Deletions of AXIN1, a Component of the WNT/wingless Pathway, in Sporadic Medulloblastomas


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Abstract

Medulloblastoma (MB) represents the most frequent malignant brain tumor in children. Most MBs appear sporadically; however, their incidence is highly elevated in two inherited tumor predisposition syndromes, Gorlin’s and Turcot’s syndrome. The genetic defects responsible for these diseases have been identified. Whereas Gorlin’s syndrome patients carry germ-line mutations in the patched (PTCH) gene, Turcot’s syndrome patients with MBs carry germ-line mutations of the adenosomatous polyposis coli (APC) gene. The APC gene product is a component of a multiprotein complex controlling β-catenin degradation. In this complex, Axin plays a major role as scaffold protein. Whereas APC mutations are rare in sporadic MBs, a hot-spot region of β-catenin (CTNNB1) mutations was identified in a subset of MBs. To find out if Axin is also involved in the pathogenesis of sporadic MBs, we analyzed 86 MBs and 11 MB cell lines for mutations in the AXIN1 gene. Using single-strand conformation polymorphism analysis, screening for large deletions by reverse transcription-PCR, and sequencing analysis, a single somatic point mutation in exon 1 (Pro255Ser) and seven large deletions (12%) of AXIN1 were detected. This indicates that AXIN1 may function as a tumor suppressor gene in MBs.

Introduction

MBs are malignant primitive neuroectodermal tumors of the cerebellum. They represent the most common malignant brain tumor of childhood with an incidence of approximately five per million children (1). Whereas most MBs occur sporadically, their incidence is highly elevated in patients carrying germ-line APC gene mutations (2).

APC is part of a multiprotein complex of the WNT/wingless pathway which induces β-catenin degradation. Axin, GSK-3β, β-catenin, PP2A, and PP2C are additional components of this complex (3–15). Activation of the WNT signal pathway blocks degradation of β-catenin and leads to its association with TCF transcription factors. The WNT/wingless signaling pathway is necessary for regulating development and organogenesis (3, 16, 17), and alterations of its signaling appear to contribute to the pathogenesis of several human cancers including colorectal, endometrial and ovarian carcinomas (18, 19). In particular, mutations of β-catenin and APC lead to constitutive stabilization of β-catenin. Mutations of β-catenin (5% of cases) and APC (4%) as components of the WNT pathway have been identified previously in sporadic MBs (20–24).

Axin was initially identified as the gene product of the mouse fused locus, which negatively regulates WNT signaling (25). Recent experiments demonstrate that Axin functions as a tumor suppressor in HCC (26). The different domains of Axin possess binding capacity for APC, GSK-3β, β-catenin, PP2A, Dishevelled, and Axin itself (14, 27). As a scaffold protein of this multiprotein complex, Axin is able to bring β-catenin and GSK-3β into close proximity, thus facilitating β-catenin phosphorylation (5, 28) and subsequent ubiquitin-mediated degradation by the proteasome system (29). Recent experiments suggest that Axin has to form dimers for its function as inhibitor of TCF/LEF1 transcription (11, 27, 30).

In this study, we identified mutations of the human homologue AXIN1 in a subset of sporadic MBs, indicating that such mutations activate the WNT pathway in these tumors.

Materials and Methods

Patients, Tumors, and Cell Lines. Biopsy samples of 86 sporadic MBs and 11 MB cell lines, including 3 that have been described previously (31), were analyzed.

In 1 patient, we were able to study both the primary and the recurrent tumor. From 57 MB samples, sufficient tumor tissue was available for RNA isolation. Constitutional genomic DNA was isolated in 61 cases from peripheral blood leukocytes. The age of the patients ranged from 1 month to 59 years.

All tumors were classified according to the revised WHO classification of brain tumors using standard histological methods, including H&E and reticulin stains, as well as immunohistochemical reactions (32). Tumor cell differentiation was assessed by immunostaining for neuron-specific enolase, synaptophysin, and glial fibrillary acidic protein. Tumor samples were obtained at the time of surgical resection, snap frozen in liquid nitrogen, and stored at −80°C.

