

Evidence That Haploinsufficiency of *Ptch* Leads to Medulloblastoma in Mice

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The *PTCH* gene encodes a putative tumor suppressor protein; germline alterations in *PTCH* have been found in patients with the nevoid basal cell carcinoma syndrome (NBCCS). Medulloblastoma, a brain tumor, develops in about 3% of NBCCS patients, and mutations in *PTCH* have also been described in a subset of sporadic medulloblastomas. The search for the causes of medulloblastoma has been hindered by the lack of an appropriate model system for this tumor type. Recently, a transgenic mouse hemizygous for the *Ptch* gene was generated by homologous recombination. Medulloblastomas were found in about 19% of these mice within the first 25 weeks after birth. The status of the wild-type *PTCH* allele in these tumors has not been investigated. For clearer definition of the role of *PTCH* as a tumor suppressor in medulloblastoma, 13 cerebellar tumors from transgenic *Ptch*^{+/-} mice were examined for alterations in the remaining *Ptch* allele. A single mutation was found in one tumor, a C-to-A substitution changing a tyrosine to a stop codon; all other tumors exhibited a wild-type sequence. Two tumors with normal *Ptch* cDNA were examined by in situ hybridization. *Ptch* cDNA was found in tumor cells but not in associated tumor stroma. We also examined the mRNA expression levels for the remaining *Ptch* allele, as well as for *Glil*, a gene known to be transcriptionally activated by *Ptch* inactivation. Blot analysis of RNA from the 13 tumors shows that *Ptch* mRNA of appropriate size is expressed in all tumors at varying levels. Expression of *Glil* was increased in tumors compared to normal cerebellum. These results suggest that deletion of one copy of *Ptch* may be sufficient to promote medulloblastoma development in mice. *Genes Chromosomes Cancer* 28:77–81, 2000. © 2000 Wiley-Liss, Inc.

INTRODUCTION

Primitive neuroectodermal tumors of the central nervous system account for 20% of pediatric brain tumors (Humphreys et al., 1982). These tumors occur most frequently in the posterior fossa, where they are called medulloblastoma, but they may also occur in the cerebrum (cerebral neuroblastoma) or pineal gland (pineoblastoma). Medulloblastoma may occur in association with two familial cancer syndromes, the nevoid basal cell carcinoma (NBCCS)—or Gorlin—syndrome and Turcot 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 (Gorlin, 1987). In addition, at least 40 cases of medulloblastoma have been reported in patients with NBCCS, indicating that about 3% of individuals with this syndrome develop medulloblastoma (Lacombe et al., 1990; Evans et al., 1991).

The gene for NBCCS has been mapped to chromosome segment 9q22.3 (Farndon et al., 1992; Gialani et al., 1992) and identified as *PTCH*, the human homolog of the *Drosophila patched* gene (Hahn et al., 1996; Johnson et al., 1996). In *Drosophila* and mammals, this locus encodes a protein

with 12 putative transmembrane domains that is capable of binding ligands of the hedgehog family (Hooper et al., 1989; Nakano et al., 1989). *Patched* has an essential role in embryonic patterning in *Drosophila*; an analogous role in man may explain the congenital anomalies associated with NBCCS. Not surprisingly, in light of the association of medulloblastoma with NBCCS, *PTCH* inactivation by deletion and mutation has been reported in a subset of sporadic medulloblastomas (Pietesh et al., 1997; Vorechovsky et al., 1997; Wolter et al., 1997; Xie et al., 1997; Zurawel et al., 1997).

In vertebrates, binding of Sonic Hedgehog (SHH) to PTCH is followed by signal transduction and induction of target genes, including *PTCH* and *GLII*. One theory states that PTCH inhibits signal transduction and that binding of Sonic Hedgehog to PTCH removes this inhibition (Stone et al., 1997).

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The search for the causes of medulloblastoma has been hindered by the lack of an appropriate model system for this tumor type. Recently, a knockout mouse was constructed for study of the role of *Ptch* in development (Goodrich et al., 1997). Although mice homozygous for the *Ptch* deletion died in utero, hemizygous mice survived. About 19% of these hemizygous mice developed medulloblastoma-like tumors of the cerebellum within the first 25 weeks after birth. We sequenced the remaining *Ptch* allele in cerebellar tumors from 13 of these hemizygous knockout mice. We also examined the mRNA expression levels for the remaining allele, as well as for *Gli1*. Our results suggest that haploinsufficiency of the *Ptch* gene, rather than mutation of the remaining *Ptch* allele, leads to tumorigenesis in the mouse cerebellum.

