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Advances in Brief

Deletions of *AXIN1*, a Component of the *WNT/wingless* Pathway, in Sporadic Medulloblastomas[1]

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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 *adenomatous 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[3] 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 ultracentrifugation 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 cosmids with GenBank accession nos. Z99754, Z81450, 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

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[3] The abbreviations used are: MB, medulloblastoma; GSK-3β, glycogen synthase kinase-3β; HCC, hepatocellular carcinoma; LOH, loss of heterozygosity; SSCP, single-strand conformational polymorphism; APC, adenomatous polyposis coli; PP, protein phosphatase; RT-PCR, reverse transcription-PCR; SNP, single nucleotide polymorphism; TCF, T-Cell Factor; DIX, for Dishevelled and Axin proteins; RGS, regulator of G-protein signaling.

Table 1 *Primer sequences for deletion analysis of AXIN1 and LOH marker CA461a8*

Deletion analysis	
AXIN1 a	F 5′-GCG CTC ATT GTT CAT TGA CGC-3′
	R 5′-AAC ACT CTC TGA GTA GCC TCG-3′
AXIN1 b	F 5′-AGG CTA CTC AGA GAG TGT TGG-3′
	R 5′-GAG ACA AGC TGT GTT GAA GG-3′
LOH	
CA461a8 (35)	F 5′-TTT GTG TAA AAC AAA GGA AAA ATA-3′
	R 5′-GGA GTA AAA TGA CTT TTT ACC CTA-3′

10 μl PCR reaction mix contained 20 ng of genomic DNA, 1–2 mM $MgCl_2$, 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 (acrylamide:bisacrylamide ratio 1:79, 1:29) gels and run 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 $MgCl_2$, 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 at 9000 *g* for 10 min. The extracted cDNA was diluted, and the *AXIN1* fragment was reamplified, purified, and finally sequenced (see above).

LOH. We used the microsatellite marker D16S521 adjacent to *AXIN1* and a primer set (CA461a8), which flanks a CA repeat within 1 Mb of the *AXIN1* gene (Table 1; Ref. 35). In addition, we used the C94A polymorphism in exon 1 (Table 2) and searched for LOH with the SSCP method. Products were resolved on denaturing polyacrylamide gels and stained with silver as described previously (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 codes for Arg419Cys (G 0.99; A 0.01), pos. C1317T codes for Thr439Met (C 0.99; T 0.01), pos. C1600G codes for Asp533Glu (C 0.99; G 0.01), and pos. G2637A codes 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 NH_2- 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

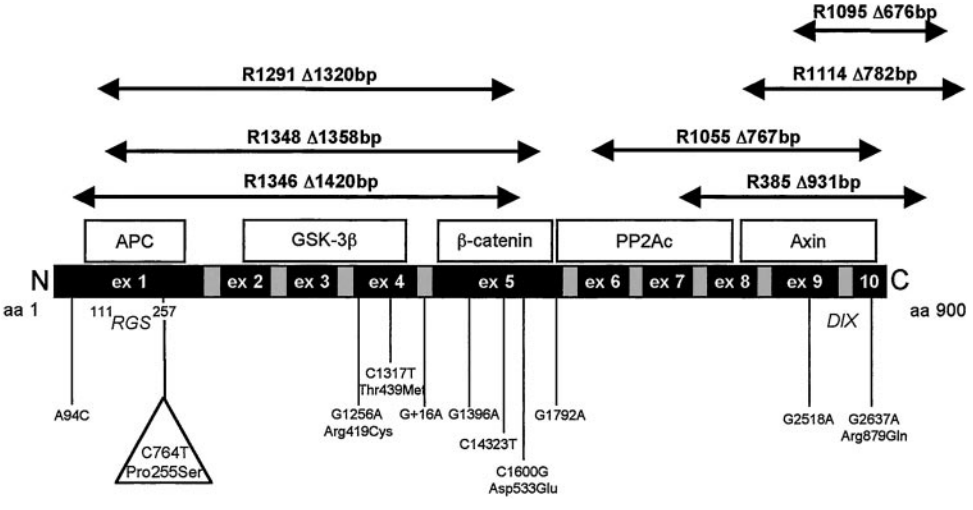

Fig. 1. Genetic alterations of the *AXIN1* gene in sporadic MBs. Deletions are indicated by *two-headed arrows*. Polymorphisms are symbolized by *vertical lines*. The point mutation is framed with a *triangle*.

