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Molecular Genetic Correlates of p16, cdk4, and pRb Immunohistochemistry in Glioblastomas

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Abstract. The vast majority of glioblastomas have *CDKN2A, CDK4,* or *RB* gene alterations that perturb the p16-cdk4-pRb cell cycle regulatory cascade. To explore whether immunohistochemical methods provide an alternative means of assessing this pathway, we studied 25 glioblastomas using a combination of molecular genetic and immunohistochemical assays. Homozygous deletion of the *CDKN2A* gene was detected in 12 of 25 (48%) cases, *CDK4* amplification in 4 of 25 (16%) tumors, and loss of heterozygosity at the *RB* gene in 8 of 22 (36%) informative cases. Five of 25 (20%) glioblastomas had diffuse p16 immunohistochemical positivity. Significantly, all of these had either *CDK4* amplification or *RB* LOH, suggesting that p16 immunopositivity only occurs in those tumors with alterations of another component in the pathway. Nineteen (76%) cases were uniformly immunonegative for p16, and 12 (48%) had *CDKN2A* homozygous deletions, but the remaining 7 cases lacked *CDKN2A* deletions, mutations and promoter methylation. All glioblastomas stained diffusely for cdk4, irrespective of *CDK4* gene amplification status. Extensive pRb staining was present in most cases that maintained both *RB* alleles, and absent in most cases with *RB* loss, but there were notable discrepancies. Thus, p16 and pRb immunohistochemistry cannot replace molecular genetic analysis of this critical regulatory cascade; instead, the combined results hint at complex regulation of this cell cycle checkpoint. From a practical point of view, although p16 immunonegativity does not necessarily indicate *CDKN2A* deletion, diffuse positive p16 immunostaining strongly suggests either *CDK4* amplification or *RB* loss and excludes *CDKN2A* deletion.

Key Words: cdk4; CDKN2A; Immunohistochemistry; Glioblastoma; p16; pRb; RB.

INTRODUCTION

The p16 protein (encoded by the *CDKN2A* gene on chromosome 9p21), cyclin dependent kinase 4 (cdk4, encoded by the *CDK4* gene on chromosome 12q13), and the retinoblastoma protein (pRb, encoded by the *RB* gene on chromosome 13q14) are integral components of a regulatory pathway that controls the G1 to S transition of the cell cycle. Current models suggest that p16 negatively regulates cdk4-cyclin D complexes, which in turn negatively regulate pRb activity (1). Thus, decreased expression of p16 or pRb, or increased activity of cdk4, should promote entry into S phase and accelerate cellular proliferation.

Alterations of this pathway are common in human cancers, suggesting that abrogation of this cascade is critical to cell cycle deregulation in human neoplasia (1). The majority of glioblastoma multiforme (GBM) have molecular genetic alterations of at least one component of this pathway (2–6). Such genetic changes are, however, uncommon in grade II astrocytomas, implying that deregulation of the p16-cdk4-pRb–mediated checkpoint is important in malignant progression of astrocytic gliomas (7). The most common alteration of this pathway in GBMs is inactivation of p16, usually by homozygous deletion (HD) of the *CDKN2A* gene, which occurs in one-third to two-thirds of cases (2, 3, 5, 6, 8, 9). Rarely, however, *CDKN2A* point mutations (5, 6, 10, 11) or transcriptional silencing due to *CDKN2A* promoter methylation (12–14) may also inactivate or downregulate p16 in GBM. The absence of functional pRb due to point mutation of the *RB* gene and loss of heterozygosity (LOH) of the remaining *RB* allele are the next most common alterations, occurring in 30–40% of GBM (4–6, 15). Finally, *CDK4* amplification and cdk4 overexpression occur in 10–20% of cases (3, 4, 6, 16–19). Studies of this pathway at the protein level have been less extensive and largely restricted to western blotting (4, 19, 20), since immunohistochemical approaches have been hampered until recently by the lack of available antibodies that perform well in fixed, embedded tissues.

Immunohistochemistry has proved a convenient and popular means for assessing expression of oncogenes and tumor suppressor genes in brain tumors. Unfortunately, correlations between molecular genetic alterations and immunohistochemical expression have not always been straightforward. For example, experience with p53 immunohistochemistry has revealed an often bewildering complexity, with interpretation of immunohistochemical results being highly dependent on tumor type and *TP53* gene status (21). The present study sought to address whether immunohistochemical methods provide an alternative means of assessing the p16-cdk4-pRb pathway in GBM. We therefore performed a detailed molecular genetic analysis of the *CDKN2A, CDK4,* and *RB* genes, coupled with immunohistochemical evaluation of the p16, cdk4, and pRb proteins, in 25 GBM.

