[CANCER RESEARCH 58, 896–899, March 1, 1998]

Advances in Brief

Sporadic Medulloblastomas Contain Oncogenic β-Catenin Mutations[1]

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Abstract

The β-catenin, glycogen synthase kinase 3β (GSK-3β), and adenomatous polyposis coli (APC) gene products interact to form a network that influences the rate of cell proliferation. Medulloblastoma occurs as part of Turcot's syndrome, and patients with Turcot's who develop medulloblastomas have been shown to harbor germ-line APC mutations. Although APC mutations have been investigated and not identified in sporadic medulloblastomas, the status of the *β-catenin* and *GSK-3β* genes has not been evaluated in this tumor. Here we show that 3 of 67 medulloblastomas harbor β-catenin mutations, each of which converts a GSK-3β phosphorylation site from serine to cysteine. The β-catenin mutation seen in the tumors was not present in matched constitutional DNA in the two cases where matched DNA was available. A loss of heterozygosity analysis of 32 medulloblastomas with paired normal DNA samples was performed with four microsatellite markers flanking the *GSK-3β* locus; loss of heterozygosity with at least one marker was identified in 7 tumors. Sequencing of the remaining *GSK-3β* allele in these cases failed to identify any mutations. Taken together, these data suggest that activating mutations in the *β-catenin* gene may be involved in the development of a subset of medulloblastomas. The *GSK-3β* gene does not appear to be a target for inactivation in this tumor.

Introduction

Brain tumors are the most common solid tumors in children; PNETs[3] of the central nervous system account for 20% of these (1). PNETs most often occur 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, the nevoid basal cell carcinoma, or Gorlin's syndrome and Turcot's syndrome. Turcot's syndrome is composed of colorectal polyposis, basal cell carcinomas, sebaceous cysts, pigmented skin spots, and central nervous system tumors, either glioblastoma multiforme or PNET (2). Turcot's patients with PNETs have been shown to harbor germ-line APC mutations (3). Interestingly, data from two studies suggest that *APC* gene alterations do not occur in sporadic PNETs (3, 4).

APC functions to bind β-catenin, sequestering this protein and targeting it for degradation. The binding of β-catenin by APC requires phosphorylation of β-catenin by GSK-3β on three serines and one threonine residue, all of which are encoded in exon 3 of the *β-catenin* gene (5, 6). Hypophosphorylated β-catenin associates with members of the T-cell factor (Tcf) family. This complex functions as a transactivator for genes whose products promote cell proliferation (5, 7, 8).

Because the APC/β-catenin pathway is implicated in the development of PNETs by the occurrence of PNETs in Turcot's syndrome, we have investigated a series of spontaneous PNETs for oncogenic mutations in β-catenin and for inactivation of GSK-3β. Our results indicate that activating mutations of β-catenin may be important in the development of a subset of medulloblastomas. Inactivation of GSK-3β was not observed.

Materials and Methods

Sequencing. The primers used to amplify exon 3 of β-catenin from genomic tumor DNA were (5′ to 3′): forward, TGGAACCAGACAGAAAAGCG; and reverse, ACAGGACTTGGGAGGTATCC. Samples were amplified through 35 cycles in a DNA Thermal Cycler 480 (Perkin-Elmer, Norwalk, CT) at 95°C denaturation for 30 s, 55°C annealing for 30 s, and 72°C extension for 45 s. Each reaction consisted of 50 ng of DNA template, 10 pM forward and reverse primers, 0.2 mM each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl. PCR products were visualized on a 1.5% agarose gel with ethidium bromide. After pretreatment 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. For those samples that demonstrated mutations, the mutation was confirmed by RFLP using *XmnI* and *Hin*fI. To resolve the 54/61-bp doublet resulting from *Hin*fI digestion, the PCR amplification was done in the presence of [^{32}P-α]dCTP, and the products were run on a nondenaturing, 12% polyacrylamide gel.

