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Analysis of APCL, a Brain-specific Adenomatous Polyposis Coli Homologue, for Mutations and Expression in Brain Tumors

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We recently identified a novel homologue of the adenomatous polyposis coli (*APC*) tumor suppressor gene, *APCL*, whose abundant and specific expression in the central nervous system indicated an important role in neuronal proliferation and differentiation. To investigate possible involvement of *APCL* alterations in brain tumors, we first analyzed the expression of *APCL* mRNA in seven glioma tissues by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis, and in nine glioma cell lines by northern blotting. *APCL* expression was reduced significantly in most of the glioma tissues and all nine cell lines in comparison with normal brain tissue. However, single-strand conformation polymorphism (SSCP) analysis and DNA sequencing of the entire coding region of *APCL* detected no mutations in any of the glioma cell lines, or in any of the 35 astrocytic gliomas and five medulloblastomas examined. Our results suggested that some epigenetic mechanism is responsible for the decrease in *APCL* expression in our panel of brain tumors.

Key words: APC homologue — APCL — Brain tumor

We recently isolated a novel gene, *APCL*, that showed significant sequence similarity to the adenomatous polyposis coli (*APC*) tumor suppressor gene. That it is in fact a functional homologue of APC was indicated by evidence that APCL protein was able to deplete the intracellular pool of β-catenin.[1] As the *APCL* gene is expressed abundantly and specifically in the central nervous system, it is presumed to play an important role in neuronal proliferation and differentiation.

Germline mutations of *APC* have been found in some patients with Turcot's syndrome, a disorder characterized by development of primary neuroepithelial tumors of the central nervous system (glioblastoma multiforme and medulloblastoma) as well as numerous adenomatous colorectal polyps.[2,3] No somatic mutations of *APC* have been reported in sporadic brain tumors, but somatic mutations of β-catenin, a protein whose cytoplasmic level is regulated by the *APC* gene product, have been documented in medulloblastomas.[4] If the APC-β-catenin pathway is involved in the development of some brain tumors, alterations of the brain-specific *APC* homologue, *APCL*, also may be associated with tumorigenesis in the brain. To address this possibility, we analyzed the levels of mRNA expression and the DNA sequence of *APCL* in brain tumors and glioma cell lines.

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MATERIALS AND METHODS

Tumor specimens and DNA extraction Samples of tissue from 35 astrocytic gliomas and five medulloblastomas obtained from surgical specimens were classified morphologically according to the World Health Organization (WHO) system. The glioma panel consisted of 12 grade-II astrocytomas, seven grade-III anaplastic astrocytomas, and 16 grade-IV glioblastomas multiforme. The brain tumors and matched normal tissues had been quickly frozen in liquid nitrogen and stored at −80°C until preparation of DNA. Genomic DNAs were extracted by standard methods.

Cell lines and RNA extraction Nine human glioma or astrocytoma cell lines (T98G, U373MG, U251MG, U87MG, A172, SW1783, SW1088, YKG-1, and DBTRG-05MG) were obtained from the American Type Culture Collection and a primary normal astrocyte cell line from Clontech (Palo Alto, CA). Total RNA was extracted using Trizol Reagent (Gibco BRL, Grand Island, NY) according to the manufacturer's protocol, and poly(A) fractions were separated by means of a Poly(A) Quick mRNA Isolation Kit (Stratagene, La Jolla, CA).

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis of glioma tissues and cell lines RNA was prepared with Trizol Reagent from seven of the glioma tissues, normal brain tissue, and the cell lines according to the manufacturer's protocol. After DNase treatment, each sample of total RNA was heated for 10 min at 70°C in the presence of random hexamers,