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MATERIALS AND METHODS

Materials

Tumor tissues and blood samples were obtained from 24 patients at the time of biopsy; tumor tissue and normal brain were acquired at autopsy from one patient. All tumors were diagnosed as glioblastoma, according to WHO criteria (22); the autopsy case had regions of gliosarcoma. DNA was extracted from frozen tumor tissues and from blood/normal brain samples according to standard phenol-chloroform procedures (23). Prior to DNA extraction, all tumor tissues were examined by frozen section to ensure that they consisted of viable tumor tissue. In one case, distinct p16-immunopositive and -immunonegative areas were microdissected from formalin-fixed, paraffin-embedded sections, and DNA was extracted from these separate regions using a published protocol (24, 25).

Homozygous Deletions of *CDKN2A*

To assay for HD of the *CDKN2A* gene, we used a comparative multiplex PCR technique (5). The products were separated by electrophoresis on 2% agarose gels and visualized under ultraviolet light by ethidium bromide staining. As previously described in titration experiments, we require marked differences between *CDKN2A* and reference gene products to score homozygous deletion (5). Each assay was repeated at least twice and paired normal-tumor DNA samples were examined in all cases to ensure that the alterations were tumor specific. To confirm the specificity and sensitivity of the comparative multiplex assay, we studied 8 GBM that had sufficient DNA with Southern blotting using a *CDKN2A* probe (23).

Loss of Heterozygosity of Chromosome 9p21

In cases without *CDKN2A* HD, matched samples of constitutional and tumor DNA were examined for LOH of chromosome 9p21 at two microsatellite loci flanking the *CDKN2A* region, IFNA and D9S171, as described previously (11).

Single Strand Conformation Polymorphism Analysis (SSCP) of *CDKN2A*

In the 13 cases not showing *CDKN2A* HD, SSCP analysis was performed on all 3 coding exons of the *CDKN2A* gene, following a published protocol (11). A PCR-RFLP (restriction fragment length polymorphism) assay was used on 4 normal-tumor pairs with identical mobility shifts that were most likely due to a known polymorphism (11). Since this C to G polymorphism in base 494 of the 3′ untranslated region ablates an *Msp*I site, *CDKN2A* exon 3 amplicons were digested with *Msp*I. The digested products were separated on a 2.5% agarose gel and visualized with ethidium bromide and ultraviolet light.

Methylation-specific PCR of the *CDKN2A* Promoter

The methylation status of the *CDKN2A* promoter region was studied in tumors that lacked *CDKN2A* HD but which showed loss of p16 expression. Methylation was assessed by methylation-specific PCR according to a published protocol (26), with minor modification of the PCR cycle to include a touch-down of the annealing temperature from 67°C to 60°C, followed by 20 cycles at 60°C. The procedure entails bisulfite modification of DNA followed by amplification with primer pairs specific for methylated and unmethylated DNA. DNA samples from the colon carcinoma cell lines HT-29 and CaCo2 were used as positive controls for methylated DNA. The PCR products were run on a 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

CDK4 Gene Amplification

CDK4 amplification was evaluated using a differential PCR assay (5, 25). The products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light, and scored as previously described (5). Each assay was repeated at least twice. Paired normal-tumor DNA samples were examined in all cases to ensure that the alterations were tumor specific. To confirm the specificity and sensitivity of the differential PCR assay, we studied 8 GBM that had sufficient DNA with Southern blotting using a *CDK4* probe (23).

Allelic Loss of the *RB* Gene

Allelic loss of chromosome 13q14 at the *RB* gene was assessed by LOH analysis of the RB 1.20 polymorphism in intron 20 of the *RB* gene, as detailed elsewhere (15).

p16 Immunohistochemistry

The JC8 anti-p16 mouse monoclonal IgG2a antibody was generated in the Massachusetts General Hospital Cancer Center and recognizes an epitope in the first ankyrin repeat (amino acids 1-32) of the p16 protein. The antibody detects a single 16kD band on western blots of human tissues, including brain tumors (J. Koh, unpublished data). In breast carcinomas, good correlations have been noted between western blotting and immunohistochemical results (E. Schmidt, J. Koh, D. N. Louis, unpublished data). In the process of determining which anti-p16 antibodies were optimal for immunohistochemistry on formalin-fixed, paraffin-embedded specimens, we evaluated 5 non-commercial mouse monoclonal antibodies (J. Koh, unpublished data) as well as 2 commercial antibodies (Santa Cruz sc-468 rabbit polyclonal IgG anti-p16, Pharmingen G175-405 mouse monoclonal IgG1 anti-p16). The results with the JC8 were superior to the other tested antibodies.