Microsatellite Analysis. Normal and tumor DNAs were isolated and purified as described previously (9). 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 following amplification in the presence of [^{32}P-α]dCTP (Amersham Life Sciences). The PCR reactions for these determinations, as well as those used in the assessment of LOH on 3q, consisted of 10–100 ng of DNA, 8–10 pM forward and reverse primers (Research Genetics, Huntsville, AL), 0.8 μCi of [^{32}P-α]dCTP, and 0.2–0.35 units of Taq polymerase (Promega Corp., Madison, WI) or AmpliTAQ Gold (Perkin-Elmer, Foster City, CA) in 10–15 μl of aqueous buffer containing 200 μM of dG- dA- 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 amplified in a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, CT). Samples were run on 6% acrylamide sequencing gels containing 8 M urea and 15% formamide. Gels were dried and exposed to single-sided Kodak XAR film for 4–48 h. Autoradiographs were assessed for loss of heterozygosity by visual comparison of tumor and blood band intensities. Amplimers for this study were *D3S1303, D3S1769, D3S3515, D3S3683, DS31558, D3S1278, D3S3675,* and *D3S3513.*

BAC Sequencing. The complete cDNA sequence of GSK-3β was amplified by PCR. This cDNA probe was then used to screen chromosome 3q-specific BACs (Genome Systems, Inc., St. Louis, MO). Fourteen BAC clones bound the probe. We purified DNA from these BACs using the Mkb-100 BAC plasmid preparation kit (Genome Systems, Inc.) and sequenced these directly using primers within the GSK-3β cDNA. The PCR conditions were the same as for β-catenin sequencing, except that we used 50 PCR cycles as per Genome Systems, Inc. protocols. The primer pairs used for exon amplification are as follows (5′-3′): exon 1, GATTCGCGAAGAGAGTGATC and TGCTAACTTTCATGCTGCC; exon 2, ATTTAATTCCTTACAGTTCTCGTG and GGATATGTATTACGACAGCAAC; exon 3, GTTCAATATGATGAATAGCTGTTATCC and CAGTACACACTTTGTGTCAAACC; exon 4, CTCTAATTT-

Received 11/12/97; accepted 12/31/97.

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[1] This work was supported in part by the Pediatric Brain Tumor Foundation of the United States.

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[3] The abbreviations used are: PNET, primitive neuroectodermal tumors; APC, adenomatous polyposis coli; Tcf, T-cell factor; BAC, bacterial artificial chromosome; LOH, loss of heterozygosity.

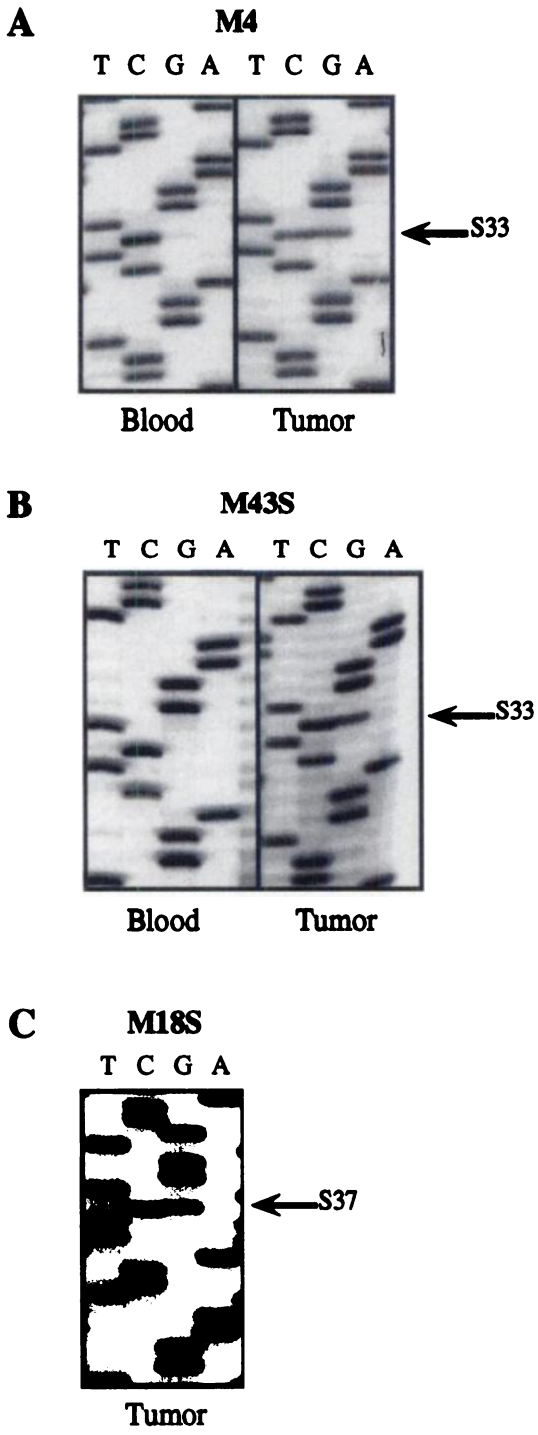

Fig. 1. Oncogenic mutation of one *β-catenin* allele in medulloblastoma. Direct sequencing of tumor (*A–C*) and matched normal (*A* and *B*) DNA reveals a C-to-G substitution in one *β-catenin* allele of the tumor but not the paired normal DNA. In the case of M18S, matched normal DNA was not available for comparison. In each case, 50 ng of genomic DNA was amplified by PCR and sequenced on both the sense and antisense strands three times with the same results.