MATERIALS AND METHODS

RT-PCR

Total RNA was isolated from 13 mouse tumors removed from 13 different mice using TRIzol reagent (Gibco-BRL, Grand Island, NY) according to the manufacturer's enclosed protocols. For the reverse-transcriptase reaction, MuLV-RT and random hexamers were used to generate *Ptch* cDNA. The primers used for PCR amplification of *Ptch* were designed to amplify overlapping fragments so as not to conceal any mutations within the primer sequences. Samples were amplified through 35 cycles in a DNA Thermal Cycler 480 (Perkin Elmer, Norwalk, CT) at 95°C denaturation for 30 sec, 55°C annealing for 30 sec, and 72°C extension for 45 sec. Each reaction consisted of 100-ng RNA template, 10-pM forward and reverse primers, 0.2 mM each dNTP, 1.5-mM MgCl₂, 50-mM KCl, 10-mM Tris-HCl. PCR products were visualized on a 1.5% agarose gel with ethidium bromide.

Sequencing

After pretreatment of the RT-PCR products with exonuclease I and shrimp alkaline phosphatase to digest the primers and inactivate the nucleotides used during the PCR reaction, PCR products were sequenced directly by Thermo Sequenase radiolabeled terminator cycle sequencing (Amersham Life Sciences, Arlington Heights, IL). Sequencing reactions were run on 8% polyacrylamide-urea gels, dried, and exposed to single-sided Biomax film.

RNA Blotting

Probes for the *Ptch* and *Gli1* RNA blots were full-length *Ptch* cDNA and an 817-bp RT-PCR

product for *Gli1*, respectively. Total RNA was isolated as described above, and 10 µg from each tumor was applied to a 1% agarose/5% formaldehyde gel and, after electrophoresis, was transferred to a nylon membrane and UV-cross-linked (Stratalinker 1800, Stratagene, La Jolla, CA). Probes used to hybridize the blot were prepared by the random oligonucleotide priming method with [³²P]-dCTP. The blots were hybridized overnight at 42°C in hybridization solution (10 × Denhardt's solution, 10% Dextran, 2.5 × SSC, 0.1% SDS), washed for 20 min in 2 × SSC/0.1% SDS (× 2), then washed in 0.4 × SSC/0.1% SDS (× 2). Autoradiographs were produced on Kodak XAR film.

In Situ Hybridization

The probes for the in situ experiments were amplified by PCR and include exons 1 and 2 of murine *Ptch*, bases 1 to 352 (Genbank accession number U46155). The products were cloned in both orientations into the pCR 2.1 vector (Invitrogen, Carlsbad, CA). Sense and antisense riboprobes were transcribed using the T7 promoter in the presence of ³⁵S-labeled UTP. Tumors were removed from mice and immediately fixed in 4% paraformaldehyde. Tissue was then processed in paraffin blocks, and 5-µm sections were placed onto glass slides and hybridized to ³⁵S-labeled riboprobes in buffer containing 10% dextran sulfate, 50% deionized formamide, 50 × Denhardt's, 20-mM DTT, 300-µg/mL herring sperm DNA, and 150-µg/mL wheat germ tRNA. The slides were then coated with autoradiography emulsion (Kodak, Rochester, NY) and incubated at 4°C for 7 days before developing and counterstaining with hematoxylin and eosin.

RESULTS

Because *PTCH* has been reported to function as a tumor suppressor gene in both basal cell carcinoma (BCC) and medulloblastoma, the remaining *Ptch* allele in brain tumors that arose in *Ptch*^{+/-} mice was sequenced for better definition of the involvement of the *Ptch* locus in this new animal model of medulloblastoma. Total RNA was extracted from 10 frozen and 3 fresh tumors removed from 13 different mice, and RT-PCR was performed using primers that span the entire coding region of the murine *Ptch* cDNA. The *Ptch* cDNA was amplified in four fragments; the PCR products were then sequenced directly. In only one tumor was a mutation identified (Fig. 1). This mutation, C2760A (GenBank accession number U46155), changes a tyrosine residue to a stop codon, which

