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RAPID COMMUNICATION

Characterization of C4-2 as a Tumor-suppressor Gene in Human Brain Tumors

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Background: Brain tumors claimed the lives of 13,300 people in 1995. Our objective was to isolate and characterize unique tumor-suppressor genes from human brain tumors derived from patients in the United States.
Methods: Differential display-polymerase chain reaction was used to isolate tumor suppressor genes.
Results: Clone C4-2 was isolated and is expressed in normal adult human brain, but not in brain tissue from glioblastoma multiforme tumors. C4-2 has 66% homology to the previously isolated ARPP-16 (cAMP-regulated phosphoprotein of Mr = 16,000) based on limited sequencing. C4-2 is expressed at high levels in normal brain and is not expressed or expressed at low levels in several brain tumor cell lines. Expression of C4-2 was also either not expressed or expressed at low levels in meningioma, B-cell lymphoma, recurrent glioma, LNCAP (prostate tumor cell line), breast tumor, or prostate tumor tissue.
Conclusion: We conclude that C4-2 may function as a potential tumor-suppressor gene. J. Surg. Oncol. 64:102–108

KEY WORDS: brain neoplasms; differential display-polymerase chain reaction; polymerase chain reaction; phosphoproteins

INTRODUCTION

Brain tumors claimed the lives of 13,300 people in the United States in 1995 [1]. The number of deaths caused by such tumors is increasing each year in the United States, with more than 17,900 deaths estimated in 1996 from the same cause. The increased incidence of brain tumors is not only evident in the young, but also in adults [1]. It has been documented that a significant increase in mortality has occurred in adult primary malignant brain tumors between 1982 and 1996 [1]. Glioblastomas, astrocytomas, and meningiomas are the most common brain tumors that affect adults. Glioblastoma multiforme are high grade astrocytomas that grow very rapidly and contain malignant cells [2]. The molecular basis for the genesis of glioblastoma multiforme may involve systematic events at the chromosomal or gene expression level.

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These events may include inactivation of tumor-suppressor genes, activation of oncogenes, or specific translocations at the chromosomal level. Genetic changes at the chromosomal and gene expression levels are well documented for other brain tumors [3].

Tumor-suppressor genes play an important role in normal cell growth, differentiation, and progression through the cell cycle [3]. Mutations that cause change in the expression of tumor-suppressor genes may lead to cell transformation in vitro and tumor development in vivo [3]. It has been documented that loss of tumor-suppressor genes at chromosome 10, mutations in p53, or overexpression of epidermal growth factor receptor may be major events leading to glioblastoma multiforme [3–5]. The exact series of events involving tumor-suppressor genes that leads to initiation and progression of glioblastoma are not presently known. We sought to isolate potential tumor-suppressor genes that are overexpressed in normal brain tissue (NBT) as compared to glioblastoma multiforme tissue (GMT) tumors using the technique of differential display-polymerase chain reaction (DD-PCR) [6]. These genes might then serve as potential candidates for brain tumor therapy.

DD-PCR is a modified PCR technique first developed in 1992 [6]. This technique is more sensitive and reproducible than previously documented techniques of differential hybridization and subtractive library construction [6]. In the past, PCR has been utilized to isolate differentially expressed genes in a number of different applications [7–9]. Recently, DD-PCR has been used extensively to isolate genes that are differentially expressed in several different kinds of tumors [8,9]. The DD-PCR technique has been modified and improved recently to increase its specificity and efficiency [10,11]. Using the technique of DD-PCR on NBT and GBT tissue, we have isolated a number of genes that are overexpressed either in NBT or in GMT. We herein report our results with respect to C4-2, which is expressed in NBT but not brain tumor tissue.

