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RESEARCH ARTICLE

# Homozygous Deletions of the *CDKN2C*/p18$^{INK4C}$ Gene on the Short Arm of Chromosome 1 in Anaplastic Oligodendrogliomas

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**Allelic deletions of the short arm of chromosome 1 are common in oligodendrogliomas and have been correlated with chemosensitivity and better prognosis in patients with high-grade oligodendrogliomas. In these tumors, 1p loss is also inversely related to deletions of the *CDKN2A* gene on 9p, which encodes the key cell cycle regulatory molecule p16$^{INK4A}$. Because the *CDKN2C* gene, which encodes the homologous p18$^{INK4C}$ cell cycle regulatory protein, maps to chromosomal band 1p32, *CDKN2C* is an attractive candidate for the oligodendroglioma suppressor gene on chromosome 1. To evaluate this possibility, we studied 39 high-grade oligodendrogliomas for homozygous deletions and point mutations of the *CDKN2C* gene, as well as for allelic loss of 1p. Although no mutations were detected in the *CDKN2C* coding region, two tumors had homozygous deletions that involved *CDKN2C*. Interestingly, these cases did not have *CDKN2A* gene deletions. Coupled with the recent report of rare point mutations of *CDKN2C* in oligodendrogliomas, these findings suggest that *CDKN2C* inactivation may be oncogenic in a small percentage of human oligodendrogliomas.**

## Introduction

Among human malignant gliomas, oligodendrogliomas have attracted much attention over the past few years because of their unique clinical, pathological and genetic features (17, 20). For instance, unlike other glioma types, oligodendrogliomas are often chemosensitive tumors, with approximately two-thirds of high-grade cases responding to procarbazine, CCNU and vincristine (PCV) chemotherapy (3). In addition, allelic losses of particular chromosomes are powerful predictors of such chemosensitivity and of longer survival in patients with high-grade oligodendrogliomas (4). For these reasons, there has been a concerted effort to identify the genetic changes that underlie the formation and progression of oligodendroglial tumors, and to understand the clinical significance of these genetic alterations.

The cardinal molecular genetic alterations in oligodendrogliomas are allelic losses of chromosomal arms 1p and 19q, which occur in 50-70% of tumors (1, 23, 24, 30). These two genetic events are tightly linked with one another, so that nearly all tumors with 1p loss also suffer 19q loss (14, 23). Significantly, those tumors with combined loss of the short arm of chromosome 1 and the long arm of chromosome 19 are remarkably chemosensitive lesions and have overall survivals that far exceed those of histologically similar tumors lacking these genetic changes (4).

While the tumor suppressor locus on 19q has been relatively well-defined (24, 30), mapping of the oligodendroglioma gene on 1p remains fairly crude. At least three candidate regions on the short arm of chromosome 1 have been suggested: 1p36, 1p34 and 1p32 (1, 22, 27, 28). Allelic losses at 1p36.3 occur in approximately 60% of oligodendrogliomas (25), and one fluorescent in situ hybridization (FISH) study has suggested even higher percentages (9). On the other hand, most oligodendroglial tumors lose the majority of the short arm of chromosome 1, with few tumors displaying losses confined to the distal 1p36 region (27). Thus, more proximal regions such as 1p32 remain viable candidates for harboring an oligodendroglioma gene and putative tumor suppressor genes at 1p32 have not yet been screened in oligodendrogliomas.

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One particularly attractive candidate gene at 1p32 is *CDKN2C*, which encodes p18$^{INK4C}$ (2) and which is mutated in several human tumor types and tumor cell lines (5, 11, 21). *CDKN2C*/p18$^{INK4C}$ is a cyclin-dependent kinase (CDK) inhibitor that is structurally and functionally homologous to the cell cycle regulators *CDKN2A*/p16$^{INK4A}$, *CDKN2B*/p15$^{INK4B}$, and *CDKN2D*/p19$^{INK4D}$ (8, 10, 12, 18). *CDKN2A* is homozygously deleted in many human cancers, including in approximately 20% of high-grade oligodendrogliomas (4). Interestingly, loss of the short arm of chromosome 1 appears inversely related to *CDKN2A* deletions in high-grade oligodendrogliomas (4), raising the possibility that the chromosome 1 glioma gene functions in a similar manner to *CDKN2A*. For these reasons, *CDKN2C*, as a CDK inhibitor that maps to 1p32, is a strong candidate for an oligodendroglioma suppressor gene. We therefore evaluated *CDKN2C* as a candidate tumor suppressor gene at 1p32 in a series of high-grade oligodendrogliomas.