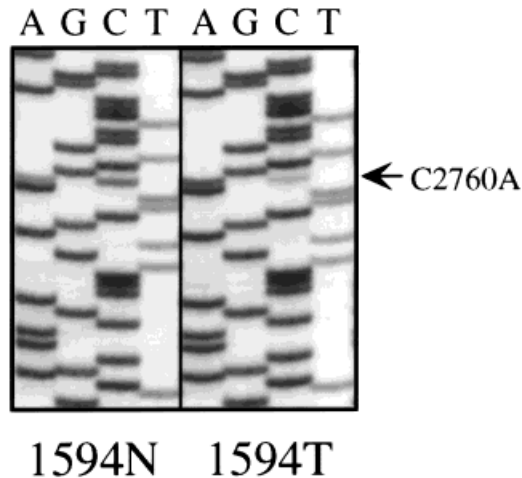


Figure 1. Mutation in *Ptch* in cerebellar tumor sample. Sequencing of RT-PCR products from normal cerebellum (1594N) and tumor (1594T) RNA reveals an A-for-C substitution converting codon 920 (TAC, tyrosine) to a stop codon (arrow). Because the tumor sample contains stromal cells, faint bands from normal tissue can be seen in the tumor lane. Similarly, because the tumor is invasive and grows large in comparison to the size of the murine cerebellum before the tumor can be detected, the normal tissue is contaminated by a small amount of tumor, as seen by the faint bands in the normal cerebellum lane.

truncates the protein at amino acid 920 in the third intracellular loop following the sixth transmembrane domain. The cDNA sequence from the remaining 12 tumors was wild-type.

To rule out the possibility that the wild-type *Ptch* sequence detected in the tumors was due to normal tissue contamination, we analyzed tumor sections by using in situ hybridization. Two additional tumors were obtained; half of each was fixed and processed for sectioning, and the other half was used for RNA extraction. Sequencing of *Ptch* message from these tumors revealed a wild-type sequence in both tumors. The remaining tumor tissue was sectioned and hybridized to a *Ptch*-specific ³⁵S-labeled riboprobe. This probe consisted of part of *Ptch* exon 1 (including the putative start codon) and all of exon 2. This region corresponds to the portion of the cDNA deleted by homologous recombination during the generation of the transgenic mouse. Thus, only mRNA derived from the wild-type allele will be detected with this probe. Figure 2a shows labeling of tumor cells with the antisense probe, indicating that these cells express wild-type *Ptch* message from the wild-type allele. Hybridization with the sense riboprobe gave no signal (Fig. 2b).

These hemizygous *Ptch*^{+/-} mice have been shown to express *Ptch* in nontumor tissue at about 50% of the level of wild-type mice (Goodrich et al., 1997). Using a full-length *Ptch* cDNA probe, RNA

