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CDKN2/p16 or *RB* Alterations Occur in the Majority of Glioblastomas and Are Inversely Correlated[1]

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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. Two additional GBMs and one anaplastic astrocytoma had allelic loss of chromosome 9p, as assessed by microsatellite polymorphisms flanking the *CDKN2/p16* region. Single-strand conformation polymorphism and DNA sequencing analysis of all three coding exons of *CDKN2/p16* revealed 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 G_1 to S in the mammalian cell cycle is under intricate regulatory control. One G_1-S control pathway involves a complex of regulatory molecules, primarily p16, cdk4,[3] 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) at and 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 comparative multiplex PCR techniques 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′-ATTTCTGCCTGGAGACAGTGG-3′ and 5′-AGGCCTTGGGAGCCT-CAGG-3′; generously provided by Dr. Alan J. Buckler, Massachusetts General Hospital). PCR amplification was performed with a Programmable Thermal cycler (M.J. Research) in 10-μl reaction volumes at pH 8.4, including 200 μM dNTPs, 1.5 mM $MgCl_2$, 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 possibility of differential amplification with varying concentrations of starting DNA template, we studied serial dilutions of normal DNA, ranging from 50 to 0.05 ng of template DNA. Each tumor assay was repeated at least three times. For the positive and some negative cases, paired blood-tumor DNA samples were examined to highlight that the alterations were tumor specific.

LOH of Chromosome 9p. Blood-tumor DNA samples were examined for LOH at two microsatellite loci flanking the *CDKN2/p16* region, *IFNA* and *D9S171*, as described previously (11, 22).

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, 23). Cases with mobility shifts on SSCP were directly sequenced with $Vent_R$(exo-) DNA polymerase and the Circum-Vent Thermal

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[3] The abbreviations used are: cdk4, cyclin-dependent kinase 4; pRb, retinoblastoma; GBM, glioblastoma multiforme; STS, sequence-tagged site; LOH, loss of heterozygosity; SSCP, single-strand conformation polymorphism.

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* or *RB* alterations, using a differential PCR assay (24, 25). A 119-bp fragment of *CDK4* was amplified with the primers 5′-CTGGTTGGATAGGAGTGA-3′ and 5′-GGAATAGGAAGAATG-GATGG-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 $MgCl_2$, 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 argue 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

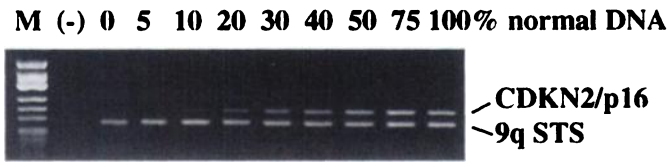

Fig. 1. *CDKN2/p16* homozygous deletion assay. Titration of normal DNA and cultured glioma DNA with a homozygous deletion of *CDKN2/p16*. Loss or marked reduction of amplification of the *CDKN2/p16* amplicon is noted when normal DNA constitutes <30% of the total DNA. Decreased amplification of the *CDKN2/p16* amplicon relative to the control 9q STS amplicon is also seen at 30% normal DNA (compare with 100% normal lane). *M*, size marker, φx174 digested with *HinfI*; (−), no DNA control.

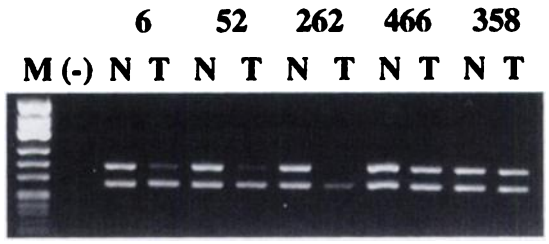

Fig. 2. *CDKN2/p16* homozygous deletion assay. Corresponding normal (*N*) and tumor (*T*) DNA samples from five GBM cases. Homozygous deletions of *CDKN2/p16* (reduction or loss of upper band) are noted in three GBMs (cases 6, 52, and 262). All of the constitutional DNA samples (*N*) and the two other tumors (cases 466 and 358) show equal amplification of the *CDKN2/p16* and 9q STS (lower band) amplicons. *M*, size marker, φx174 digested with *HinfI*; (−), no DNA control.