MATERIALS AND METHODS

Human Tissues and Cell Lines

Brain and nonbrain tumors and normal tissues were procured from the tissue bank maintained by Pacific Northwest Cancer Foundation, Northwest Hospital (Seattle, WA) and from the Mayo Clinic (Rochester, MN). Brain tumor cell lines CCF-STTG1 (astrocytoma grade IV), SW 1783 (astrocytoma grade III), IMR-32 (neuroblastoma), D283 Med (medulloblastoma), Hs 683 (glioma), PFSK-1 (primitive neuroectodermal tumor), and DBTRG-05MG (glioblastoma multiforme) cell lines were purchased from ATCC (American Type Culture Collection, Rockville, MD). Fetal normal human astrocytes (FNHA) were purchased from Clonetics (San Diego, CA). All the cell lines were cultured under the conditions recommended by ATCC or Clonetics.

Differential Display-Polymerase Chain Reaction

We isolated and cloned differentially expressed genes from NBT and GMT using a protocol described previously [11]. NBT and GMT were obtained from the same regions of the brain. Total RNA was isolated using the GITC/$CsCl_2$ protocol described previously by Shambrook et al. [12]. Five μg of total RNA was treated with Dnase I (Amersham, Arlington Heights, IL) (2 u/μl) for 30 minutes at 37°C. RNA was then extracted and precipitated using 3 M sodium acetate. First-strand cDNA synthesis was carried out using the advantage 1st strand cDNA synthesis kit from Clontech (San Diego, CA) using BT3-2 primer (5′T(T)18NG3′). Approximately 125 ng of 1st strand cDNA synthesis product was used for carrying out the PCR reactions. The DD-PCR reaction was carried out using (γ P^{32}) end labelled BT3-2 primer and BT8 (5′NTACTGATCCATGACA3′), BT10 (5′NGCTGCTCTCATACT3′) or BT 12 (5′NTGATCTAAGGCACATA3′) primers using cDNA from NBT or GMT tissue in duplicate using the conditions described previously [11]. PCR products were then electrophoresed on a 6% sequencing gel. The bands that showed differential expression were cut out and DNA was eluted. PCR was then carried out as described for DD-PCR using appropriate primers. The PCR product was then cloned into the PCRII vector from Invitrogen (San Diego, CA). Positive clones were screened by PCR and sequenced using the Sequenase version 2.0 sequencing kit from Amersham/U.S. Biochemicals.

Gene Specific Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

To confirm the differential expression of C4-2, we used the RT-PCR technique described recently [13]. In brief, 5 μg of total RNA was treated with DNase I and first-strand synthesis was carried out using the same conditions as described previously. First-strand cDNA was used as template to carry out PCR using primers BT-63 (5′TGATCCATGACATTCAGTG3′) and BT-64 (5′GGAATGCAGAGTATTGAAG3′) for C4-2, BT41 (5′CTCAGTGTTAACGGATAAT3′) and BT42 (5′TGTTGAGAAGAGTACATCTT3′) for D2-2. For comparison, a housekeeping gene D1-2, which is expressed in both NBT and GMT, was used as an internal control. PCR for D1-2 was carried out using BT-59 (5′CGGAGCAATATGAAATGATCT3′) and BT-60 (5′GCAAATACAGCTCCTATTG3′). RT-PCR was performed using Gene Amp PCR kit from Perkin Elmer (Branchburg, NJ) under the following conditions: 4 μl of dNTP mix, 2 μl (100 ng/μl) each of D1-2 or D2-2 specific primers, 4 μl of 25 mM $MgCl_2$, 125 ng of cDNA template, and 5 units of Amplitaq (Perkin Elmer) DNA