## Materials and Methods

***DNA samples.*** Thirty-nine high-grade oligodendrogliomas and 3 glioblastomas (cases 1648, 1654, 1670), along with corresponding peripheral blood samples, were derived from patients treated at the London Regional Cancer Centre, Ontario, Canada, or at Massachusetts General Hospital, Boston, MA, USA. All tumors were classified according to World Health Organization (WHO) criteria (13). Histological, clinical and genetic data on the oligodendroglial tumors have been published elsewhere (4). The percentage of 1p LOH among these oligodendrogliomas was 67% (24 of 36 informative cases) (4). DNA was extracted from blood leukocytes, from microdissected, formalin-fixed, paraffin-embedded sections of the 39 oligodendrogliomas, and from fresh, frozen tissue on case 1796 and the glioblastoma cases, according to published methods (16).

***Single strand conformation polymorphism (SSCP) analysis.*** *CDKN2C* is comprised of 3 exons (2). Exon 1 does not include coding sequence and was therefore not screened for mutations. Oligonucleotide primer pairs used to amplify *CDKN2C* exon 2 were 5′TGATCGTCAGGACCCTAAAG3′ and 5′CTGCAGCGCAGTCCTTCC3′ (6), generating a 154 bp product after 30 cycles with an annealing temperature of 55°C. PCR was performed in 10 µl - reaction volumes, containing 1 µl (50 ng/µl) template DNA, 5.45 µl $H_2O$, 1 µl 10x buffer containing 2 mM $MgCl_2$, 1 µl 2mM dNTPs , 2 x 0.5 µl *CDKN2C* primers with a concentration of 20 pmol each, 0.5 µl (5 µCi) $^{32}$PαdCTP and 0.05 µl (0.25 U) Taq polymerase. For *CDKN2C* exon 3, the sequence was divided into three amplicons (3a-1, 3a-2, 3b); new intraexonic primers were designed and flanking intronic primers were used according to previous studies (11). The sizes of the amplicons from 5′ to 3′ for 3a-1, 3a-2 and 3b were 157 bp, 169 bp and 181 bp, respectively. The corresponding primer pairs for amplicon 3a-1 were 5′AGGATTCTACCATTTCTACTTCTTT3′ and 5′GTAAAGTGTCCAGGAAACCT3′; for amplicon 3a-2, 5′ATTCATGATGCGGCCAGAGC3′ and 5′CATTGCTGGCCGTGTGCTTC3′, and for amplicon 3b, 5′GGCTGCCAAAGAAGGTCACC3′ and 5′TTATTGAAGATTTGTGGCTCCCCCA3′. Amplification of exon 3 was carried out as follows: for 3a-1, 30 cycles with an annealing temperature of 55°C; for 3a-2, 30 cycles with an annealing temperature of 61°C; and for 3b, 30 cycles with an annealing temperature of 55°C. The Mg concentration of the buffer used for all amplicons was 1 mM $MgCl_2$, and PCR was otherwise carried out as described for exon 2. SSCP analysis was carried out as published (15, 26), with the products separated on 8% non-denaturing polyacrylamide gels containing 10% glycerol at 5-6 W for 24 hrs, followed by autoradiographic detection.

***Comparative multiplex PCR for homozygous CDKN2C deletions.*** Comparative multiplex PCR to detect homozygous deletions at both exon 2 and exon 3 was adapted from published assays (19, 29), using the *APEX* (apurinic/apyrimidinic endonuclease) gene as a control. The *APEX* nuclease gene maps to 14q11.2-14q12, a site that is not frequently altered in oligodendrogliomas. Primers for amplification of the *APEX* gene fragment were 5′CCTTTCGCAAGTTCCTGAAG3′, and 5′AACCTGTCAGCCAGTGGCAC3′ (19). To amplify *CDKN2C* exons 2 and 3, primers were used as indicated for PCR-SSCP. For *CDKN2C* exon 2 and the control gene *APEX*, 30 PCR cycles were performed with a touch-down of annealing temperatures from 62 to 53°C. PCR was performed in 10 µl -reaction volumes, containing 1 µl (50 ng/µl) template DNA, 4.9 µl $H_2O$, 1 µl 10x buffer containing 1.5 mM $MgCl_2$, 1 µl 2 mM dNTPs, 2 x 0.5 µl *CDKN2C* primers and 2 x 0.5 µl control primers with a concentration of 20 pmol/µl each, and 0.1 µl (0.5 U) Taq polymerase. Comparative multiplex PCR for *CDKN2C* exon 3 and *APEX* was performed using amplicon 3a-1 with a touch-down of annealing temperatures from 62 to 53°C, using the PCR

conditions for exon 2, except for a buffer concentration of 1 mM Mg.