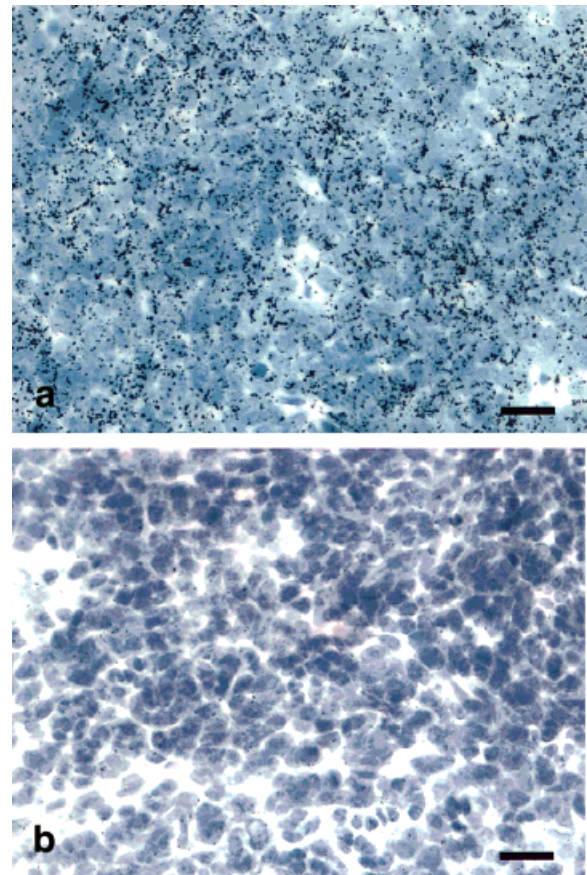


Figure 2. *Ptch* message expression in tumor cells. Paraffin sections of two murine tumors were hybridized with ³⁵S-labeled antisense riboprobes specific for the region of the *Ptch* gene deleted by homologous recombination during construction of the *Ptch*^{+/-} mouse. Hybridization with sense riboprobes of the same sequence was done in parallel. Slides were coated with photographic emulsion and developed after 10 days. **a**: Antisense probe, showing *Ptch* message being expressed from the remaining allele in tumor cells. **b**: Sense probe, demonstrating background labeling, indicating specificity of the probe. Bars: 20 μ m.

blot analysis of total tumor RNA from 11 tumors revealed a *Ptch* transcript of the expected size in all tumors. However, the amount of *Ptch* transcript varied considerably between tumors (Fig. 3a). When compared with the normal cerebellum control, levels of *Ptch* expression were higher in three tumors (1594, 310, 595), unchanged in five tumors (638, 1409, 1550, 1596, 1703), and lower in three tumors (1808, 351, 1900).

One effect of *Ptch* inactivation is increased transcription of *Gli1* (Epstein et al., 1996). To ascertain whether the levels of *Gli1* transcript were elevated in these tumors due to *Ptch* hemizygous inactivation, tumor RNA from 11 tumors was probed for *Gli1* expression. Blotting was performed as described with an 817-bp RT-PCR product for *Gli1* as the probe. As shown in Figure 3b, *Gli1* mRNA

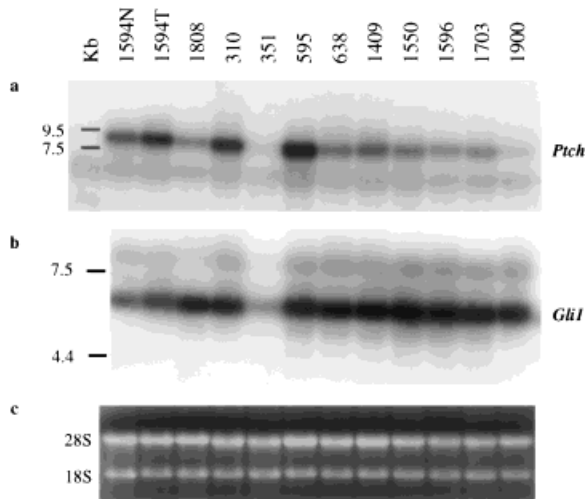


Figure 3. Expression of *Ptch* and *Gli1* in cerebellar tumors from transgenic *Ptch*^{+/-} mice. **a:** RNA blotting shows that *Ptch* mRNA of expected size (8.5 kb) is expressed in all tumors. As in Figure 1, the lane labeled 1594N represents expression in normal cerebellum. **b:** The blot shown in Figure 3a was stripped and reprobed for *Gli1*. Increased expression of *Gli1* is seen in all tumors except 351, where *Ptch* expression is very low. **c:** Ethidium bromide staining of the gel showed that all lanes were loaded equally with 10- μ g RNA.

levels were increased compared to those in normal cerebellum in all tumors but one (351), regardless of the amount of *Ptch* mRNA. The levels of expression of both *Ptch* and *Gli1* in the tumor with the mutation in the remaining allele (1594) did not differ substantially from those of tumors that did not contain mutations.

DISCUSSION

The data presented here suggest that medulloblastoma formation in *Ptch*^{+/-} mice may be due to haploinsufficiency at the *Ptch* locus. The classical two-hit paradigm for a tumor suppressor gene involves inactivation of both copies of the gene in order for tumorigenesis to occur. Data from the present study indicate that, in the *Ptch*^{+/-} mouse, brain tumors arise despite the presence of a wild-type *Ptch* allele. The RT-PCR, in situ hybridization, and RNA blot results show that transcription of the wild-type *Ptch* allele is not being repressed by methylation, mutation of the promoter region, or by genomic imprinting. However, other mechanisms resulting in the inactivation of the wild-type *Ptch* allele, such as altered message or protein stability, or improper regulation of expression, either spatially or temporally, could compromise *Ptch* protein function. These hemizygous *Ptch*^{+/-} mice have been shown to express *Ptch* in nontumor tissue at about 50% of the level in wild-type mice (Goodrich et al., 1997). Clearly, the wild-type *Ptch*

sequences obtained from the tumors are not the result of normal tissue contamination, because the in situ hybridization results demonstrate the presence of *Ptch* mRNA transcribed from the wild-type allele in tumor cells.