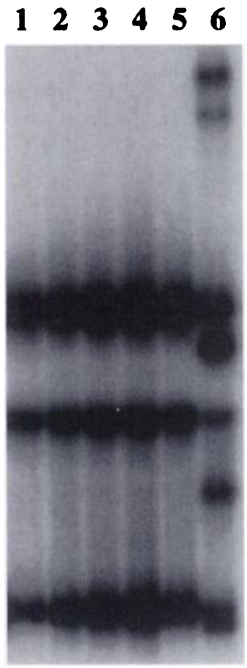

Fig. 3. SSCP analysis of *CDKN2/p16* exon1 in six GBMs. A striking migration shift is seen in *Lane 6*. DNA sequencing of this case showed a 4-bp deletion (see "Results and Discussion").

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

Table 1 *CDKN2/p16 and RB analysis in GBM*[a]

	CDKN2/p16	
	Δ[b]	wt.
RB		
Δ	4	10
wt.	22	6

[a] *CDKN2/p16* and *Rb* alterations are relatively exclusive ($P = 0.003$).
[b] Δ, alteration; wt., wild-type.

19–26 (1) [or nucleotides 43–50 by Okamoto *et al.* sequence (28)] in the tumor DNA. This same deletion has been reported in a pancreatic adenocarcinoma (29) and, given the loss of a repeated sequence, may be secondary to a "slipped-mispairing" mechanism (30). The resultant 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 protein product. Prior studies of the *CDKN2/p16* gene in gliomas have yielded only two cases that conform to the classical tumor suppressor gene scenario, in which a mutation is accompanied by allelic loss: a GBM with a G to A nonsense mutation at codon 102 (8); and a GBM with a C to T transition at codon 106, resulting in an amino acid change of proline to leucine (11). Therefore, of these three *CDKN2/p16* glioma mutations (8, 11, and this report), two result in truncated proteins, which is consistent with the majority of *CDKN2/p16* mutations reported in other human tumors (17, 31, 32). However, other mechanisms can also result in functional inactivation of *CDKN2/p16*. 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

1. Serrano, M., Hannon, G. J., and Beach, D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature (Lond.), *366:* 704–707, 1993.
2. Medema, R. H., Herrera, R. E., Lam, F., and Weinberg, R. A. Growth suppression by $p16^{ink4}$ requires functional retinoblastoma protein. Proc. Natl. Acad. Sci. USA, *92:* 6289–6293, 1995.
3. Koh, J., Enders, G. H., Dynlacht, B. D., and Harlow, E. Tumour-derived p16 alleles encoding proteins defective in cell-cycle inhibition. Nature (Lond.), *375:* 506–510, 1995.
4. Lukas, J., Parry, D., Aagaard, L., Mann, D. J., Bartkova, J., Strauss, M., Peters, G.,

and Bartek, J. Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. Nature (Lond.), *375:* 503–506, 1995.