Table 2 *Mutational analysis of the AXIN1 gene in MBs*

A. Case	Age (yr)/sex	Histology	aa positions[a]	Δbp	Δ positions[b]	Exons	LOH on 16p
Deletions							
R1291 (D811II)	28/male	Classic	89–528[c]	1320	266–1585	1–5	ni[d]
R1348 (D378)	8/male	Classic	118–571	1358	355–1712	1–5	No
R1346 (D243II)	9/male	Classic	59–532	1420	178–1597	1–5	ni
R1095 (D83II)	6/female	Classic	809	676	2426–3101	9–10	No
R1114 (D398)	23/female	Desmoplastic	775	782	2325–3106	8–10	No
R1055 (D228II)	9/male	Classic	646–TGA[e]	767	1938–2704	6–10	na
R385 (D82)	7/female	Desmoplastic	723	931	2168–3098	7–10	na

B. Case	Age (yr)/sex	Histology	Exon	Base change[a]	aa change	LOH 16p	Allelic freq.
Point mutations							
D 210 II	34/female	Classic	1	C764T	Pro255Ser	ni	
Polymorphisms			1	C94A	No		C 0.80 A 0.20[f]
			Intron 4	G+16A	No		G 0.90 A 0.10
			5	G1396A	No		G 0.81 A 0.19
			5	C1432T	No		C 0.99 T 0.01
			5	G1792A	No		G 0.99 A 0.01
			9	G2518A	No		G 0.99 A 0.01
			4	C1317T	Thr439Met		C 0.99 T 0.01
			4	C1256T	Arg419Cys		C 0.99 T 0.01
			5	C1600G	Asp533Glu		C 0.99 G 0.01
			10	G2637A	Arg879Gln		G 0.99 A 0.01

[a] Amino acid positions according to GenBank accession no. AF009674.
[b] Base positions according to AF009674.
[c] Deletion is in frame.
[d] ni, not informative; na, not analyzed.
[e] Deletion terminates just behind TGA stop codon.
[f] Allelic frequency in 205 normal control DNAs from healthy Caucasians: A 0.78 C 0.22.

spanning from exon 1 to the middle of exon 5, generating severely truncated proteins.

Similar but smaller deletions of *AXIN1* have been identified in HCCs, which predict truncated proteins lacking the binding site for β-catenin. In these tumors, most cases showed a loss of the second allele (26). We were able to test for LOH in 5 of 7 MBs with deletions. Three of them (R1348, R1095, and R1114) did not show LOH, and the other 2 cases (R1291 and R1346) were not informative (Table 2).

Some truncated mutants of Axin may bind to and inactivate endogenous wild-type Axin (11). In *Xenopus* experiments, ΔRGS-Axin, in which the RGS (aa 220–340) domain was deleted, acted as a dominant-negative molecule, which activates the *WNT* pathway (25). This was confirmed by studies with similar Axin fragments in *Xenopus* embryos that also lacked the RGS domain (AxΔ251–351). These fragments induced a clear increase in β-catenin levels *in vivo*, and this effect was dominant (11). The effect could be reduced by deletion of the COOH-terminal 100 amino acids which contain the DIX domain, necessary for *AXIN1* multimerization (see below). Expression of $Axin^{\Delta GSK\text{-}3\beta}$ with a deleted GSK-3β binding site in L cells induced the accumulation of β-catenin in the absence of Wnt-3a and did not suppress the stabilization of β-catenin by Wnt-3a (28). Additionally, $Axin^{\Delta GSK\text{-}3\beta}$ acts via a dominant-negative mechanism (28). Taken together, these results strongly suggest that the in-frame deletion in R1291 may play a role in tumor development.

In addition, we discovered four COOH-terminal deletions in MBs, spanning from exon 6 to exon 10 behind the TGA stop codon. The COOH-terminal half of Axin deleted in these cases contains the DIX domain (25, 27, 39) and an oligomerization domain (30). Because the DIX domain is necessary but not sufficient for homo-oligomerization of Axin (27), both domains appear to be crucial for oligomerization (27, 30). Bridging of GSK-3β and β-catenin is necessary but not sufficient for the inhibitory effect of Axin on TCF-dependent transcriptional activity (30). Moreover, oligomerization of Axin molecules through the COOH-terminus seems to be crucial for inhibition of TCF transcription (30). It was reported that COOH-terminal deleted rat Axin (Myc-rAxin aa 1–717) was not able to promote the degradation of β-catenin and to reduce cell growth in SW480 cells (27). Instead Myc-rAxin appeared to act via a dominant-negative mechanism to inhibit endogenous Axin activities in SW480 cells (27). Taken together, these data support the hypothesis that the DIX domain of Axin 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