and cooled. Reverse transcription experiments were performed at 42°C for 1 h in a 20-μl volume containing 1× first-strand buffer, 1 m*M* dNTP, 10 m*M* dithiothreitol (DTT), 10 units of RNase inhibitor (TaKaRa, Otsu) and 200 units of Superscript II (Gibco BRL). Using forward primer 5′-GGAATCTGTCTGCACACAGCAC-3′ and reverse primer 5′-CTTGGAGTGCACCAGATTACGCAG-3′, we performed PCR for 30 cycles as follows: initial denaturation at 94°C for 3 min, then 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 72°C for 3 min, on the Gene Amp PCR system 9600 (Perkin Elmer-Cetus, Norwalk, CT). PCR products were separated by 2% agarose gel electrophoresis, transferred to membranes, and hybridized with a [^{32}P]ATP-labeled oligonucleotide internal primer (5′-GCACGCTCTGGAACCTGT-3′). After having been washed in 6× standard saline citrate (SSC), the membranes were exposed to X-ray film for 3 h at −80°C. RT-PCR products were normalized using the PCR product of glyceraldehyde 3-phosphate dehydrogenas (GAPDH) as a quantitative control.

Northern-blot analysis A 0.5-μg aliquot of poly(A) from each cell line, and poly(A) of normal brain tissue supplied by Clontech, were separated on 1% agarose gel containing formaldehyde and transferred to nylon membranes. After hybridization overnight at 42°C with [^{32}P]dCTP-labeled probes for APCL or β-actin, the blots were washed once in 2× SSC at room temperature and twice for 30 min at 55°C in 0.1× SSC containing 0.1% sodium dodecyl sulfate (SDS), then exposed to X-ray film for 3 days at −80°C.

PCR single-strand conformation polymorphism (SSCP) analysis The *APCL* gene consists of 13 small exons and one large exon at the 3′ end.[1] Thirty-one primer pairs (Table I) were designed to amplify the entire coding region of *APCL* from genomic DNA. The PCR-SSCP experiments were performed as follows: each 25-μl reaction mixture contained 10–50 ng of genomic DNA, 16.6

Table I. Primers for Amplification and Exon-intron Boundaries of *APCL*

Exon	Forward primers	Reverse primers	Size (bp)	Accession No.
1	5′-CCCAGACCATCAGCTGAACC-3′	5′-GAAGCCATCAGCCACTGCA-3′	279	AB022518
2	5′-AGCCCAGTGCAGTGGCTGAT-3′	5′-GGCGGAGGTCAGCGGCCCT-3′	217	AB022519
3	5′-AAGGCAGGCAGGGCCGCTGACCT-3′	5′-AGGCAGTCATGCTCCAGCC-3′	300	AB022519
4	5′-ATATCAAAATAAACACACACGGCG-3′	5′-CGCTTCCCGCTCCCTGAGA-3′	227	AB022520
5	5′-ACTCAGGGTGCGGGAAGCG-3′	5′-TGATTTGCCCACTGGCGCAG-3′	263	AB022520
6	5′-GTCATCCCAGGGAGAGGCG-3′	5′-TCTGCCTGGGCCGACACCA-3′	206	AB022521
7	5′-TCATCACGGGTGAGCAGACTGGT-3′	5′-TCCAGCCCAGACAGCTGCG-3′	210	AB022522
8	5′-TCCGACCGGGTTTCCAGGT-3′	5′-GAAGCCCTAGCTGCACGTTAA-3′	523	AB022523
9	5′-TCGGGAGTCACCTGGGACATT-3′	5′-CTTTAGCGGCCGAGGAGCCA-3′	229	AB022524
10	5′-TCTGGGCAAGGGAGTGAGG-3′	5′-CTGGAAGACTGGATGAGGAAA-3′	271	AB022525
11	5′-CACAGTCTCCCTTGTGTCCC-3′	5′-TCTGCAGAGAGTCGGACG-3′	243	AB022526
12	5′-CCCTAGTCCCACCACACACTTG-3′	5′-CTTCCAGTGACTGAAGAAGCAT-3′	201	AB022527
13	5′-TTCACTGAATGTGAGCGTGGGA-3′	5′-GACAGTGCCCAGCCCAGCCCTGT-3′	366	AB022528
14-1	5′-TTTACCTGCCACATGGTG-3′	5′-TTGGAGTGCACCAGATTACGCAG-3′	273	AB022529
14-2	5′-GCACGCTCTGGAACCTGT-3′	5′-CCCTGCTTCTCCAGGTGCT-3′	298	AB022529
14-3	5′-TGCCCAGCCTGTACGTGCG-3′	5′-CCCCTGCAGGAAGGGGCT-3′	296	AB022529
14-4	5′-TGCACCGTCATCCCTGGCT-3′	5′-CTCCTGTCCCGGGTCTCCA-3′	301	AB022529
14-5	5′-TCGACCAGCTGGTGGAGGA-3′	5′-CGGCGCGAAGCCAGTGCG-3′	301	AB022529
14-6	5′-TCTCCAACGACAGCCTCAAC-3′	5′-AGATTCCAGGCCCCGCGA-3′	300	AB022529
14-7	5′-TACCTATCAGCACGTGCCAC-3′	5′-TGCTGTCCAGCTCAGCCTC-3′	347	AB022529
14-8	5′-TGGTGACCTGGATGACAGTGA-3′	5′-AGCGGTGTCTCCTGCACGTA-3′	210	AB022529
14-9	5′-TAACCGAGGCCGGGGCCT-3′	5′-AAGCGCTTCACGTAGCTCTC-3′	327	AB022529
14-10	5′-AAGACGCCACCGCTGGCG-3′	5′-CAAAGTGGAAGCCGGCGCC-3′	313	AB022529
14-11	5′-GAGCACTACGTGCAGCAGG-3′	5′-CACCAACATGTAGACGGGCA-3′	279	AB022529
14-12	5′-GAGTGCCTGGGAGCCGC-3′	5′-GCCTGTCTGGCAGAGGTG-3′	281	AB022529
14-13	5′-AGCGGGCAGGCAAAGACCCA-3′	5′-CTGGGCGAGCTGTCGCGT-3′	597	AB022529
14-14	5′-CGGCCGTCCATCCACGAG-3′	5′-TGCAGCCACGTGACAATTGAAT-3′	407	AB022529
14-15	5′-TGTCAGTGGGATCCACCCTACA-3′	5′-TGGAGGAGGGGGTCTTGGCGA-3′	453	AB022529
14-16	5′-TACACCGGCCTGCCAAGACC-3′	5′-CGAGGAGCAGAGGAGGAAGACG-3′	595	AB022529
14-17	5′-TGCCCGCCGTCTTCCTCT-3′	5′-TCCCTGGTCTCCAGGCTCG-3′	541	AB022529
14-18	5′-CGAGCCTGGAGACCAGGGA-3′	5′-GGAGAGAACGTTCCAGAAGG-3′	327	AB022529