DNA and cDNA Preparation. Individual tissue samples were reviewed microscopically on frozen sections to exclude contaminating necrotic or normal brain tissue. Genomic DNA from tumors and peripheral blood leukocytes was extracted by standard proteinase K digestion and phenol/chloroform extraction (33). Total cellular RNA was either prepared by lysis in guanidinium isocyanate and ultra-centrifugation through a cesium chloride cushion or by using the TRIZol reagent (Life Technologies) following the manufacturer’s protocols. Before reverse transcription using the SuperScript preamplification system (Life Technologies) with random hexamers as primers, contaminating residual genomic DNA was removed by RNase-free DNase (Roche) digestion.

Mutational Analysis of the AXIN1 Gene. We screened the coding sequence of the AXIN1 gene with the SSCP method using 23 sets of primers published previously (26). Primer sequences were designed from cosmid with GenBank accession nos. Z99754, Z11450, Z69667, and Z98272. PCR was performed in a Robocycler (Stratagene) thermocycler. The samples were denatured at 94°C for 2 min followed by 38 cycles (94°C × 40 s, 48°C–60°C × 50 s, 72°C × 50 s) and a final incubation for 5 min at 72°C. A single
Table 1  Primer sequences for deletion analysis of AXIN1 and LOH marker CA461a8

<table>
<thead>
<tr>
<th>Deletion analysis</th>
<th>AXIN1 a</th>
<th>AXIN1 b</th>
<th>LOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>F 5’-GGC CTC ATT GTT CAT TGA CGC-3’</td>
<td>R 5’-AAC ACT CTC TGA GTA GCC TCG-3’</td>
<td>R 5’-GAG ACA GAG TGT GAA GGA GG-3’</td>
<td></td>
</tr>
<tr>
<td>F 5’-AGG CTA CTC AGA GAG TGT GTG-3’</td>
<td>F 5’-TTT GTG TAA AAC AAA GGA AAA ATA-3’</td>
<td>F 5’-GGA GTA AAA TGA CTT TTT ACC CTA-3’</td>
<td></td>
</tr>
</tbody>
</table>

10 µl PCR reaction mix contained 20 ng of genomic DNA, 1–2 mm MgCl₂, 0.2 mm each deoxynucleotide triphosphate (Fermentas), 5 pmol of each primer, and 0.25 units of Taq Polymerase (Life Technologies). Some reactions were run with 10% DMSO. For SSCP analysis, products were loaded onto 10 and 14% polyacrylamide gels (acylamide:bisacrylamide ratio 1:79, 1:29) and gels and runs were at room temperature (60 V) or at 4°C (80 V). Single and double strands of PCR products were visualized by silver staining (34).

Aberrantly migrating bands were excised and eluted in aqua dest, and the DNA was reamplified. The resulting PCR products were purified using the QIAquick PCR purification Kit (Qiagen) and finally sequenced. We used the Applied Biosystems PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit on a GeneAmp PCR system 9600 (Perkin-Elmer), using 20 ng of PCR product as a template. Finally, the sequencing reaction products were separated on an Applied Biosystems 373A or an Applied Biosystems 377 sequencer.

**Deletion Analysis by RT-PCR.** We used two sets of primers (Table 1) to amplify large fragments of the AXIN1 gene (AF009674). PCR reactions were carried out on a Robocycler (Stratagene). Denaturation at 94°C for 5 min was followed by 42 cycles (94°C × 60 s, 54°C × 70 s, 72°C × 70 s) and a final incubation step at 72°C for 10 min for both primer sets. The 10 µl PCR reaction mix contained the ingredients described above (with 1 mm MgCl₂, 10% DMSO).

Products were visualized on a 2% agarose gel containing ethidium bromide. Aberrant cDNA bands were excised, placed on the perforated bottom of a microtube located in a larger collection tube, and then centrifuged at room temperature (600 g) at room temperature (60 V) or at 4°C (80 V). Single and double strands of PCR products were visualized by silver staining (34).

