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. By DNA sequencing analysis of all three coding exons of CDKN2/p16, we identified a frameshift mutation (four-bp deletion) in one of the three GBMs that had 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 (1–4). 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.

MATERIALS AND METHODS

Materials. Tumor tissues and blood samples were obtained from patients operated on at the Massachusetts General Hospital (Boston, MA) and at the University Hospital (Zürich, Switzerland). All tumors were examined by a neuropathologist and graded according to WHO criteria (20). The 55 astrocytic gliomas were classified as 42 WHO grade IV GBMs, 8 WHO grade III anaplastic astrocytomas, and 5 WHO grade II astrocytomas.

Homozygous Deletions of CDKN2/p16. To assay for homozygous deletions of the CDKN2/p16 gene, we used a modification of a comparative multiplex PCR technique described previously (10, 21). Oligonucleotide primers were designed to amplify a 235-bp fragment of the 5′ end of CDKN2/p16 exon 2 (11) and to amplify a control 180-bp STS on the long arm of chromosome 9 (5′-ATTTCTGCCCTGGAGACAGTG-3′ and 5′-AGGCCCTTGGAAGCTTCGGAAGG-3′; generously provided by Dr. Alan J. Buckler, Massachusetts General Hospital). PCR amplification was performed with a Programmable Thermal Cycler (MJ Research) in 10-μl reaction volumes at pH 8.4, including 200 μM dNTPs, 1.5 mM MgCl2, 2 μM control primers, 1 μM CDKN2/p16 primers, 2 units 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 62 to 55°C: 2 cycles each at 62, 60, and 59°C; 3 cycles each at 58, 57, and 56°C; and 13 cycles at 55°C. The products were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. To titrate the assay, we evaluated serial mixtures of normal human DNA and DNA from a cultured glioma with a homozygous deletion of CDKN2/p16 (as assessed by both PCR and Southern blotting: data not shown), ranging from 100% normal DNA to 100% CDKN2/p16-deleted DNA. To determine whether the ratio of amplified CDKN2/p16 to the control 9q STS varied among individuals, we studied 30 normal human DNA samples. Finally, to exclude the co-amplification of the 50-kb CDKN2/p16 and the 9.5-kb control 9q STS, we performed Southern blotting on gel-purified bands from methylated human DNA samples.

SSCP and DNA Sequencing of CDKN2/p16. SSCP analysis was performed on all three coding exons of the CDKN2/p16 gene, as published previously (11, 22). Cases with mobility shifts on SSCP were directly sequenced with Vent(exo-) DNA polymerase and the Circum-Vent Thermal Sequencing Kit (Clontech). DNA sequencing was performed in the laboratory of Dr. Alan J. Buckler, Department of Molecular Neuro-Oncology, Massachusetts General Hospital.
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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 FLT1 (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 that showed neither CDKN2/p16 nor RB alterations, using a differential PCR assay (24, 25). A 119-bp fragment of CDK4 was amplified with the primers 5'-CTGGTTGGATAGGAGAGTGA-3' and 5'-GGAATAGGAAGAATGATTACCAT-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, 2 U 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 appropriately 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 TGGC sequence from a short direct repeat of TGGCTGGC at CDKN2/p16 nucleotides.

Fig. 1. CDKN2/p16 homozygous deletion assay. Titration of normal DNA and cultured glioma DNA with a homozygous deletion of CDKN2/p16. Loss or marked reduction of amplification of the CDKN2/p16 amplicon is noted when normal DNA constitutes <30% of the total DNA. Decreased amplification of the CDKN2/p16 amplicon relative to the control 9q STS amplicon is also seen at 30% normal DNA (compare with 100% normal lane). M, size marker, dxt174 digested with HinfI; (−), no DNA control.

Fig. 2. CDKN2/p16 homozygous deletion assay. Corresponding normal (N) and tumor (T) DNA samples from five GBM cases. Homozygous deletions of CDKN2/p16 (redaction or loss of upper band) are noted in three GBMs (cases 6, 52, and 262). All of the constitutional DNA samples (N) and the two other tumors (cases 466 and 358) show equal amplification of the CDKN2/p16 and 9q STS (lower band) amplicons. M, size marker, dxt174 digested with HinfI; (−), no DNA control.

Fig. 3. SSCP analysis of CDKN2/p16 exon 1 in six GBMs. A striking migration shift is seen in Lane 6. DNA sequencing of this case showed a 4-bp deletion (see "Results and Discussion").

Table

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pitS glioma mutations (8, 11, and this report), two result in truncated proteins reported in other human tumors (17, 31, 32). However, other GBM with a G to A nonsense mutation at codon 102 (8); and a GBM gene scenario, in which a mutation is accompanied by allelic loss: a frameshift leads to a stop at codon 17 (1) [or codon 25 by Okamoto et al. sequence (28)] in exon 1 and presumably to a grossly truncated frameshift, which is consistent with the majority of CDKN2/p16 mutations. For instance, hypermethylation of 5' CpG islands may lead to transcriptional silencing of this gene, including in some GBMs (33), and thus, it is possible that the two remaining cases with LOH have CDKN2/p16 inactivation by such a mechanism.

LOH at the RB gene was found in 14 of 42 GBMs (33%) and in 2 of 8 anaplastic astrocytomas but in none of 5 astrocytomas. Deletion mapping, using flanking polymorphisms, showed that allelic losses targeted the 13q14 region that includes RB, with some deletions maintaining the centromeric markers and others maintaining the telomeric markers. SSCP and direct sequencing analysis of all 27 exons of RB revealed 3 inactivating mutations, all in GBMs with LOH of chromosome 13q. Finally, immunohistochemistry demonstrated loss of pRb expression in 2 GBMs, with both cases having LOH of chromosome 13q as well as inactivating mutations of RB. These data strongly suggested that the RB gene was the glioma tumor suppressor on chromosome 13q, and that chromosome 13q loss presumably reflects RB inactivation even in those cases without SSCP-detectable point mutations in RB. However, it remained possible that a second chromosome 13q glioma tumor suppressor was responsible in those cases without SSCP-detectable RB mutations. The details of the RB gene and pRb analyses have been published previously (14).

Of the 42 GBMs, 36 (86%) had abnormalities of either CDKN2/p16 or RB; 26 GBMs had CDKN2/p16 alterations (24 homozygous deletions; 1 frameshift mutation with LOH of 9p; and 1 LOH of 9p without a detectable mutation) and 14 GBMs had RB abnormalities (14 with LOH at Rb 1.20; 3 of these 14 with RB mutations; and 2 of these 3 with loss of pRb expression (Table 1). Significantly, these two genetic changes were relatively exclusive (P = 0.003, two-tailed Fisher exact), with only four cases having alterations at both genetic loci. However, there was no correlation between the molecular genetic findings and the age or sex of the patients. Of the eight anaplastic astrocytomas, one had CDKN2/p16 deletion alone, one had RB LOH alone, and one had both changes; these numbers were too small for statistical analysis. The segregation of GBMs into those with CDKN2/p16 and those with RB abnormalities supports the hypothesis that p16 and pRb operate in the same pathway, and that inactivation of either component has a similar oncogenic effect.

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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REFERENCES

4. Lukas, J., Parry, D., Aagaard, L., Mann, D. J., Bartkova, J., Strauss, M., Peters, G.,
CDKN2/p16 OR RB ALTERATIONS IN GBM


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

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