polymerase. PCR conditions were performed as follows: 94°C, 50°C, and 72°C for 1 minute each for 35 cycles. PCR product was then run on a 2% agarose gel. DNA was transferred on to MSI magnacharge membrane using the standard Southern Blotting conditions as described by Shambrook et al. [12]. The membrane was prehybridized for 12 hours in a prehybridization buffer [12]. Hybridization was at 42°C using C4-2, D1-2, or D2-2 specific probes. C4-2, D2-2, and D1-2 specific probes were prepared by multiprime labelling (Amersham Megaprime Labelling Kit). (5′TTGTGCAAATACGATATGTTGCCTTAGGCATATCTTTTGT3′), D2-2 (5′CCAAACTGGACATCAAGGAATTGCTACACAGAAGAACCACCATCCAGGATAGAA3′) or D1-2 (5′TAGGCCTGACTGGCATTGTATTAGCAAACTCATCACTAGA3′) specific primers. These primers are internal to the primers used for PCR, and they do not carry any of the primer sequences used in the PCR. Primer sequences were checked for homologous sequences using the DNA BLAST program of the National Center for Biotechnology Information (NCBI) (NIH, Bethesda, MD) prior to use. Quantitation of the signal on Southern blot was carried out using ImageQuaNT™ program of the Molecular Dynamics (Sunnyvale, CA) Phosphor Imager. This protocol was also used to quantitate expression of C4-2, D2-2 or D1-2 in brain tumor cell lines, FNHA and tumor tissues.

Cloning and Sequencing of C4-2

DD-PCR product for clone C4-2 was 250 bases long. It was cloned into the PCRII vector from Invitrogen. Clone C4-2 was sequenced using Sequenase 2.0 kit from Amersham. Using the 250 bp fragment, we screened a human brain library and isolated a 2.0Kb insert. This clone was partially sequenced to confirm its identity. A sequence search for the CD-4 clone was carried out using the BLAST database of the NCBI (NIH).

Northern Blot Analysis

To investigate the expression of C4-2 in brain and normal tissues, we used Multiple Tissue Blots (MTB) from Clontech. These blots have 2 μg of pure polyA pure mRNA blotted onto them. MTBs were prehybridized in expresshybridization solution (Clonetech, Palo Alto, CA) for 3–4 hours. Hybridization was done with multiprime labelled 2.0 Kb C4-2 probe. After autoradiographic exposure, the C4-2 probe was washed from the blot and then hybridized with human β actin probe. Quantitation of expression of D2-2 and β actin was done by ImageQuaNT™ program of the Molecular Dynamics Phosphor Imager.

Quantitation of Northern and Southern Blots

Quantitation of Northern and Southern blots was performed using the ImageQuaNT™ volume quantitation program from the Molecular Dynamics Phosphor Imager. Volume quantitation calculates the volume under the surface created by a 3-D plot of pixel locations and pixel values. We quantitated the volume (the integrated intensity of all the pixels in the spot excluding the background) of C4-2 bands in Northern or Southern blots. These pixel values were then normalized with pixel values in the bands of housekeeping genes (D1-2, β actin) and are referred to as relative expression in the figures. The subjective terms of low, medium, and high relative expression are based on C4-2 expression in normal brain as high, or, in brain tumor tissue, as low.

The D1-2 gene was found to be consistently expressed in a wide variety of nonneoplastic and neoplastic tissues and cell lines (Figs. 2, 4, 5). Thus D1-2 served as an internal control for gel loading.

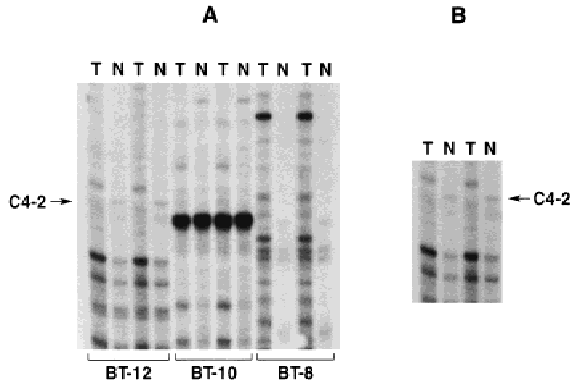

Fig. 1. Identification of differentially expressed genes from glioblastoma multiforme tumor tissue and normal brain tissue using differential display-polymerase chain reaction (DD-PCR) showing that the gene C4-2 is expressed in normal adult brain tissue but not in glioblastoma multiforme tissue. **A** is an autoradiogram of DD-PCR using the 5′ specific primers as indicated at the bottom of the panel. Note gene C4-2 expression (arrow) in the normal (N) lane but not in the tumor (T) lane. **B** shows details of the C4-2 region from A.