GAATATTTAAAGACCAGTTTC and AAAGAGGCTCTCCTTGGTTCAA; exon 5, AATCTGTTACTTTATTGACTGCTTAAGG and GGAGAGGGACAGTAGCTTAC; exon 6, GGATAGCAGTCTAAGTTCTTTAAGG and GTAGTACTAAATTACCAAAATCAAGAAGC; exon 7, CTCAAGCTATGAAGTATTAATAAGGC and TCCAGTTCAAATATTCTGATTCAACC; exon 8, GGAAAAGATGGCAATAACTTGC and AGAAACCTGTTTTAGTTAACTACTG; exon 9, GTCATTATTCACAGGGTATTTGC and GTGAAGAGGCTAAGTGTTTGG; exon 10, GCATACAAAGTTAAGAGTGGACTC and GCCTCCCAAAGTGCTGGG; and exon 11, CAGCCTGCAGTTGAAACAGTGT and AGCTGGCTCGTCGGGACTGT.

All amplified products for sequencing are less than 300 bp. Standard PCR conditions (55°C annealing temperature and 1.5 mM $MgCl_2$) were used for all exons except exon 4, which requires a higher $MgCl_2$ concentration of 3.5 mM. Only the protein coding regions of exons 1 and 11 were sequenced.

Results

Because all four GSK-3β phosphorylation sites are located in exon 3 of β-catenin, exon 3 from 67 sporadic medulloblastomas was investigated by direct sequencing. Two tumors were found to have point mutations in codon 33 (tumors M4 and M43S), and one tumor (M18S) had a point mutation in codon 37. In each case, the alteration results in the substitution of a cysteine for a serine at a GSK-3β phosphorylation site (Fig. 1). Sequencing of exon 3 in the constitutional DNA for these patients failed to show a mutation, indicating that the mutation identified is tumor specific.

The mutations detected by direct sequencing were confirmed using a RFLP analysis. Exon 3 PCR amplimers from each tumor with a β-catenin mutation were digested with either *Hin*fI (for mutations at Ser^{33}) or *Xmn*I (for Ser^{37}). Loss of the *Hin*fI restriction site at Ser^{33} results in a 61-bp restriction fragment, rather than the 54-bp fragment found in normal DNA (Fig. 2). Loss of the *Xmn*I site at Ser^{37} results in a 150-bp fragment, rather than the 85- and 65-bp fragments seen in normal DNA. In all three tumors, the larger DNA fragment resulting from loss of the restriction site, along with the normal length product derived from the unaltered allele, is seen after digestion (Fig. 2). Constitutional DNA from two of the samples was available for analysis and revealed only the normal restriction pattern, indicating that the β-catenin mutations are tumor specific.

To determine the possible involvement of inactivating mutations in GSK-3β in sporadic medulloblastoma, 32 tumor and matched constitutional DNA samples were subjected to microsatellite LOH analysis. Seven tumors demonstrated LOH, and two tumors were not informative for every interpretable marker (Table 1 and Fig. 3). Of the seven tumors with LOH, two had loss on both sides of the *GSK-3β* locus, whereas five had loss on one side of the locus but retained heterozygosity on the other side. BAC clones containing the *GSK-3β* gene

