CDKN2/p16 or RB Alterations Occur in the Majority of Glioblastomas and Are Inversely Correlated

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ABSTRACT

p16 is involved in a cell cycle regulatory cascade that includes cyclin-dependent kinase 4 (cdk4), cyclin D1, and pRb (retinoblastoma). Alterations of each of these components have been described in primary human glioblastoma multiforme (GBM) or in GBM cell lines. Because perturbation of any component in this pathway may have similar oncogenic effects, we studied the relationship between abnormalities of CDKN2/p16 and RB, the two commonly involved tumor suppressor genes, in 55 astrocytic gliomas (42 GBMs, 8 anaplastic astrocytomas, and 5 astrocytomas). By using comparative multiplex PCR, homozygous deletions of the CDKN2/p16 gene were detected in 24 GBMs (57%) and in 2 anaplastic astrocytomas. These data indicate that the vast majority of GBMs probably have lost the remaining 9p allele. Allelic loss of chromosome 13q at the RB gene, RB gene mutations, or loss of pRb expression was noted in 14 GBMs (33%) and 2 anaplastic astrocytomas. Thirty-six of 42 GBMs (86%) had alterations of either CDKN2/p16 (n = 22), RB (n = 10), or both (n = 4); these two genetic changes, however, were relatively exclusive (P = 0.003). Furthermore, of the six GBMs without either CDKN2/p16 or RB gene abnormalities, one case had CDK4 gene amplification. These data indicate that the vast majority of GBMs probably have inactivation of the p16-cdk4/cyclin D1-pRb pathway. The findings also provide corroborative evidence that CDKN2/p16 and RB are the critical glioma tumor suppressor genes on chromosomes 9p and 13q, respectively.

INTRODUCTION

The transition from G1 to S in the mammalian cell cycle is under intricate regulatory control. One G1-S control pathway involves a complex of regulatory molecules, primarily p16, cdk4, cyclin D1, and pRb. The simplest schema suggests that p16 inhibits the cdk4/cyclin D1 complex, preventing cdk4 from phosphorylating pRb, and so ensuring that pRb maintains its brake on the cell cycle. Alterations of these individual components have been implicated in GBM tumorigenesis. For instance, homozygous deletions of chromosome 9p involving the region of the CDKN2/p16 gene occur in about one-half to two-thirds of GBMs (5–10). Although inactivating mutations of the CDKN2/p16 gene are uncommon in gliomas (6, 8, 11), recent expression (12) and in vitro (13) data implicate this gene in GBM. Allelic loss of chromosome 13q occurs in approximately one-third of GBMs and is accompanied by inactivating mutations in the RB gene (14). CDK4 gene amplification and cyclin D1 overexpression also occur but are less common (9, 15, 16).

Disruption of this pathway, with subsequent deregulated progression into S phase, may occur if p16 or pRb are inactivated or if cdk4 or cyclin D1 are overexpressed, suggesting that perturbation of any individual component will have a similar oncogenic effect (1–4). In this regard, an inverse relationship of p16 and pRb inactivation has been found in a number of non-GBM tumor cell lines (17–19). On the other hand, in some GBMs and GBM cell lines, CDK4 amplification and cyclin D1 overexpression appear to be alternative events to CDKN2/p16 deletions because these genetic changes only rarely occur in the same tumors (9, 16). Direct comparison of CDKN2/p16 and RB genetic abnormalities, however, has not been performed in primary GBMs. In the present study, we investigated these two common genetic changes to determine whether CDKN2/p16 and RB alterations represent alternative pathways to loss of cell cycle control in GBM tumorigenesis.
Cycle Sequencing kit (New England BioLabs, Beverly, MA), using the SSCP primers and 5% DMSO.

**RB Gene Mutations, Chromosome 13q Loss, and pRb Expression.** These procedures have been detailed elsewhere (14). Briefly, allelic loss of chromosome 13q was assessed by LOH studies at the pRb 1.20 polymorphism within intron 20 of the RB gene (13q14) and at the flanking microsatellite polymorphisms FLTI (13q12), D13S71 (13q21), and D13S193 (13q32). RB gene mutations were detected by SSCP for all 27 exons and flanking intronic sequences and characterized by DNA sequencing. pRb expression was evaluated on available fixed, embedded tumor sections by immunohistochemistry using the polyclonal antibody Rb-WL-1.

**CDK4 Amplification.** CDK4 amplification was assessed in those six cases which showed neither CDKN2/p16 or RB alterations, using a differential PCR assay (24, 25). A 119-bp fragment of CDK4 was amplified with the primers 5'-CTGGTrGGATAGGAGAGTGA-3' and 5'-GGAATAGGAAGAATGG-3' along with an 82-bp fragment of the IFN-γ gene (24). PCR amplification was performed in 10-μl reaction volumes at pH 8.4, including 200 μM dNTPs, 1.5 mM MgCl₂, 2 μM CDK4 primers, 1 μM γ-IFN primers, 2U Taq polymerase, 5–10 ng genomic DNA and 5% DMSO. For each PCR, the annealing temperature was gradually decreased in a "touch-down" protocol from 59 to 52°C: 2 cycles each at 59, 57, and 56°C; 3 cycles each at 55, 54, and 53°C; and 13 cycles at 52°C. The products were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. Preferential amplification of CDK4 relative to IFN-γ suggested CDK4 gene amplification.