RESULTS

Isolation of Clone C4-2 Using Technique of Modified DD-PCR

We used the modified technique of DD-PCR to isolate genes that are differentially expressed either in NBT or GMT. Using one 3′ primer and three 5′ primers, we performed the technique of DD-PCR on GMT and NBT (Fig. 1). We isolated 18 bands that showed differential expression either in GMT or NBT. Fourteen of these bands were expressed at higher levels in GMT and four in NBT. All of these bands were isolated, DNA eluted, reamplified, and cloned into the PCR II vector. Sequence analysis of these clones indicates that the majority of these genes had no homology to known sequences in the NCBI database (NIH). In this report we describe the characterization of clone C4-2, which appears to be unique because it shows a higher expression in NBT than in GMT (Fig. 1A,B). In the past, Northern Blot analysis

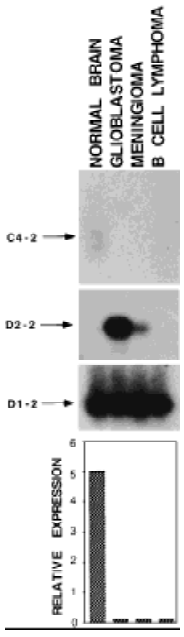

Fig. 2. Clone C4-2 is differentially expressed at high levels in normal brain, but not in glioblastoma or meningioma primary brain tumor tissue or B-cell lymphoma. Shown is an autoradiogram of a reverse transcription-polymerase chain reaction (RT-PCR) for C4-2 (upper panel), D2-2 (middle panel), which is a gene overexpressed in glioblastoma [16]. D1-2 (a housekeeping gene) is equally expressed in a wide variety of tissues (lower panel) and is used as an internal control for gel loading. The bar graph represents the relative expression of C4-2 in these tissues after correction for gel loading based on D1-2 expression.

and in situ hybridization were used to confirm differential expression of genes isolated by DD-PCR. Because of limited brain tissue availability, we used the technique of RT-PCR to confirm the expression of DD-PCR clones. This technique was used recently to demonstrate differential expression of genes isolated by DD-PCR [13]. We modified this technique and made it more specific than previously documented. Using this gene-specific RT-PCR technique, we demonstrated that C4-2 is expressed 50 times higher in NBT than in GMT (Fig. 2). D2-2, a gene not expressed in NBT but overexpressed in GMT, was not detected in a B-cell lymphoma, or meningioma tumor samples. This result demonstrated that C4-2 is differentially expressed in NBT and GMT. As a control, we demonstrate that the expression of a tumor-associated gene (D2-2) is high in GMT compared to NBT (Fig. 2) [16]. D1-2, a control gene that was isolated from a similar batch of DD-PCR, is expressed consistently at the same levels in normal and tumor samples.

Sequence Analysis of Clone C4-2

Clone C4-2 isolated by DD-PCR, was 250 base pairs in length and had a long polyA tail. This indicates that the C4-2 sequence is at the 3′ end of the gene. Clone C4-2 sequence homology analysis indicated that it had a strong homology to ARPP-16 (Fig. 3). Northern blot analysis of C4-2 on brain tissue showed that there were two natural transcripts of 1.6 and 6.0Kb. ARPP-16 is a phosphoprotein that is expressed in cells with increased cAMP levels (14,15). ARPP-16 has a homology to another protein of a different molecular weight and is referred to as ARPP-19 [14,15]. Expression of ARPP-16 and 19 involves a combination of alternative promoters and splicing rather than the conventional differential splicing of an identical primary transcript. Both of these proteins are expressed in brain and are enriched in the basal ganglia [14,15]. The exact function of these proteins is not known, but they are thought to mediate the action of neurotransmitters or hormones that raise cAMP levels in cells [14,15]. Using a 250 bp C4-2 fragment as a probe, we screened a human brain library (Stratagene, La Jolla, CA) and isolated a clone with a 2.0 Kb insert. Sequence analysis showed that this clone may partially correspond to the 6.0 Kb natural transcript.