5. Jen, J., Harper, W., Bigner, S. H., Bigner, D. D., Papadopoulos, N., Markowitz, S., Willson, J. K. V., Kinzler, K. W., and Vogelstein, B. Deletion of p16 and p15 in brain tumors. Cancer Res., *54:* 6353–6358, 1994.
6. Giani, C., and Finocchiaro, G. Mutation rate of the *CDKN2* gene in malignant gliomas. Cancer Res., *54:* 6338–6339, 1994.
7. Dreyling, M. H., Bohlander, S. K., Adeyanju, M. O., and Olopade, O. I. Detection of CDKN2 deletions in tumor cell lines and primary glioma by interphase fluorescence *in situ* hybridization. Cancer Res., *55:* 984–988, 1995.
8. Moulton, T., Samara, G., Chung, W., Yuan, L., Desai, R., Sisti, M., Bruce, J., and Tycko, B. MTS1/p16/CDKN2 lesions in primary glioblastoma multiforme. Am. J. Pathol., *146:* 613–619, 1995.
9. Schmidt, E. E., Ichimura, K., Reifenberger, G., and Collins, V. P. *CDKN2* (*p16/MTS1*) gene deletion or CDK4 amplification occurs in the majority of glioblastomas. Cancer Res., *54:* 6321–6324, 1994.
10. Walker, D. G., Duan, W., Popovic, E. A., Kaye, A. H., Tomlinson, F. H., and Lavin, M. Homozygous deletions of the multiple tumor suppressor gene 1 in the progression of human astrocytomas. Cancer Res., *55:* 20–23, 1995.
11. Ueki, K., Rubio, M-P., Ramesh, V., Correa, K. M., Rutter, J. L., von Deimling, A., Buckler, A. J., Gusella, J. F., and Louis, D. N. *MTS1/CDKN2* gene mutations are rare in primary human astrocytomas with allelic loss of chromosome 9p. Hum. Mol. Genet., *3:* 1841–1845, 1994.
12. Nishikawa, R., Furnari, F., Lin, H., Arap, W., Berger, M. S., Cavenee, W. K., and Huang, H-J. S. Loss of $p16^{INK4}$ expression is frequent in high grade gliomas. Cancer Res., *55:* 1941–1945, 1995.
13. Arap, W., Nishikawa, R., Furnari, F. B., Cavenee, W. K., and Huang, H-J. S. Replacement of the *p16/CDKN2* gene suppresses human glioma cell growth. Cancer Res., *55:* 1351–1354, 1995.
14. Henson, J. W., Schnitker, B. L., Correa, K. M., von Deimling, A., Fassbender, F., Xu, H-J., Benedict, W. F., Yandell, D. W., and Louis, D. N. The retinoblastoma susceptibility (*Rb*) gene is involved in the malignant progression of human astrocytomas. Ann. Neurol., *36:* 714–721, 1994.
15. Reifenberger, G., Reifenberger, J., Ichimura, K., Melzter, P. S., and Collins, V. P. Amplification of multiple genes from chromosomal region 12q13–14 in human malignant gliomas: preliminary mapping of the amplicons shows preferential involvement of CDK4, SAS, and MDM2. Cancer Res., *54:* 4299–4303, 1994.
16. He, J., Allen, J. R., Collins, V. P., Allalunis-Turner, M. J., Godbout, R., Day, R. S. I., and James, C. D. CDK4 amplification is an alternative mechanism to *p16* gene homozygous deletion in glioma cell lines. Cancer Res., *54:* 5804–5807, 1994.
17. Okamoto, A., Demetrick, D. J., Spillare, E. A., Hagiwara, K., Hussain, S. P., Bennet, W. P., Forrester, K., Gerwin, B., Serrano, M., Beach, D. H., and Harris, C. C. Mutations and altered expression of $p16^{INK4}$ in human cancer. Proc. Natl. Acad. Sci. USA, *91:* 11045–11049, 1994.
18. Otterson, G. A., Kratzke, R. A., Coxon, A., Kim, Y. W., and Kaye, F. Absence of $p16^{INK4}$ protein is restricted to the subset of lung cancer lines that retains wildtype RB. Oncogene, *9:* 3375–3378, 1994.
19. Tam, S. W., Shay, J. W., and Pagano, M. Differential expression and cell cycle regulation of the cyclin-dependent kinase inhibitor $p16^{INK4}$. Cancer Res., *54:* 5816–5820, 1994.