***Southern blotting.*** Ten μg/sample of *Hind* III-digested genomic DNA (100 U Hind III/10 μg DNA, 24 h at 37C°) of case 1796 and various control DNAs (normal blood, cases 1648, 1654, 1670) were run in a 0.7% TAE-agarose gel at 15 V for 70 h, and blotted onto a nylon membrane. Hybridization of the probes was performed according to standard procedures, with approximately 150 ng DNA per probe radioactively double-labeled with $^{32}$PαdCTP and $^{32}$PαdATP and purified using Sephadex G50 columns. The same membrane was probed with gel-purified PCR products of *CDKN2C* exon 2 (154 bp) and the *APEX* gene fragment (187 bp). The PCR product for *CDKN2C* exon 2 was sequenced to confirm its origin from the *CDKN2C* gene. Densitometric analysis of the Southern blot autoradiographs was performed, using the Image-Pro Plus Analysis software program (Media Cybernetics, Silver Spring, Maryland), by calculating the ratio of *CDKN2C*:*APEX* density values.

## Results

***Comparative multiplex PCR for homozygous CDKN2C deletions.*** Since the *CDKN2C* homologue *CDKN2A* on chromosome 9 is often inactivated by homozygous deletions (29), we examined the 39 anaplastic oligodendrogliomas for homozygous deletions of *CDKN2C*. Comparative multiplex PCR using *CDKN2C* exon 2 disclosed two cases (5.1%) with homozygous deletions (cases 1748 and 1796, Figure 1). The assay was repeated three times, with the same results. As a control, we performed the assay with *CDKN2C* exon 3, amplicon 3a-1, and again demonstrated deletions in cases 1748 and 1796 but not in any of the other tumors (data not shown). Significantly, neither of these cases had homozygous deletions of the *CDKN2A* gene on chromosome 9, using a similar comparative multiplex PCR assay specific for *CDKN2A* exon 2 (4).

***Southern hybridization.*** To confirm the comparative multiplex results, we performed Southern blotting with DNA from case 1796 and DNA from 3 glioblastomas as controls; unfortunately, frozen tissue was no longer available for case 1748. Hybridization of *CDKN2C* exon 2 showed only a barely discernible signal in case 1796, in contrast to the strong signals noted in the glioblastomas (Figure 2A), consistent with a true homozygous deletion of *CDKN2C* in case 1796. The faint remaining hybridization signal probably represents hybridization with DNA from normal, non-neoplastic cells within the tumor, such as endothelial cells and lymphocytes. We also hybridized the same Southern blot with a fragment of the *APEX* gene. As shown in Figure 2B, hybridization of the *APEX* gene in case 1796 showed a signal similar in intensity to the control tumor DNAs. Densitometric analysis revealed a *CDKN2C*:*APEX* density ratio of 0.52 in case 1796, whereas the normal and the glioblastoma controls averaged density ratios of 2.3, with a range of 1.7 - 2.9. With the average value of the controls set to 1, the *CDKN2C* signal in case 1796 is fivefold weaker, consistent with a homozygous deletion.

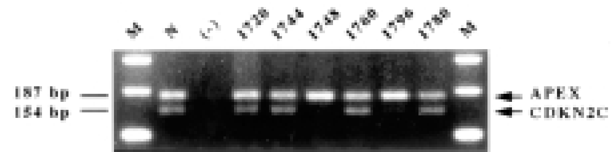


**Figure 1.** Comparative multiplex PCR for *CDKN2C* exon 2 (154 bp) and control (*APEX* gene fragment, 187 bp). Homozygous deletions of *CDKN2C* exon 2 are noted in cases 1748 and 1796. Lanes: M, DNA molecular size marker (100 bp ladder); N, normal DNA control; (-), negative control ($H_2O$); cases 1720 - 1796, representative panel of anaplastic oligodendroglioma samples.

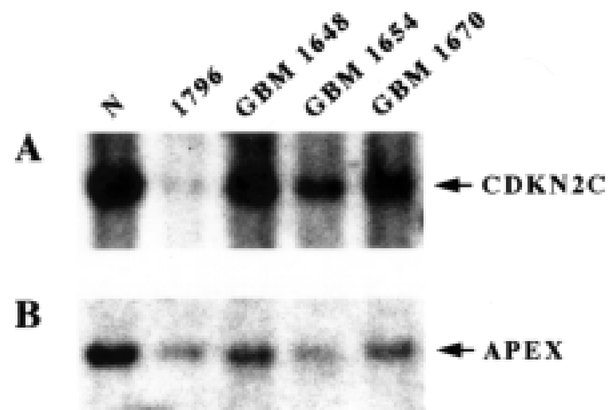


**Figure 2.** Hybridization of *CDKN2C* exon 2 and control cDNAs onto a Southern blot of Hind III-digested genomic DNA from case 1796 and controls (normal DNA, glioblastoma DNAs). Lanes: N, normal DNA; 1796, anaplastic oligodendroglioma with evidence for homozygous deletion of the *CDKN2C* gene in the multiplex PCR; 1648-1670, glioblastoma DNAs. In case 1796, the *CDKN2C* gene is barely detected (**A**), but the *APEX* gene is present (**B**). Densitometric analysis of case 1796 revealed a *CDKN2C*:*APEX* ratio of 0.52, whereas the *CDKN2C*:*APEX* ratio for the normal control was 1.7, and GBMs (glioblastomas) 1648, 1654 and 1670 were 2.4, 2.9 and 2.3, respectively.