m*M* NH_4SO_4, 67 m*M* Tris-HCl (pH 8.8), 2 m*M* $MgCl_2$, 10 m*M* 2-mercaptoethanol, 6.7 μM ethylenediaminetetraacetic acid (EDTA), 5% dimethylsulfoxide (DMSO), 1.5 m*M* of each dNTP, 0.5 μM of each appropriate primer, and 1 unit of Ex-Taq polymerase (TaKaRa). PCR amplifications were performed in a thermocycler (Perkin Elmer-Cetus 9600) with denaturation at 96°C for 4 min, then amplification in 35 cycles of 96°C for 30 s, 58–62°C for 30 s, 72°C for 30 s, and final extension at 72°C for 3 min. The PCR products of exons 8 and 14-13, 14-14, 14-15, 14-16, and 14-17 were digested with appropriate restriction enzymes to achieve higher sensitivity of SSCP analysis. That is, the 523-bp PCR product for exon 8 was digested with *Sph*I into 291-bp and 234-bp fragments, the 597-bp product for exon 14-13 with *Not*I into 303 bp and 298 bp, the 407-bp product for exon 14-14 with *Cpo*I into 245 bp and 165 bp, the 453-bp product for exon 14-15 with *Sma*I into 176 bp and 277 bp, the 595-bp product for exon 14-16 with *BssH*II into 212-, 156-, and 229-bp fragments, and the 541-bp product for exon 14-17 with *Eco52*I into 180-, 127-, and 237-bp fragments.