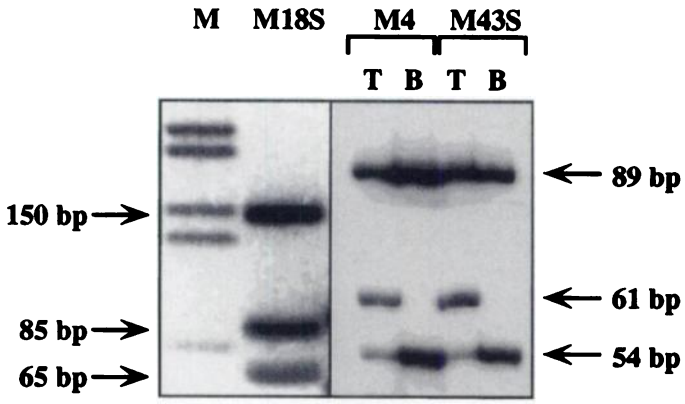

Fig. 2. Confirmation of mutation as demonstrated by RFLP. The 150-bp region of β-catenin harboring a mutation shown in Fig. 1 was reamplified from each tumor and digested with either *Xmn*I (tumor M18S) or *Hin*fI (tumors M4 and M43S). In the case of M18S, the digested sample was run on a 2.5% agarose gel with a DNA standard (*M*). The 150-bp fragment represents the complete PCR product that is uncut due to the presence of a mutation in the *Xmn*I recognition site in one allele. The remaining wild-type allele is cut into two fragments of 85 and 65 bp. For tumors M4 and M43S, it was necessary to label the products during the PCR reaction with [^{32}P]dCTP and run the digested sample on a 10% nondenaturing polyacrylamide gel to resolve the 54- and 61-bp bands resulting from mutation of one allele. Because the two *Hin*fI recognition sites in this region are only seven bases apart and only one is eliminated by the mutation, these seven bases are contained in a 61-bp fragment in the tumor (*T*) lanes. The 7-bp band runs off the gel in the normal (*B*, blood) lanes.

Table 1 *Summary of LOH analysis in sequenced tumors*

	Microsatellite[a]							
Tumor	D3S1278	D3S3683	D3S3675	D3S1769	D3S1558	D3S1303	D3S3515	D3S3513
M5	−	ND	−	ND	−	ND	ND	ND
M6	ND	NI	NI	ND	NI	NI	ND	ND
M7	−	−	NI	ND	+	−	−	−
M10	+	−	+	ND	−	+	+	ND
M12	+	NI	+	+	ND	NI	NI	NI
M14	−	−	NI	−	−	−	−	−
M16	ND	ND	−	−	+	+	−	ND
M17	+	−	+	−	+	+	+	+
M24	+	−	+	ND	ND	−	NI	+

[a] Microsatellite markers are ordered from centromeric (left) to telomeric (right) with the *GSK-3β* gene located between *D3S1769* and *D3S1558*. +, tumor maintenance of heterozygosity; −, tumor LOH; NI, noninformative; ND, not determined.

were obtained (Genome Systems, St. Louis, MO), and intron/exon boundaries were determined. Eleven exons containing the entire coding region of the gene were identified. Intronic primers flanking each exon were generated, and all GSK-3β exons from all nine tumors that may have lost one *GSK-3β* allele was amplified and sequenced. No mutations were identified in any tumor, although we did note that the published sequence for GSK-3β (GenBank L33801) may contain an error. All DNA samples sequenced in this study contain CTA (Lys) at codon 350, rather than CAT (His). The homologous rat and *Xenopus* proteins both have a lysine at this location (GenBank X53428 and U31862, respectively).

Discussion

In this study, we have examined the status in PNETs of two genes, *β-catenin* and *GSK-3β*, involved in the APC/β-catenin pathway. APC regulates the activity of β-catenin by binding the phosphorylated form of the protein; APC and phosphorylated β-catenin can be coimmunoprecipitated (10). The binding of β-catenin by APC prevents the association of β-catenin with members of the Tcf family and targets β-catenin for destruction (11). The binding of hypophosphorylated β-catenin to Tcf family members results in the formation of a transcriptional transactivator. Transcription by the β-catenin/Tcf complex is increased by Wnt-1 signaling (12–14). A similar developmental pathway has been defined in Drosophila; the Drosophila GSK-3β homologue, *shaggy*, regulates binding of the β-catenin homologue, *armadillo*, to APC; armadillo/Tcf transcription is increased by signaling of the Wnt-1 homologue, *wingless* (15).

Recently, activating mutations in β-catenin have been described in some colorectal carcinoma cell lines that do not have mutated APC (5, 8). The oncogenic mutations in each case eliminated a GSK-3β phosphorylation site in exon 3 of β-catenin. Because these mutations prevent binding of β-catenin to APC, they promote constitutive β-catenin/Tcf-mediated transcription. Cotransfection experiments using a β-catenin/Tcf reporter construct and activated β-catenin demonstrated that β-catenin mutations exerted a dominant effect, rendering β-catenin/Tcf transcription resistant to APC-mediated down-regulation (5). Transcriptional levels of reporter constructs in cells with wild-type APC transfected with mutant β-catenin were at least six times higher than in the same cells transfected with wild-type β-catenin. Gel-shift analysis also demonstrated that free β-catenin was constitutively bound to Tcf-4 in nuclear extracts from cells containing mutant β-catenin, even in the presence of wild-type APC. Taken together, these data indicate that mutations eliminating a GSK-3β phosphorylation site from β-catenin result in a protein that is no longer regulated by APC but that continues to function as a transactivator when complexed with Tcf.