Expression of C4-2 in Tumor Tissues

Since C4-2 was overexpressed in NBT, of which three were analyzed, as compared to the three GMT which we analyzed, we were interested in studying its expression in other tumor tissues. As shown in Figure 4, C4-2 was expressed at very low levels in glioblastoma, meningiomas, colon cancer metastatic to the brain, recurrent glioma, B-cell lymphoma, breast tumors, prostate tumors, and a prostate tumor cell line (LNCAP). Clone D2-2 was used as a control overexpressed gene from brain tumors to illustrate the integrity of the experiments [16]. This experiment confirmed that low expression of C4-2 was not only confined to brain tumor tissues but also to other tumor types.

Expression of C4-2 in Brain Tumor Cell Lines and Fetal Normal Human Astrocytes (FNHA)

We investigated the expression of C4-2 in cell lines derived from different human brain tumors and FNHA. As shown in Figure 5, C4-2 is expressed at very low levels in cell lines derived from glioblastoma, grade IV

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ARPP-16     TAGCAGTACC  CATGACATTC  AGTGGCGTTG  TGCAAATATG  GTATGTTGC-
C4-2        GTACTG-ATC  CATGACATTC  AGTGGCCTTG  TGCAAATACG  ATATGTTGCC

ARPP-16     TTAGGCATAT  CTTTTGTCCT  AGGCAGAACG  TTTCATTTTG  ACTTTTAT-G
C4-2        TTAGGCATAT  CTTTTGTCCT  ATGCCAGAAC  CTTTATTTTG  ATTTTTTTCG

ARPP16      AAAATTACTG  TTCATATAGT  TTATATAAAC  TTTTTTAATG  --TAGAAACT
C4-2        AAAGTTGCAA  TTCATGTAAT  TTATATAAAC  TTTTTAAATA  GCTAGAAACT

ARPP-16     TTTTACTTGC  ACAGTCAATT  TAGGGGACAC  -TAGAATAAA  AGACTTTGCC
C4-2        TTTTACTTCC  ACACTCAGTT  TTGGAGACCC  CTAGAATAAA  AGGCTTCAAT

ARPP-16     TCTTGTGGCC  CCTCCCTTCT  TTTTTTTTGC  TTCTT
C4-2        ACTC-TG---  CATTCCCGAA  AAAAAAAAAA  AAAAA
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Fig. 3. Sequence homology of clone C4-2 with ARPP-16. The sequence of clone C4-2 was matched with ARPP-16 using the DNA BLAST database of the National Center for Biotechnology Information. The shaded boxes represent areas of strong homology. C4-2 has a 66.8% identity to ARPP-16, a cyclic AMP regulated phosphoprotein.

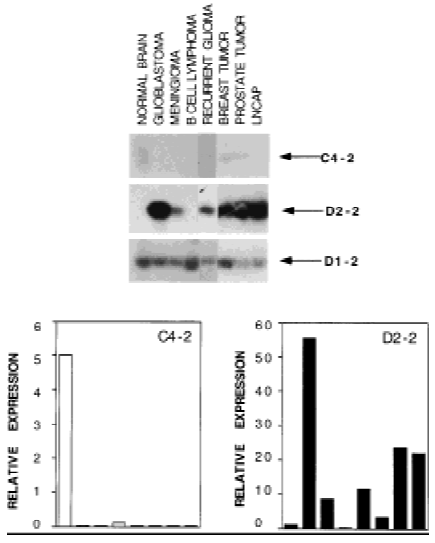

Fig. 4. C4-2 is not expressed or minimally expressed in a variety of tumor tissues compared to another gene, D2-2, which is overexpressed in tumor tissue. Total RNA was isolated from several normal and tumor tissues. Reverse transcription-polymerase chain reaction (RT-PCR) for C4-2, D2-2, and D1-2 was performed as described in detail in Materials and Methods. The upper panel shows the suppression of C4-2 in tumor tissues, the middle panel shows the overexpression of D2-2 in tumor tissues, and the lower panel shows D1-2 expression in tumors used as internal control for gel loading. The bar graphs represent the relative expression of C4-2 (left) and D2-2 (right) in these tissues after correction for gel loading based on D1-2 expression.

