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

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 mM dNTP, 10 mM dithiothreitol (DTT), 10 units of RNase inhibitor (TaKaRa, Otsu) and 200 units of Superscript II (Gibco BRL). Using forward primer 5′-GGAACTCTACGTGAGACC-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 1 min, and cooled. Reverse transcription experiments were performed as follows: each 25-µl reaction mixture contained 10 ng of genomic DNA, 16.6

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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 [32P]dCTP-labeled oligonucleotide internal primer (5′-GCAGCTTCGGAACCTG-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 dehydrogenases (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 [32P]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′-CCCCACCATCCACGTGACC-3′ | 5′-GAAGCCATCATCCCGACTGCA-3′ | 279 | AB022518 |
| 2 | 5′-AGCCTGTCATGCTGCCAT-3′ | 5′-GGCGGGATCAAGGAGGCGG-3′ | 217 | AB022519 |
| 3 | 5′-AAAGCAGCGACGGGCGCTGACCT-3′ | 5′-AGCGCTATGCTGCCTCAACCC-3′ | 300 | AB022519 |
| 4 | 5′-ATATCCAAATGACCAACACCGGCG-3′ | 5′-CGCTTTGCTGCTTCGCCAGA-3′ | 227 | AB022520 |
| 5 | 5′-ACTGACCGGTTCCGGAAGG-3′ | 5′-TGATTTGCCCTACCTGCGAG-3′ | 263 | AB022520 |
| 6 | 5′-CTGCTCCAGGGCCGACCCAC-3′ | 5′-TCTGCGGGCAGGAGCAAG-3′ | 206 | AB022521 |
| 7 | 5′-TACACTGCGTTCGAGCAGACTGTT-3′ | 5′-TCCAGCCGAGCACGTCG-3′ | 210 | AB022522 |
| 8 | 5′-TCCGACCGGTTTTCGAGT-3′ | 5′-GAAGCCCTAGCTGCTAGTAAA-3′ | 523 | AB022523 |
| 9 | 5′-TCCGGAATTCACCGACTGAC-3′ | 5′-CTTTAGGCGGCGGAGGCGCA-3′ | 229 | AB022524 |
| 10 | 5′-CAGCTGGCACCCTGGTATG-3′ | 5′-CTGCGAAGACTGAGTGGAA-3′ | 271 | AB022525 |
| 11 | 5′-CAGCTCCTCTGGTTCCTTTT-3′ | 5′-TCTGCGAAGACTGAGTGACC-3′ | 243 | AB022526 |
| 12 | 5′-CAGCTCGATCCACACCAACAT-3′ | 5′-CTTCGAGACGTCCAGAAAGGT-3′ | 201 | AB022527 |
| 13 | 5′-TCTCGAATGACCGGGTGAGA-3′ | 5′-TGAATTCAGCGGGGATG-3′ | 366 | AB022528 |
| 14 | 5′-TTAGACCTGGCACTGATAG-3′ | 5′-TTGAGGTCGACGAGTACG-3′ | 273 | AB022529 |
| 15 | 5′-GCGCCCGCCTGCTGTTCTG-3′ | 5′-CCCGCTTTCTCAGGTCG-3′ | 298 | AB022529 |
| 16 | 5′-TGCCGAGCTTGTGAGGAG-3′ | 5′-CCTGTCGATCGTGCTCA-3′ | 296 | AB022529 |
| 17 | 5′-GTCGACCGCAGCGCAC-3′ | 5′-CCCGCTTTCTCAGGTCG-3′ | 296 | AB022529 |
| 18 | 5′-GTCGACCGCAGCGCAC-3′ | 5′-CCCGCTTTCTCAGGTCG-3′ | 296 | AB022529 |
| 19 | 5′-GAGAAGCCCACCCGCTCGG-3′ | 5′-CCCGCTTTCTCAGGTCG-3′ | 296 | AB022529 |
| 20 | 5′-GAGAAGCCCACCCGCTCGG-3′ | 5′-CCCGCTTTCTCAGGTCG-3′ | 296 | AB022529 |
| 21 | 5′-GAGAAGCCCACCCGCTCGG-3′ | 5′-CCCGCTTTCTCAGGTCG-3′ | 296 | AB022529 |
| 22 | 5′-GAGAAGCCCACCCGCTCGG-3′ | 5′-CCCGCTTTCTCAGGTCG-3′ | 296 | AB022529 |
| 23 | 5′-GAGAAGCCCACCCGCTCGG-3′ | 5′-CCCGCTTTCTCAGGTCG-3′ | 296 | AB022529 |
| 24 | 5′-GAGAAGCCCACCCGCTCGG-3′ | 5′-CCCGCTTTCTCAGGTCG-3′ | 296 | AB022529 |
| 25 | 5′-GAGAAGCCCACCCGCTCGG-3′ | 5′-CCCGCTTTCTCAGGTCG-3′ | 296 | AB022529 |

**Analysis of APCL for Mutations and Expression in Brain Tumors**
mM NH₄SO₄, 67 mM Tris-HCl (pH 8.8), 2 mM MgCl₂, 10 mM 2-mercaptoethanol, 6.7 µM ethylenediaminetetraacetic acid (EDTA), 5% dimethylsulfoxide (DMSO), 1.5 mM 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 SphI into 291-bp and 234-bp fragments, the 597-bp product for exon 14-13 with NotI into 303 bp and 298 bp, the 407-bp product for exon 14-14 with CpoI into 245 bp and 165 bp, the 453-bp product for exon 14-15 with SmaI into 176 bp and 277 bp, the 595-bp product for exon 14-16 with BssHII into 212-, 156-, and 229-bp fragments, and the 541-bp product for exon 14-17 with Eco52I 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 mM 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).

**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.
Analysis of APCL for Mutations and Expression in Brain Tumors

To determine 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.

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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 others8) 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 astrocytomas9); RB or p16 alterations occur in the majority of glioblastomas10); and PTEN/DMBT11–13) 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) astrocytes16) 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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Fig. 3. LOH of a telomeric locus at 19p13.3, D19S878. Only astrocytic gliomas T5 and T29 showed LOH for this microsatellite marker.

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