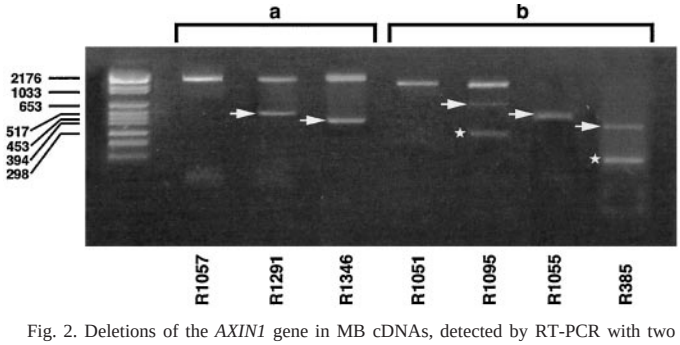

Fig. 2. Deletions of the *AXIN1* gene in MB cDNAs, detected by RT-PCR with two primer sets spanning exon 1 to exon 5 (*a*) and exon 5 to exon 10 (*b*). In addition to the wild-type product of 1781 bp (*R1057*) and 1317 bp (*R1051*), respectively, smaller products representing the deleted *AXIN1* gene product can be detected. Deletions of *AXIN1* were identified by excision, reamplification, and sequencing. Bands which represent *AXIN1* deletions are indicated by *arrows*; the smaller bands marked with ∗ were unspecific PCR products.

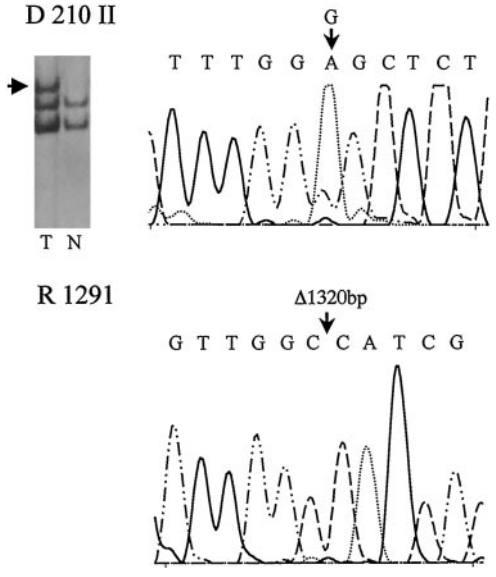

Fig. 3. Point mutation in tumor D210II and deletion in tumor R1291. The somatic mutation in D210II was identified by SSCP screening. An aberrant band of the tumor sample (*T*), which is not present in the blood sample (*N*), is marked by an *arrow*. DNA sequencing of the excised and reamplified DNA products reveals a somatic C→T transition in codon 255 leading to a substitution of proline to serine. The deletion in sample R1291 was discovered by RT-PCR, excision of aberrant bands (see Fig. 2), and reamplification. Sequencing uncovered a 1320-bp in-frame deletion starting at base 266.

(35). We now have identified genetic alterations of *AXIN1* in MBs. The presence of *AXIN1* mutations suggests that different alterations in the *WNT* pathway may lead to an overactivation of *WNT* signaling in MBs. In MBs of the desmoplastic variant, mutations in the human homologue of the *Drosophila patched* gene have been observed by our group (43).

We found that three MB samples with *PTCH* (*patched*) mutations did not carry *AXIN1* mutations (data not shown). *AXIN1* mutations in our MB panel occurred in both subtypes of MBs classic and desmoplastic. A recent mutational analysis of *β-catenin* in MBs by our group (24) also revealed mutations in both variants, indicating that *WNT* overactivation can contribute to the pathogenesis of both subtypes of MBs. The frequency of *AXIN1* deletions in MBs was 12%. This figure resembles the frequency of genetic alterations of *AXIN1* in HCCs (26). It appears that *AXIN1* plays a role as tumor suppressor in various kinds of tumors.

In conclusion, our results indicate that *AXIN1* deletions may contribute to the pathogenesis of a subset of sporadic MBs via inappropriate activation of the *WNT* signaling pathway. Additional studies will be required to elucidate the function of mutated Axin variants in cerebellar cells and to determine whether other components of the *WNT* pathway, such as *conductin/AXIN2*, carry mutations in MBs.

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