**Results and Discussion.**

**SSCP Screening.** Almost the entire coding sequence of the AXIN1 gene was analyzed by SSCP in our panel of 97 MB DNA samples. The target sequence ranged from exon 1, nucleotide position 31 of the Axin mRNA (AF009674), to exon 10 position 2723, 19 nucleotides downstream of the TGA Stop Codon.

We identified 10 SNPs by altered migration on SSCP gels of both the tumor DNA sample and DNA from normal tissue of individual patients (Fig. 1). We confirmed 3 previously published silent SNPs (35, 36). Seven of these 10 polymorphisms represent novel SNPs:

Three rare polymorphisms were silent, at positions (according to GenBank accession no. AF009674) C1432T for Pro 477 (C 0.99; T 0.01), G1792A for Gln 597 (G 0.99; A 0.01), and G2518A for Arg 820 (G 0.99; A 0.01). Four SNPs resulted in the following amino acid substitutions because of base exchanges in the first or second position of the coding triplets: pos. G1256A for Arg419Cys (G 0.99; A 0.01), pos. C1317T for Thr439Met (C 0.99; T 0.01), pos. C1600G for Asp533Glu (C 0.99; G 0.01), and pos. G2637A for Arg879Gln (G 0.99; A 0.01). Because these alterations were present in both the patient’s tumor and constitutional DNA, they likely represent rare polymorphisms.

Additionally, we discovered a somatic point mutation in D210II, a classic MB, resulting in an amino acid exchange of Pro255 to Ser. Pro255 is located in exon 1, which encodes the binding site for APC. Considering the importance of proline for the secondary structure of proteins, this mutation may decrease the ability of Axin to bind APC. This interaction is required for down-regulation of β-catenin (37, 38).

**LOH.** We searched for allelic losses at the AXIN1 locus with markers D16S521 and CA461a8 located within 1 Mb of AXIN1 on chromosome 16p (35). In addition, we also used the frequent C94A polymorphism (Table 2) in exon 1 to analyze LOH with the SSCP method. Of the 65 cases tested for LOH, 52 were informative with at least one of the tested markers. We identified two samples with allelic loss (D354 and D491) using marker C94A and CA461a8, respectively.

**Deletion Analysis of AXIN1.** Deletion analysis was carried out in the NH₂- and COOH-terminal region of the AXIN1 gene with two primer sets (Table 1). We identified 7 MBs with deletions of the AXIN1 gene (Table 2; Figs. 1 and 2). Three of these included parts of exon 1 and extended to exon 5. The other 4 deletions were COOH-terminal.

Deletion R1291 (Fig. 3) was in-frame resulting in the loss of 440 amino acids encoded by most of exon 1 to exon 5. The truncated protein lacks binding sites for APC, GSK-3β, and β-catenin. Interaction of Axin with GSK-3β and β-catenin has been shown to be necessary to down-regulate β-catenin and to decrease cell proliferation (5, 28, 30, 37). R1348 and R1346 had large frameshift deletions...
In vitro, Myc-rAxin appeared to act via a dominant-negative mechanism (28). Taken together, these data support the hypothesis that the DIX domain of AXIN1 is necessary for degradation of β-catenin and suppression of cellular proliferation.

Frameshift mutations have also been identified in conductin/AXIN2, a homologue of AXIN1 (4). Such mutations resulted in the elimination of the DIX domain and seemed to be a causal link for tumor development in colorectal cancer with defective mismatch repair (40). Experiments with a TCF reporter assay cotransfecting 293 cells with a frameshift mutant of AXIN2 and wild-type AXIN2 suggested a dominant-negative effect of mutant AXIN2 (40). A dominant role of COOH-terminal deletions in either AXIN1 or AXIN2 may lead to an overactivation of WNT signaling and may explain why truncating mutations may be functional and not neutralized by redundancy of wild-type AXIN1 or AXIN2.

An activation of the WNT signaling pathway by amino acid substitutions and deletions in its important components β-catenin and APC has been demonstrated in various tumors, such as melanomas (41), lung adenocarcinomas (42), and MBs (20–22, 24). AXIN1 mutations were reported previously in HCCs (26) and colon carcinomas spanning from exon 1 to the middle of exon 5, generating severely truncated proteins.

