Sporadic Medulloblastomas Contain PTCH Mutations

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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, 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 alteration in 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 uracil microsatellite primer D10S224 to identify working concentrations that would yield similar autoradiographic intensities after amplification in the presence of [α-32P]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 μM forward and reverse primers (Research Genetics, Huntsville, AL), 0.8 μCi [α-32P]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 MgCl2. Samples were placed in 96-well plates, overlayed with mineral oil, and amplified in a twin-block thermal cycler (Eppendorf, 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 μM urea and 15% formamide and electrophoresed for 1.5—3 h at 75 W. Gels were dried and assessed for LOH by visual comparison of tumor and blood-band intensities. Losses were confirmed by repeated assays and by exposing dried gels to Phosphorlmager screens and quantitating allele count intensities with ImageQuan 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 PstI. 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 heating and blotting to 80°C for 2 h. Filters were hybridized as described previously (13) and hybridized using 25 ng of [α-32P]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.

Received 12/5/96; accepted 1/17/97.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by National Cancer Institute Grants CA57572 and CA59005.

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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**

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* 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): GGGATTCACCTCTAAAACA, ACCACACTGGGGACTTTT (D9S106); TTTCCTGCTCCACCA, CATGCGCAAGACTGCGCT (D9S180); AGCTTCTTAAAACAA, ATCCACAGACCTGCGGAG (D9S280); M15, M20, and M21, and at D9S176 only for Mi5 (patient was uninformative for this RFLP marker within PTCH), were used to examine the tumors for PTCH mutations. 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. 3A): 27 bp in tumor M15 (3146ins27) and 19 bp in tumor M21 (3112ins19; Fig. 3B). 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. 3C) 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 remaining five tumors was informative. The mutations were identical in exons 16 and 17 in the tumors M7, M10, and M12, and both loci for specimen M7 demonstrated tumor maintenance of heterozygosity.

**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 PstI 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.)

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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 cubitis interruptus genes. Hedgehog also up-regulates patched, which in turn down-regulates hedgehog, cubitis interruptus, wingless, and, interest-
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 wt-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.

References

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*Cancer Res* 1997;57:842-845.

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