**RESULTS AND DISCUSSION**

Titration experiments, with mixtures of normal human DNA and DNA from a cultured glioma with a homozygous deletion of CDKN2/p16, showed that the loss or marked reduction of the CDKN2/p16 amplicon was readily detected when normal DNA constituted <30% of the total DNA (Fig. 1). This corresponds with the in vivo situation in which a tumor with a homozygous deletion is contaminated with <30% nonneoplastic cells. Previous studies with the tumor DNA samples used in the present study, which were obtained after histological examination of frozen tissues, have suggested that normal contamination is minimal (22, 26), accounting for <15% of cells and, thus, making false-negative results unlikely. False-positive results for multiplex PCR assays, on the other hand, could occur if the technique were so sensitive that hemizygous deletions (i.e., LOH) were detected. However, our titration experiments demonstrate that the assay will be negative at 50% wild-type DNA, the situation expected with a hemizygous deletion with no normal DNA contamination. The ratio of CDKN2/p16 to control 9q STS amplification was equivalent in 30 normal DNA samples and in samples in which the starting DNA template concentration was varied from 0.5 to 50 ng (data not shown). These findings show that the assay is appropriate, sensitive and specific in detecting CDKN2/p16 homozygous deletions and that the assay is not affected by variations between individuals or variations in tumor DNA concentrations.

Homozygous deletions of CDKN2/p16 were detected in 24 of 42 GBMs (57%; Fig. 2) and in 2 of 8 anaplastic astrocytomas but in none of the 5 astrocytomas. These findings are consistent with the previous Southern blot, fluorescent in situ hybridization, and multiplex PCR estimates of homozygous CDKN2/p16 deletions in primary and xenografted GBMs, which have ranged from 33 to 68% (5–10). The data also support previous observations that homozygous CDKN2/p16 deletions occur less commonly in anaplastic astrocytomas and rarely, if ever, in astrocytomas, implying that these genetic changes are associated with malignant progression of astrocytic gliomas (9, 10).

Those GBMs and anaplastic astrocytomas without CDKN2/p16 homozygous deletions were examined for LOH at chromosome 9p markers that flank the CDKN2/p16 region. Two of 18 GBMs and 1 of 6 anaplastic astrocytomas showed LOH. Although CDKN2/p16 point mutations are uncommon in gliomas (5, 6, 8, 11, 12, 27), the three cases with LOH of 9p and the remaining tumors without homozygous deletions were studied for CDKN2/p16 gene point mutations or small deletions/insertions by using SSCP analysis. Only case 312, a GBM with LOH of chromosome 9p, showed a mobility shift in the exon 1 tumor DNA but not in the patient’s constitutional DNA (Fig. 3). Direct sequencing of the PCR products from tumor 312 and constitutional DNA revealed a 4-bp deletion of one TGCC sequence from a short direct repeat of TGCC-TCGC at CDKN2/p16 nucleotides...
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<th>CDKN2/p16 Alteration</th>
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CDKN2/p16 and RB alterations are relatively exclusive ($P = 0.003$).
ΔΔ, alteration; wt., wild-type.

A debate has centered on whether CDKN2/p16 is the primary glioma tumor suppressor gene on chromosome 9p. Homozygous deletions of CDKN2/p16 occur more frequently in cell lines than in primary tumors, and point mutations were initially not detected in most primary tumors (27), including astrocytomas (11), leading to the hypothesis that CDKN2/p16 was not the critical chromosome 9p tumor suppressor gene. However, deletions in primary GBMs almost always involve CDKN2/p16 (5, 7, 9), and two mutations have been described previously in primary GBMs with allelic loss of chromosome 9p (8, 11). In addition, recent data have shown reduced or absent p16 expression in some malignant gliomas without CDKN2/p16 loss (12), suggesting alternative means, such as hypermethylation (33), of inactivating the gene in GBMs. Moreover, replacement of CDKN2/p16 into GBM cell lines lacking the gene results in growth suppression but had no effect in cell lines containing the CDKN2/p16 gene (13). Our present observations provide additional corroborative evidence implicating CDKN2/p16 in gliomas: (a) the inverse relationship between CDKN2/p16 and RB alterations in primary gliomas supports the in vitro data that these molecules are involved in a single functional pathway; and (b) documentation of a third, presumably inactivating, mutation provides another rare example of CDKN2/p16 conforming to the classical tumor suppressor gene scenario.

Six GBMs had neither CDKN2/p16 nor RB alterations. Because amplification of the CDK4 gene and overexpression of cyclin D1 may have similar effects to p16 or pRb inactivation (1), these mechanisms may provide additional alternatives for progression to GBM (9). CDK4, located on chromosome 12q13–14, has been reported to be amplified in 15% of malignant gliomas (15), although this frequency may be higher among cases without CDKN2/p16 loss, reaching 50% of GBMs without CDKN2/p16 loss in one study (9). CDK4, however, is part of a relatively large amplicon that includes other oncogenes, and it has been debated whether CDK4 or another gene such as MDM2 is the critical chromosome 12q glioma oncogene (15). We attempted to assay CDK4 amplification in these tumors using a differential PCR technique (24). One of the six GBMs without CDKN2/p16 or RB abnormalities had changes that suggested amplification with this assay (data not shown). Unfortunately, adequate DNA to confirm these findings by Southern blotting or RNA or protein samples to assess cyclin D1 overexpression were not available. Nonetheless, the suggestion that one of the six cases had CDK4 gene amplification, along with previous observations that CDK4 gene amplification occurs in primary GBMs (15), that CDK4 amplification and CDKN2/p16 deletions do not occur together in GBM cell lines (16), and that some GBM cell lines overexpress cyclin D1 (16), support the hypothesis that most GBMs have a genetic alteration that interferes with this crucial cell cycle regulatory pathway.

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