A number of mechanisms can be suggested that will increase β-catenin/Tcf activity. One such mechanism involves inactivation of APC, as has been described in colorectal carcinoma (5, 7). Two reports have suggested that APC is not mutated in sporadic PNETs (3, 4). In the first of these, DNA from 91 medulloblastomas was examined for mutations in the portion of the *APC* gene where at least two-thirds of the mutations in Turcot's syndrome occur (4). No tumor had an APC mutation, although the region studied only accounts for 10% of the coding region of the gene. In the second study, DNA from 23 medulloblastomas was examined for deletions in the region of the *APC* gene using four microsatellite markers (4). No LOH was found in this region in any tumor; however, the closest marker used was 30–70 kb away from the *APC* gene.

A second way to increase free β-catenin levels is to mutate the *β-catenin* gene itself such that the gene product can no longer be bound by APC. Exon 3 of β-catenin contains four potential GSK-3β phosphorylation sites, three serines, and one threonine. Lack of phosphorylation at any one of these sites decreases sequestration of β-catenin by APC and increases β-catenin-Tcf transcriptional activity (6). Five β-catenin mutations have been described in colon carcinoma, and all alter one of these potential GSK-3β phosphorylation sites (5, 7). We show here that β-catenin mutations identical to those described in colon carcinoma occur in a subset of medulloblastomas.

A third way to increase levels of free β-catenin would involve the inactivation of GSK-3β, resulting in unphosphorylated β-catenin that is not bound by APC. In this scheme, *GSK-3β* functions as a tumor suppressor gene. In this report, 32 medulloblastomas were examined for alteration in the *GSK-3β* gene. Although we identified LOH in the

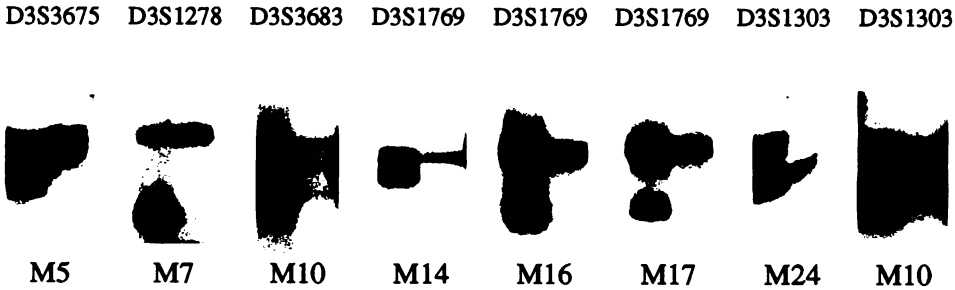

Fig. 3. Medulloblastoma LOH in the GSK-3β region as indicated by microsatellite analysis. Ten to 100-ng quantities of paired normal (*left*) and tumor (*right*) DNAs were subjected to 35 cycles of amplification in the presence of [^{32}P]dCTP and primers for the indicated loci. Reaction products were resolved by electrophoresis through a 6% sequencing gel that was subsequently dried and exposed to X-ray film. Results are a representative sample of LOH in each tumor (see Table 1). A marker demonstrating maintenance of heterozygosity in tumor M10 (*D3S1303*) is shown for comparison.

region of the *GSK-3β* locus in a subset of tumors, none of these tumors had an alteration in the remaining *GSK-3β* allele. Although it is possible that other mechanisms of *GSK-3β* inactivation, such as methylation or mutation outside of the coding region, may be involved in the inactivation of *GSK-3β*, these results suggest that this gene is not functioning as a tumor suppressor in PNET.

A subset of the tumors used in this report have been studied previously for mutations in the *Patched* (*PTC*) gene (16). None of the tumors with *PTC* mutations contained a β-catenin mutation. Whether these mutations can be linked to a common site in developmental pathways, the disruption of which leads to medulloblastoma, is unclear at this time.

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Cancer Res 1998;58:896-899.