A 3-μl aliquot of each PCR product was added to 6 μl of loading buffer (95% formamide, 10 m*M* EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol) and denatured for 10 min at 90°C. The samples were electrophoresed at 4°C in non-denaturing polyacrylamide gels (5% polyacrylamide, 0.5× Tris-HCl-boric acid-EDTA (TBE) and 5% glycerol). After electrophoresis, the gels were stained with SYBER Green II (FMC Bioproducts, Rockland, ME) and visualized with an FMBIO II Multi-View fluorescent image analyzer (TaKaRa).

Loss of heterozygosity (LOH) at the *APCL* locus DNAs from the 40 tumors and their corresponding normal tissues were amplified with two microsatellite markers, *D19S886* and *D19S878*, which flank *APCL* in the telomeric region of *19p13.3*. For *D19S886*, the forward primer was 5′-TGGATCTACACTCCGGC-3′ and the reverse primer was 5′-ATTTTACTGGCTGGCACTTG-3′; for *D19S878*, the forward primer was 5′-GCCTGGGCGACAGAGAAT-3′ and the reverse primer was 5′-GGTTGCCCGCAGAG-AGTG-3′. Annealing temperatures for the PCR reaction were 56°C for *D19S886* and 58°C for *D19S878*. One primer of each pair was labeled with [^{32}P]ATP, and PCR reactions were performed for 35 cycles in a 20-μl volume containing 10–50 ng of genomic DNA, 5 pmol of labeled primer, 5 pmol of unlabeled primer, and 50 nmol of each deoxynucleotide, with 1 unit of Ex-Taq polymerase (TaKaRa). PCR products were separated on 6% denaturing polyacrylamide gels and visualized by autoradiography.

RESULTS AND DISCUSSION

To investigate whether *APCL* plays a role in the etiology of brain tumors, we analyzed the expression of *APCL* mRNA in seven glioma specimens by semi-quantitative RT-PCR (Fig. 1) and in nine glioma cell lines by northern blotting and semi-quantitative RT-PCR (Fig. 2). *APCL* expression was below that of normal brain tissue and normal astrocytes in all of the glioma cell lines and in most of the glioma specimens examined. These results suggested that down-regulation of *APCL* may be associated with transformation and/or progression of brain tumors.

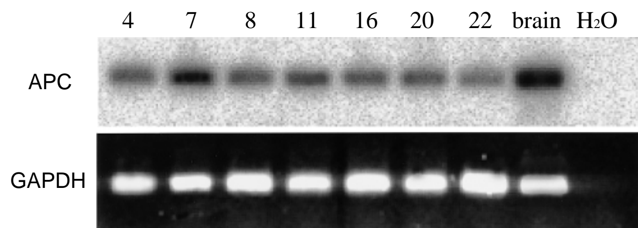

Fig. 1. Semi-quantitative RT-PCR analysis of seven glioma tissues. Expression of *APCL* in most of these gliomas was reduced in comparison to normal brain tissue. RT-PCR products were normalized using the PCR product of GAPDH as a quantitative control.

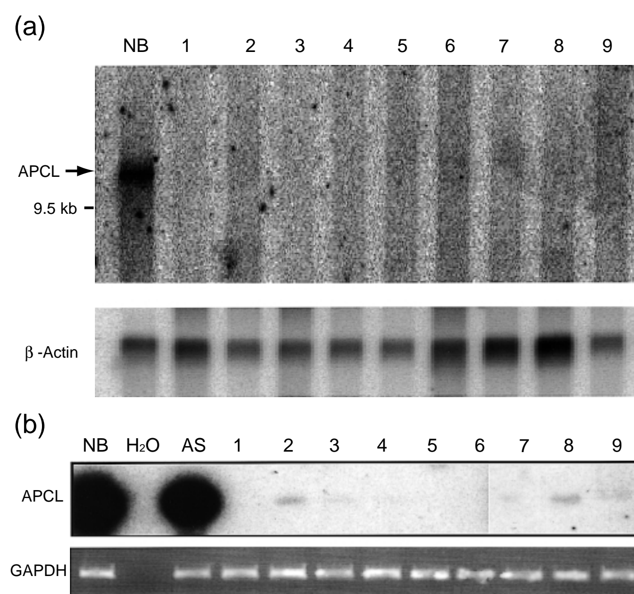

Fig. 2. (a) Northern-blot analysis of *APCL* in nine glioma cell lines and normal brain tissue. NB, normal brain. Lanes 1, T98G; 2, U373MG; 3, U251MG; 4, U87MG, 5, A172; 6, SW1783; 7, SW1088; 8, YKG-1; 9, DBTRG-05MG. (b) Semi-quantitative RT-PCR analysis of nine glioma cell lines. Expression of *APCL* in most of these gliomas was reduced in comparison to normal brain and primary normal astrocyte cell line. RT-PCR products were normalized using the PCR product of GAPDH as a quantitative control. AS, primary normal astrocyte cell line.

