as revealed through RFLP analysis. Normal tumor DNA pairs were treated with restriction enzyme *Pst*I, resolved by electrophoresis through a 0.8% agarose gel, blot-transferred to a reinforced nitrocellulose membrane, and sequentially hybridized with probes for the indicated loci. The *PTCH* probe was generated by amplification of genomic DNA with primers flanking exon 6 of *PTCH* (8). The results show LOH in tumor M21, consistent with the results associated with the microsatellite analysis (Fig. 1). Densitometric analysis of signal intensities revealed ~50% signal reduction of the *PTCH* restriction fragment in tumors *M11* and *M15* after normalization of signal responses with a control probe from the short arm of chromosome 9, D9S19. Specimens *M7*, *M10*, and *M12* show tumor maintenance of heterozygosity, results that are also consistent with the microsatellite analysis (Table 1).

remaining 19 tumors without LOH failed to reveal any additional mutations.

Discussion

In this study, 5 of 24 medulloblastomas showed evidence of allelic loss with markers closely linked to the *PTCH* gene. Before the identification of *PTCH* as the gene involved in NBCCS, two studies reported loss of genetic markers mapped to 9q in medulloblastoma. In the first study, 16 patients were examined with 12 microsatellite markers mapping between 9q13 and 9q34 (17). Two tumors (12.5%) showed LOH with microsatellite markers that are now known to flank the *PTCH* locus. In the second study, medulloblastoma from 20 patients, 17 with sporadic tumors and 3 with NBCCS, were investigated with seven microsatellite markers mapped to 9q22.3–9q31 (18). Both informative tumors from patients with NBCCS showed LOH for markers that flanked the *PTCH* locus; the third patient was not informative for the markers closest to *PTCH*. Three of the 17 sporadic tumors also showed LOH on 9q at markers flanking or near *PTCH*. It is interesting that all three of the tumors from patients with NBCCS in this study were designated desmoplastic medulloblastomas. The other tumors with LOH on 9q were among six desmoplastic tumors in the sporadic group; thus, all of the tumors with LOH on 9q in this study were desmoplastic. In the data presented here, 20.8% of the tumors showed LOH on 9q; only one of five was desmoplastic, and the other desmoplastic tumor did not display LOH. This result suggests that LOH in the region of *PTCH* is not solely associated with the desmoplastic variant of medulloblastoma.

PTCH mutations have been described in patients with NBCCS and in spontaneous basal cell carcinomas. To date, 43 such mutations have been reported, of which 21 are single-base substitutions, 9 are insertions ranging from a single base to 300 bases, and 13 are deletions, ranging from a single base to 37 bases (7, 8, 16, 19, 20). The described mutations are distributed fairly evenly throughout the *PTCH* gene; no "mutational hot spots" have been identified. Only one mutation has been previously reported in exon 17 (19). All three mutations detected here in sporadic medulloblastoma were in this exon. It is not known whether the subset of patients with NBCCS that develop medulloblastoma also have exon 17 mutations. Because none of the *PTCH* mutations reported here occurred in the germ line, the patients with these mutations cannot have a form of NBCCS that solely develops medulloblastoma.

The patched protein has an important regulatory role in the "hedgehog" signaling pathway (21) that is critical to embryo segmentation and other steps in *Drosophila* development. The regulatory element, hedgehog, stimulates the expression of the *wingless* and *cubitus interruptus* genes. Hedgehog also up-regulates *patched*, which in turn down-regulates *hedgehog*, *cubitus interruptus*, *wingless*, and, interest-