***SSCP analysis.*** All amplicons (exon 2, exon 3 amplicons 3a-1, 3a-2 and 3b) amplified well but failed to demonstrate tumor-specific shifts on SSCP analysis of the 39 anaplastic oligodendrogliomas. One of the exon 3 amplicons (3a-2) showed a polymorphism in 3 (7.7%) of 39 cases (cases 1722, 1762 and 1784), which was noted in the corresponding blood DNA samples as well. Among these 3 cases, the polymorphism allowed demonstration of allelic loss in 2 cases (cases 1762 and 1784).

## Discussion

Allelic loss of the short arm of chromosome 1 is an important early event in the formation of human oligodendrogliomas, and strongly suggests the presence of one or more oligodendroglioma tumor suppressor genes on 1p (1, 25, 27, 28). In addition, allelic loss of 1p correlates with chemosensitivity and prolonged survival in patients with high-grade oligodendrogliomas, further suggesting that alterations of the 1p gene are closely related to clinical behavior (4). The *CDKN2C* gene is an attractive candidate for this oligodendroglioma tumor suppressor gene since it maps to the short arm of chromosome 1 and it encodes a cell cycle regulator that is homologous to *CDKN2A*, which is primarily deleted in those high-grade oligodendrogliomas that retain both 1p alleles.

Because *CDKN2A* is typically inactivated by homozygous deletions, we initially evaluated high-grade oligodendrogliomas for homozygous deletions of *CDKN2C*. Two cases (5%) had marked preferential amplification of the control amplicon in comparative multiplex PCR, consistent with the presence of homozygous deletions. For one of these cases, the presence of a homozygous deletion was confirmed by Southern blotting. Both of these two cases had also shown allelic loss of chromosome 1 distal to 1p36 in a previous study (4). Thus, a small percentage of anaplastic oligodendrogliomas have homozygous deletions that affect the coding region of the *CDKN2C* gene, which would be expected to result in absence of the $p18^{INK4C}$ product and loss of cell cycle control. Interestingly, both cases with *CDKN2C* deletions had MIB-1 proliferation indices (28% for case 1748 and 41% for case 1796) that are substantially above the mean for this group of tumors (mean MIB-1 labeling index = 18%, range = 0-43%) (4). In addition, neither of these cases had deletions of the *CDKN2A* gene, furthering the possibility that inactivation of these two genes may have similar effects in cell cycle deregulation.

Homozygous deletions, however, do not conclusively indicate that *CDKN2C* is the primary target of allelic deletion at chromosomal band 1p32. Homozygous deletions could extend to involve adjacent genes that are directly tumorigenic, with the *CDKN2C* gene co-deleted. For this reason, we evaluated these cases for point mutations in the *CDKN2C* coding region, since this would directly implicate *CDKN2C* rather than a nearby gene (7). While we did not find any point mutations in the *CDKN2C* coding sequence, Reifenberger and colleagues have recently reported a single *CDKN2C* point mutation in an anaplastic oligodendroglioma (22). This GAA to TAA mutation in codon 113 converts a glutamic acid to a stop, thus predicting a truncated $p18^{INK4C}$ protein. Combined with our present data, this inactivating point mutation argues that *CDKN2C* is a tumor suppressor gene altered in a small fraction of high-grade oligodendrogliomas.

For a number of reasons, however, *CDKN2C* is unlikely to be the major oligodendroglioma tumor suppressor gene on the short arm of chromosome 1. The numbers of deletions and mutations detected in our study and that of Reifenberger et al. are far lower than the 50-70% of oligodendrogliomas that demonstrate loss of the short arm of chromosome 1. In addition, some deletion mapping studies have shown oligodendrogliomas with losses localized to the 1p36 region (27). Nonetheless, *CDKN2C*/$p18^{INK4C}$ inactivation may play a role in the malignant progression of oligodendrogliomas, particularly if other cell cycle regulators such as *CDKN2A*/$p16^{INK4A}$ are not altered. Certainly, the role of *CDKN2C*/$p18^{INK4C}$ deletions warrants further study in larger tumor panels.


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