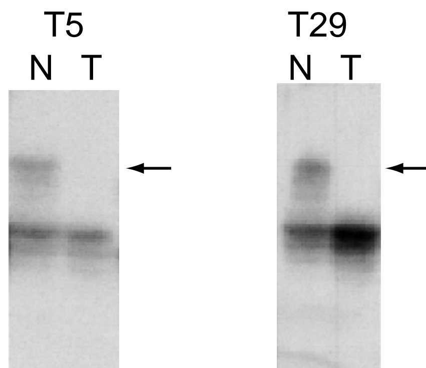

Fig. 3. LOH of a telomeric locus at *19p13.3*, *D19S878*. Only astrocytic gliomas T5 and T29 showed LOH for this microsatellite marker.

To investigate whether *APCL* might be inactivated by somatic mutations in brain tumors, we used SSCP analysis to screen DNAs isolated from 35 astrocytic gliomas, five medulloblastomas, and the nine glioma cell lines for mutations of the *APCL* gene. Since this gene consists of 13 small exons and one large exon at the 3′ end, we amplified each of the first 13 exons individually by PCR using the primer pairs shown in Table I; the last exon was divided into 18 overlapping segments for amplification. SSCP analysis revealed no structural mutations involving the coding elements of *APCL* in this panel of brain tumors. However, these results did not exclude the possibility of epigenetic inactivation of *APCL*, for example by methylation in the promoter region.[5, 6]

To determine whether the *APCL* locus, which is mapped at the telomere of *19p13.3*, was a target for LOH, we amplified two microsatellite loci adjacent to the *APCL* locus in DNAs from all 40 brain tumors of our panel. We found LOH at the *D19S878* locus in two of the 32 tumors that were informative (Fig. 3), but none of the 28 tumors informative at the *D19S886* locus had lost an allele. Ritland *et al.*[7] observed LOH of *19p* in 22 of 73 astrocytomas using other *19p* markers, and they suggested that an unknown tumor suppressor gene on *19p* could be involved in the pathogenesis of astrocytomas. However, their findings have not been confirmed by others[8] and our results also indicate that LOH of *19p13.3* is a rare event in astrocytic gliomas or medulloblastomas.

Several altered genes are known to be involved in the development or progression of brain tumors. For example, *p53* is mutated frequently in astrocytomas[9]; *RB* or *p16* alterations occur in the majority of glioblastomas[10]; and *PTEN/DMBT*[11–13] was isolated from chromosome *10q*, where frequent LOH was found in glioblastomas. Mutations or deletions in one of these genes have been found in about a quarter of glioblastomas multiforme examined.[11, 13]

The tumor suppressor gene *APC*, as well as β-catenin, which interacts with and is regulated by *APC*, can also be involved in development of brain tumors.[2–4] *APC* is highly expressed in the central nervous system although its function in the brain remains unclear. However, *in situ* hybridization experiments in rats have indicated that *APC* mRNA is expressed at high levels during development of the brain,[14] and APC protein is expressed in neurons,[15] astrocytes[16] and oligodendrocytes.[17]

Our results suggest that inactivation of the *APCL* gene by somatic mutation is not a common feature of brain tumors, nor is LOH of the *APCL* locus. Hence, we suspect that the inactivation of *APCL* expression we noted in our panel of brain tumors and cell lines reflects some other mechanism. Eventual clarification of the functions of the *APCL* gene in the central nervous system, and of the mechanism causing down-regulation of its expression in brain tumors, should help us understand the highly complicated pathway involving members of the *APC* family and β-catenin in the central nervous system.

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