In the absence of SHH, *Ptch* downregulates its own expression (Goodrich et al., 1996). Thus, as has been demonstrated in basal cell carcinomas with *PTCH* inactivation, one would predict that *PTCH* expression would be upregulated in medulloblastomas with inadequate *PTCH* activity due to loss of negative feedback (Unden, 1997). In addition, it is necessary to explain why wild-type *Ptch* message is expressed at a higher level in some tumors than in normal tissue from the same mouse if tumor formation is due to decreased *Ptch* function. Of note is that hemizygous *Ptch*^{+/-} mice have been shown to express *Ptch* in nontumor tissue at about 50% of the level of wild-type mice (Goodrich et al., 1997). The RNA blot analysis presented here indicates variable levels of *Ptch* mRNA in murine medulloblastomas. A possible explanation for these findings is that the cell type in question is susceptible to haploinsufficiency of *Ptch* only at specific, critical periods during development. Mechanistically, half-normal levels of *Ptch* protein may not be sufficient to bind all v-smo protein in the membrane, thus allowing constitutive signal transduction through unbound v-smo (Stone et al., 1996). If v-smo signaling promotes tumor formation at a specific developmental time, then later during tumor development or growth *PTCH* expression levels may be altered without consequence to tumorigenesis.

Additionally, mice overexpressing SHH, the proposed ligand for *Ptch*, develop BCC, as do NBCCS patients (Oro et al., 1997). Because the SHH study used a keratin-specific promoter, brain abnormalities were not investigated. However, the study suggested that a balance between SHH and *Ptch* protein levels was required. We have provided evidence here that, in mice, haploinsufficiency can lead to medulloblastoma.

Haploinsufficiency of *PTCH* may occur in human medulloblastoma. In this human tumor, LOH at 9q22, the location of the *PTCH* gene, has been demonstrated in tumors that do not harbor mutations in the remaining *PTCH* allele. In the two studies that report an LOH analysis for all tumors examined, the rates of 9q22 loss were 20.8% and 28.6%, whereas the rates of mutation of the remaining *PTCH* allele were only 12.5% and 14.3%, respectively (Xie et al., 1997; Zurawel et al., 1997). Similarly, mutations in the *PTCH* gene have been

reported in tumors that do not demonstrate LOH at 9q22 (Pietsch et al., 1997; Vorechovsky et al., 1997; Zurawel et al., 1999). Reasons for this may be that another tumor suppressor gene is located in this region or that one functioning copy of the gene is not sufficient to suppress tumor development in man.