Table 2  Mutational analysis of the AXIN1 gene in MBs

<table>
<thead>
<tr>
<th>A. Case</th>
<th>Age (yr)/sex</th>
<th>Histology</th>
<th>aa positions&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Δhp</th>
<th>Δ positions&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Exons</th>
<th>LOH on 16p</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1291 (D811H)</td>
<td>28/male</td>
<td>Classic</td>
<td>89–528&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1320</td>
<td>266–1585</td>
<td>1–5</td>
<td>ni&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>R1348 (D378R)</td>
<td>8/male</td>
<td>Classic</td>
<td>118–571</td>
<td>1358</td>
<td>355–1712</td>
<td>1–5</td>
<td>No</td>
</tr>
<tr>
<td>R1346 (D223HR)</td>
<td>9/male</td>
<td>Classic</td>
<td>59–532</td>
<td>1420</td>
<td>178–1597</td>
<td>1–5</td>
<td>ni</td>
</tr>
<tr>
<td>R1095 (D831H)</td>
<td>6/female</td>
<td>Classic</td>
<td>809</td>
<td>676</td>
<td>2426–3101</td>
<td>9–10</td>
<td>No</td>
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<tr>
<td>R1114 (D938)</td>
<td>23/female</td>
<td>Desmoplastic</td>
<td>775</td>
<td>782</td>
<td>2325–3106</td>
<td>8–10</td>
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<tr>
<td>R1055 (D228HR)</td>
<td>9/male</td>
<td>Classic</td>
<td>646–TGA&lt;sup&gt;e&lt;/sup&gt;</td>
<td>767</td>
<td>1938–2704</td>
<td>6–10</td>
<td>na</td>
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<tr>
<td>R385 (D822)</td>
<td>7/female</td>
<td>Desmoplastic</td>
<td>723</td>
<td>931</td>
<td>2168–3098</td>
<td>7–10</td>
<td>na</td>
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</table>

<table>
<thead>
<tr>
<th>B. Case</th>
<th>Age (yr)/sex</th>
<th>Histology</th>
<th>Exon</th>
<th>Base change&lt;sup&gt;a&lt;/sup&gt;</th>
<th>aa change</th>
<th>LOH 16p</th>
<th>Allelic freq.</th>
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<tbody>
<tr>
<td>D 210 II</td>
<td>34/female</td>
<td>Classic</td>
<td>1</td>
<td>C764T</td>
<td>Pro255Ser</td>
<td>ni</td>
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<tr>
<td>Intron 4</td>
<td>1</td>
<td>C94A</td>
<td>No</td>
<td></td>
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<tr>
<td>5</td>
<td>G1396A</td>
<td>No</td>
<td></td>
<td></td>
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<tr>
<td>5</td>
<td>G1792A</td>
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<tr>
<td>9</td>
<td>G2518A</td>
<td>No</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>4</td>
<td>C1317T</td>
<td>Thr439Ser</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>C1256T</td>
<td>Arg419Cys</td>
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<tr>
<td>5</td>
<td>C1600G</td>
<td>Asp533Glu</td>
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<tr>
<td>10</td>
<td>G2637A</td>
<td>Arg879Gln</td>
<td></td>
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</tr>
</tbody>
</table>

<sup>a</sup> Amino acid positions according to GenBank accession no. AF009674.
<sup>b</sup> Deletion is in frame.
<sup>c</sup> Deletion terminates just behind TGA stop codon.
<sup>d</sup> Allelic frequency in 205 normal control DNAs from healthy Caucasians: A 0.78 C 0.22.
<sup>e</sup> Base positions according to AF009674.


25. Kleihues, P., and Cavenee, W. K. WHO Classification of Tumors: Pathology and 


27. Strovel, E. T., Wu, D., and Sussman, D. J. Protein phosphatase 2C dephosphorylates 

28. Kleihues, P., and Cavenee, W. K. WHO Classification of Tumors: Pathology and 


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