astrocytomas, glioma, neuroectodermal and medulloblastoma tumors, and FNHA. These cell lines did show high levels of D2-2 expression, a novel tumor associated gene [16]. This experiment demonstrated that C4-2 was expressed at very low levels in the majority of brain tumor cell lines.

Expression of C4-2 in Different Regions of Normal Human Brain

To understand the function of C4-2 in brain, it is important to investigate its expression in different regions of the brain. Eight different regions of the normal human brain were studied for this purpose. As shown in Figure 6, C4-2 was expressed at high levels in the cerebellum, occipital lobe, temporal lobe, frontal lobe, putamen, and cerebral cortex. A low level of C4-2 was observed in the medulla and spinal cord. The reason for such a selective distribution of expression is not known at present.

Expression of C4-2 in Normal Human Tissue

To study the expression of C4-2 in different tissues, we performed Northern blot analysis using C4-2 as a probe. As shown in Figure 7, C4-2 was expressed at very high levels in the normal brain, at a moderate levels in the placenta, liver, kidney, and pancreas, and at low levels in a number of tissues such as heart, lung, skeletal muscle, spleen, thymus, prostate, testis, ovary, small in-

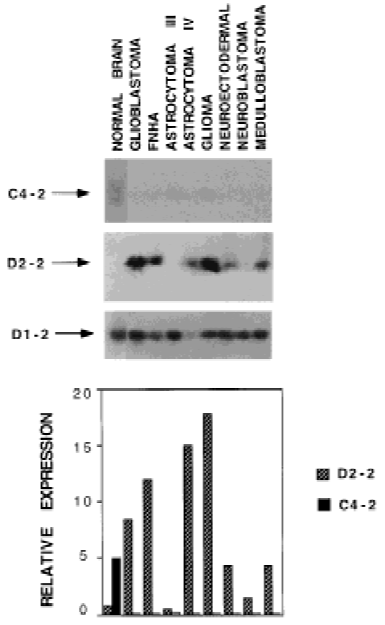

Fig. 5. C4-2 is expressed in normal adult brain but only to a minimal extent in adult brain tumor cell lines and in normal fetal human astrocytes. Human brain tumor cell lines (glioblastoma, astrocytoma III, astrocytoma V, glioma, medulloblastoma, neuroectodermal tumor, neuroblastoma) and normal fetal human astrocytes were grown to 80% confluency. Reverse transcription-polymerase chain reaction and Southern blot analysis was performed. Shown is an autoradiogram of a Southern blot of C4-2 expression (upper panel), D2-2 expression (middle panel), and D1-2 expression (lower panel), in various brain tumor cell lines and in normal fetal human astrocytes. The bar graph represents the relative expression of D2-2 in the respective cell lines after correction gel loading based on D1-2 expression.

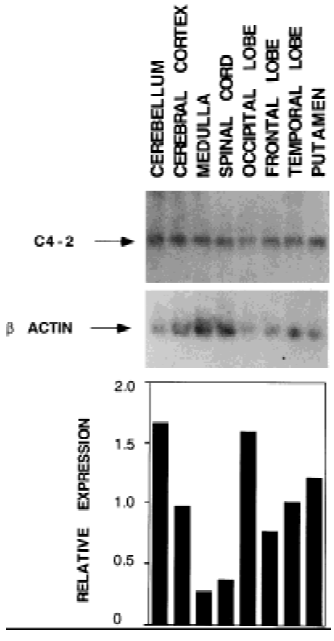

Fig. 6. C4-2 is widely expressed in different regions of normal adult brain. Shown is an autoradiogram of a Northern blot of C4-2 expression (upper panel) and of β actin expression (middle panel), which serves as an internal control for gel loading in different regions of normal adult brain. The bar graph is the relative expression of C4-2 after correction for gel loading based on β actin expression.