REFERENCES

- Epstein DJ, Marti E, Scott MP, McMahon AP. 1996. Antagonizing cAMP dependent kinase A in the dorsal CNS activates a conserved sonic hedgehog signaling pathway. *Development* 122:2885–2894.
- Evans DG, Farndon PA, Burnell LD, Gattamaneni, HR, and Birch JM. 1991. The incidence of Gorlin syndrome in 173 consecutive cases of medulloblastoma. *Brit J Cancer* 64:959–961.
- Farndon PA, Del Mastro RG, Evans DG, Kilpatrick MW. 1992. Location of gene for Gorlin syndrome. *Lancet* 339:581–582.
- Gailani MR, Bale SJ, Leffell DJ, DiGiovanna JJ, Peck GL, Poliak S, Drum, MA, Pastakia B, McBride OW, Kase R, Greene M, Mulvill JJ, Bale AE. 1992. Developmental defects in Gorlin syndrome related to a putative tumor suppressor gene on chromosome 9. *Cell* 69:111–117.
- Goodrich LV, Johnson RL, Milenkovic L, McMahon JA, Scott MP. 1996. Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog. *Genes Devel* 10:301–112.
- Goodrich LV, Milenkovic L, Higgins KM, Scott MP. 1997. Altered neural cell fates and medulloblastoma in mouse *patched* mutants. *Science* 277:1109–1113.
- Gorlin RJ. 1987. Nevoid basal-cell carcinoma syndrome. *Medicine* 66:98–113.
- Hahn H, Wicking C, Zaphiropoulos PG, Gailani MR, Shanley S, Chidambaram A, Vorechovsky I, Holmberg E, Uden AB, Gillies S, Negus K, Smyth I, Pressman C, Leffell DJ, Gerrard B, Goldstein AM, Dean M, Toftgard R, Chenevix-Trench G, Wainwright B, Bale AE. 1996. Mutations of the human homolog of *Drosophila patched* in the nevoid basal cell carcinoma syndrome. *Cell* 85:841–851.
- Hooper JE, Scott MP. 1989. The *Drosophila patched* gene encodes a putative membrane protein required for segmental patterning. *Cell* 59:751–765.
- Humphreys RP. 1982. Posterior cranial fossa brain tumors in children. In: Youmans JR, editor. *Neurological surgery*. Philadelphia: Saunders. p 2722–2752.
- Johnson RL, Rothman AL, Xie J, Goodrich LV, Bare JW, Bonifas J, Quinn AG, Myers RM, Cox DR, Epstein EH Jr, Scott MP. 1996. Human homolog of *patched*, a candidate gene for the Basal Cell Nevus Syndrome. *Science* 272:1668–1671.
- Lacombe D, Chateil JF, Fontan D, Battin J. 1990. Medulloblastoma in the nevoid basal-cell carcinoma syndrome: case reports and review of the literature. *Genet Couns* 1:273–277.
- Nakano Y, Guerrero I, Hidalgo A, Taylor A, Whittle JR, Ingham PW. 1989. A protein with several possible membrane-spanning domains encoded by the *Drosophila* segment polarity gene *patched*. *Nature* 341:508–513.
- Oro AE, Higgins KM, Hu Z, Bonifas JM, Epstein EH, Scott MP. 1997. Basal cell carcinomas in mice overexpressing sonic hedgehog. *Science* 276:817–821.
- Pietsch T, Waha A, Koch A, Kraus J, Albrecht S, Tonn J, Sorensen N, Berthold F, Henk B, Schmandt N, Wolf HK, von Deimling A, Wainwright B, Chenevix-Trench G, Wiestler OD, Wicking C. 1997. Medulloblastomas of the desmoplastic variant carry mutations of the human homologue of *Drosophila patched*. *Cancer Res* 57:2085–2088.
- Stone DM, Hynes M, Armanini M, Swanson TA, Gu Q, Johnson RL, Scott MP, Pennica D, Goddard A, Phillips H, Noll M, Hooper JE, de Sauvage F, Rosenthal A. 1996. The tumor-suppressor gene *patched* encodes a candidate receptor for sonic hedgehog. *Nature* 384:129–134.
- Uden AB, Zaphiropoulos PG, Bruce K, Toftgard R, Stahle-Backdahl M. 1997. Human patched (PTCH) mRNA is overexpressed consistently in tumor cells of both familial and sporadic basal cell carcinoma. *Cancer Res* 57:2336–2340.
- Vorechovsky I, Tingby O, Hartman M, Stromberg B, Nister M, Collins VP, Toftgard R. 1997. Somatic mutations in the human homologue of *Drosophila* in primitive neuroectodermal tumors. *Oncogene* 15:361–366.
- Wolter M, Reifenberger J, Sommer C, Ruzicka T, Reifenberger G. 1997. 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.
- Xie J, Johnson RL, Zhang X, Bare JW, Waldman FM, Cogen PH, Menon AG, Warren RS, Chen LC, Scott MP, Epstein EH Jr. 1997. Mutations of the *PATCHED* gene in several types of sporadic extracutaneous tumors. *Cancer Res* 57:2369–2372.
- Zurawel RH, Chiappa SA, Allen C, Raffel C. 1997. Sporadic medulloblastomas contain *PTCH* mutations. *Cancer Res* 57:842–845.
- Zurawel RH, Allen C, Chiappa SA, Cato W, Beigel JA, Cogen PH, de Sauvage F, Raffel C. 2000. Analysis of *PTCH/SMO/SHH* pathway genes in medulloblastoma. *Genes Chromosomes Cancer* 27:44–51.