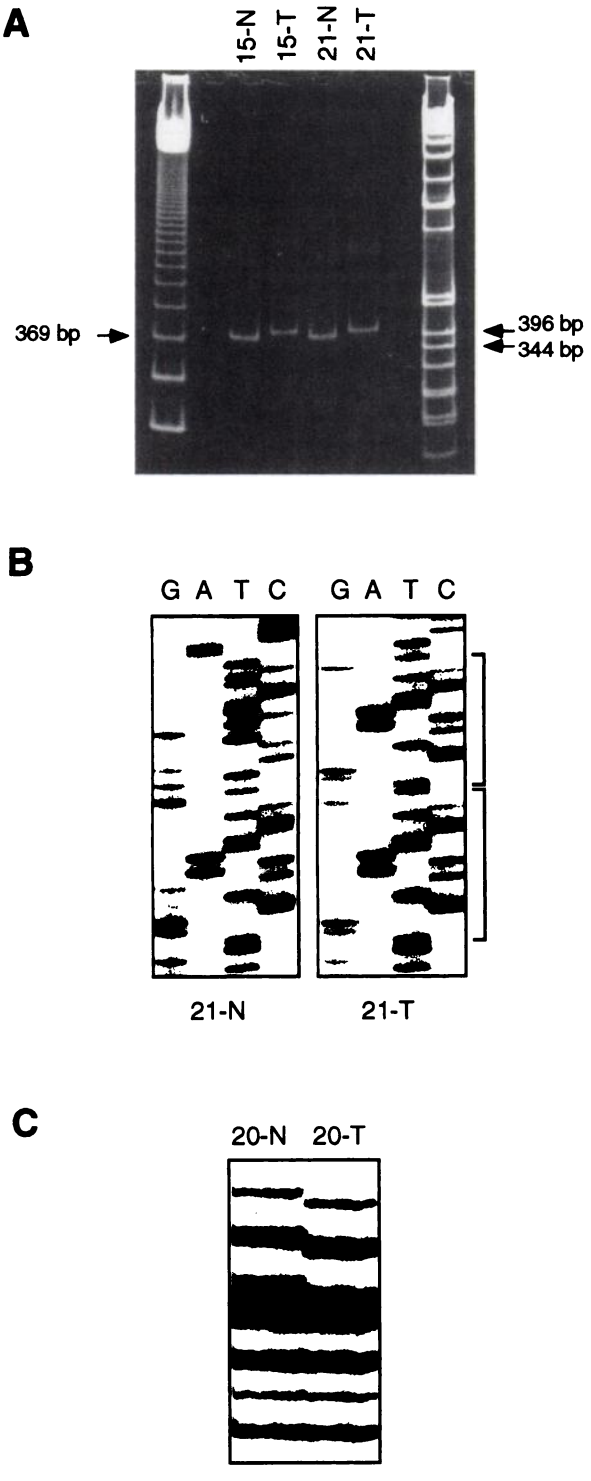

Fig. 3. Tumor-specific alteration of the remaining *PTCH* allele in specimens demonstrating LOH. *A*, exon 17 sequences were amplified (8) from corresponding normal-tumor genomic DNAs and electrophoresed though a 4–20% TBE polyacrylamide gel. The results demonstrate reduced mobility of the amplification product in each tumor specimen. Sequence analysis of the PCR products revealed 27-bp (3146ins27) and 19-bp (3112ins19, *B*) coding sequence duplications in tumors M15 and M21, respectively. *C*, single-base deletion ("A" residue, del2967) observed in tumor M20.

ingly, *patched* itself. Cubitus interruptus plays an essential role between patched and hedgehog (22). Hedgehog up-regulates *cubitus interruptus*, which in turn increases the expression of other proteins in the pathway. Conversely, patched down-regulates *cubitus interruptus*. Overexpression of *cubitus interruptus* has similar developmental effects to absence of patched function. Each of these proteins has human homologues. The hedgehog protein has three: sonic hedgehog (shh), indian hedgehog (ihh), and desert hedgehog (dhh). Shh has a tissue distribution similar to ptch (23), and the results of a recent study suggest, in fact, that ptch functions as a receptor for this protein (24). The human homologue of cubitus interruptus is a transcription factor known as gli, the gene for which is occasionally amplified in glioblastoma multiforme (25). The human homologue of wingless is wnt-1, a protein whose overexpression is associated with mammary tumors in mice (26).

Of the 24 medulloblastomas we analyzed, 19 did not show LOH in the *PTCH* region. Although these have yet to be completely examined for *PTCH* alterations, no additional mutations have yet to be identified, and, consequently, the current data suggest that *PTCH* mutations only occur in a subset of these tumors. One possible reason for the absence of *PTCH* mutations in a majority of sporadic medulloblastomas may be that other alterations in the patched/hedgehog pathway are present in these tumors. In astrocytomas, different genetic alterations have been described that presumably lead to functional inactivation of the retinoblastoma protein (27). Similarly, the aberrant expression of any of the genes normally suppressed by patched could be involved in the development of medulloblastoma, a hypothesis that awaits further investigation.

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