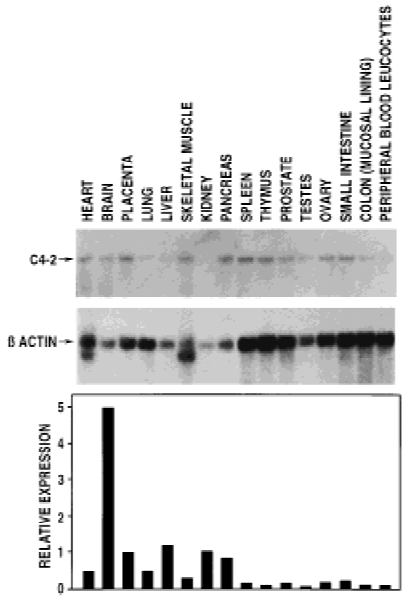

Fig. 7. Expression of C4-2 in normal human tissues. Shown is an autoradiogram of a Northern blot of C4-2 expression (upper panel), and of β actin expression (middle panel), which serves as an internal control for gel loading in various normal human tissues. The bar graph is the relative expression of C4-2 after correction for gel loading based on β actin expression. C4-2 expression in normal tissues is highest in the normal brain, the functional significance of which is not presently known.

testine, colon (mucosal lining), and peripheral blood leukocytes. High C4-2 expression in normal brain tissue suggests that it has specific functions in the brain as previously postulated, although the reason for selective distribution in placenta, liver, kidney, and pancreas is not known [14,15].

DISCUSSION

DD-PCR has wide application in the isolation of genes that are differentially expressed [7–10]. It has been used in the past not only to isolate genes that are overexpressed in tumors, but also suppressor genes that are overexpressed in normal tissues. Recently this technique was used to isolate novel cDNAs that is overexpressed in brain vor potential brain tumors [18,19]. In this study, we have used the modified technique of DD-PCR and have demonstrated the isolation and characterization of a gene designated C4-2, a potential tumor-suppressor gene.

Sequence analysis demonstrated that C4-2 has a 66.8% homology to a previously cloned gene, ARPP-16, a phosphoprotein that is thought to be expressed at high levels in cells with elevated levels of cAMP [14,15]. Northern blot analysis of ARPP-16 showed that there are two major transcripts of 6.0 or 1.9 kb. We screened a human brain library (Stratagene) to isolate the fulllength gene for C4-2. One positive clone with a 2.0 Kb insert size was isolated. Sequence analysis indicated that this clone may have a homology to the 6.0 Kb natural transcript. It is not known at present if the 6.0 Kb or 1.9 Kb transcripts encode for a similar size protein. At present it is not known if C4-2 is indeed totally identical to ARPP-16. Sequence analysis of the C4-2, 2.0 Kb fragment is currently being performed to address this question. On the basis of our current results, we feel that C4-2 may well function as a tumor-suppressor gene.

Clone C4-2 has several characteristics consistent with a brain tumor-suppressor function. C4-2 is expressed at high levels in normal brain and is essentially not detected in GMT or several cell lines derived from other brain tumor types. C4-2 has low expression, not only in the NBT tissue from which the gene was identified, but also in the majority of other brain and nonbrain tumor tissues and cell lines. Currently, we are performing experiments to express a fulllength C4-2 gene in brain tumor cell lines. The effect of C4-2 overexpression will be studied by assessing the change in cellular phenotype including thymidine incorporation and soft agar colony formation, and tumor formation in athymic nude mice.

In conclusion, C4-2 is a gene expressed in normal brain but not in brain tumors. Therefore, a putative tumor-suppression function is suggested and will be further investigated.

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