20. Kleihues, P., Burger, P. C., and Scheithauer, B. W. Histological Typing of Tumours of the Central Nervous System. Berlin, Germany: Springer-Verlag, 1993.
21. Cairns, P., Tokino, K., Eby, Y., and Sidransky, D. Homozygous deletions of 9p21 primary human bladder tumors detected by comparative multiplex polymerase chain reaction. Cancer Res., *54:* 1422–1424, 1994.
22. Louis, D. N., von Deimling, A., and Seizinger, B. R. A (CA)n dinucleotide repeat assay for evaluating loss of allelic heterozygosity in small and archival human brain tumor specimens. Am. J. Pathol., *141:* 777–782, 1992.
23. Rubio, M-P., von Deimling, A., Yandell, D. W., Wiestler, O. D., Gusella, J. F., and Louis, D. N. Accumulation of wild-type p53 protein in human astrocytomas. Cancer Res., *53:* 3465–3467, 1993.
24. Neubauer, A., Neubauer, B., He, M., Effert, P., Iglehart, D., and Frye, R. A. Analysis of gene amplification in archival tissue by differential polymerase chain reaction. Oncogene, *7:* 1019–1025, 1992.
25. Louis, D. N., Rubio, M-P., Correa, K., Gusella, J. F., and von Deimling, A. Molecular genetic alterations in pediatric brain stem gliomas. Application of PCR techniques to small and archival brain tumor specimens. J. Neuropathol. Exp. Neurol., *52:* 507–515, 1993.
26. Rubio, M-P., Correa, K. M., Ueki, K., Mohrenweiser, H. W., Gusella, J. F., von Deimling, A., and Louis. D. N. The putative glioma tumor suppressor gene on chromosome 19q maps between APOC2 and HRC. Cancer Res., *54:* 4760–4763, 1994.
27. Cairns, P., Mao, L., Merlo, A., Lee, D. J., Schwab, D., Eby, Y., Tokino, K., van der Riet, P., Blaugrund, J. E., and Sidransky, D. Rates of p16 (*MTS1*) mutations in primary tumors with 9p loss. Science (Washington DC), *265:* 415–416, 1994.
28. Okamoto, A., Hussain, S. P., Hagiwara, K., Spillare, E. A., Rusin, M. R., Demetrick, D. J., Serrano, M., Hannon, G. J., Shiseki, M., Zariwala, M., Xiong, Y., Beach, D. H., Yokota, J., and Harris, C. C. Mutations in the $p16^{INK4}$/*MTS1/CDKN2*. $p15^{INK4B}$/*MTS2*, and *p18* genes in primary and metastatic lung cancer. Cancer Res., *55:* 1448–1451, 1995.
29. Caldas, C., Hahn, S. A., da Costa, L. T., Redston, M. S., Schutte, M., Seymour, A. B., Weinstein, C. L., Hruban, R. H., Yeo, C. J., and Kern, S. E. Frequent somatic mutations and homozygous deletions of the *p16* (*MTS1*) gene in pancreatic adenocarcinomas. Nat. Genet., *8:* 27–32, 1994.
30. Hogg, A., Bia, B., Onadim, Z., and Cowell, J. K. Molecular mechanisms of oncogenic mutations in tumors from patients with bilateral and unilateral retinoblastoma. Proc. Natl. Acad. Sci. USA, *90:* 7351–7355, 1993.
31. Caldas, C., Hahn, S. A., da Costa, L. T., Redston, M. S., Shutte, M., Seymour, A. B., Weinstein, C. L., Hruban, R. H., Teo, C. J., and Kern, S. E. Frequent somatic mutations and homozygous deletions of the *p16* (*MTS1*) gene in pancreatic adenocarcinoma. Nat. Genet., *8:* 27–31, 1994.
32. Mori, T., Miura, K., Aoki, T., Nishihira, T., Mori, S., and Nakamura, Y. Frequent somatic mutation of the *MTS1/CDK4I* (multiple tumor suppressor/cyclin-dependent kinase 4 inhibitor) gene in esophageal squamous cell carcinoma. Cancer Res., *54:* 3396–3397, 1994.
33. Merlo, A., Herman, J. G., Mao, L., Lee, D. J., Gabrielson, E., Burger, P. C., Baylin, S. B., and Sidransky, D. 5′CpG island methylation is associated with transcriptional silencing of the tumor suppressor *p16/CDKN2/MTS1*. Nat. Med., *1:* 686–692, 1995.