Formalin-fixed, paraffin-embedded tissues were sectioned at 6 μm onto Probe-On Plus (Fisher Scientific) slides. After baking at 65°C for one hour, the sections were deparaffinized in xylene and rehydrated in graded ethanols. Endogenous peroxidase activity was blocked by immersing the slides in 0.5% hydrogen peroxide (H_2O_2) in methanol for 5 minutes between the 100% and 90% alcohol steps. An antigen retrieval step was employed, consisting of microwaving the slides in 0.01 M sodium citrate (pH 6.0) for 3 changes of 5 minutes each, followed by cooling in phosphate buffered saline (PBS) rinses. The sections were incubated in 10% normal horse serum in 5% milk for 20 minutes at room temperature. The JC8 anti-p16 antibody was applied at a 1:500 dilution in 1% bovine serum albumin (BSA)/PBS and incubated in a humidity chamber at room temperature for 2 hours. Following the primary antibody incubation, a secondary biotinylated horse anti-mouse antibody (Vector) was applied at a 1:1000 dilution (in 1% BSA/PBS) for

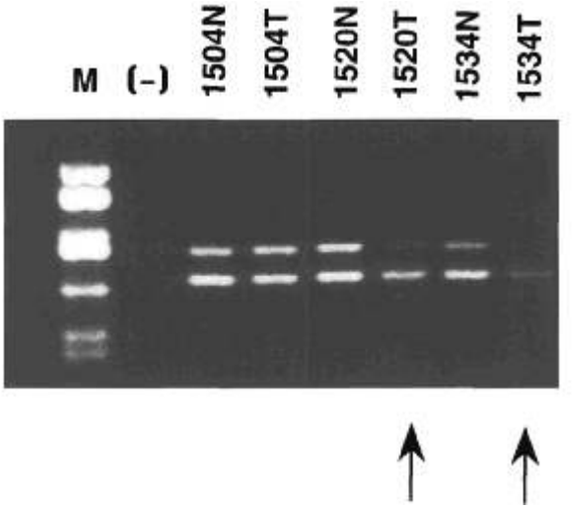

Fig. 1. Comparative multiplex analysis of the *CDKN2A* gene. Homozygous deletion of the *CDKN2A* gene is present in cases 1520 and 1534 (arrows), as evidenced by preferential amplification of the control amplicon (bottom band) with minimal amplification of the *CDKN2A* amplicon (top band). M = pUC18/*Hae*III digest size marker; (−) = "no DNA" lane; N = normal; T = tumor.

1 hour at room temperature, followed by the avidin-biotin complex kit (ABC Elite, Vector), also applied for 1 hour at room temperature. Between each of the preceding 3 steps, slides were washed in 3 changes of PBS. Following the application of 0.06% diaminobenzidine (DAB) (Sigma) with 0.01% H_2O_2 for 3 minutes, the slides were washed in distilled water and lightly counterstained in Hematoxylin Solution Gill No. 1 (Sigma). After dehydration in graded alcohols and clearing in xylene, the slides were coverslipped. Tonsil tissue served as a control in which nuclear and cytoplasmic staining was noted specifically in histiocytic cells in germinal centers and epithelial cells of the mucosal lining. Negative controls were performed by omitting the primary antibody and by using an irrelevant mouse monoclonal antibody.

cdk4 Immunohistochemistry

cdk4 immunohistochemistry followed a similar protocol with the following modifications. The sections were not microwaved, but were digested with 0.01% trypsin at 37°C for 15 minutes. Blocking was performed with 10% normal goat serum (NGS) in PBS for 30 minutes at room temperature. The primary anti-cdk4 antibody was a rabbit polyclonal antibody (sc-260, Santa Cruz Biotechnology, Inc.) diluted 1:2500 in 1% BSA/PBS with 10% NGS. The sections were incubated overnight at 4°C, followed by incubation for 30 minutes at room temperature with a biotinylated goat anti-rabbit antibody (Vector Laboratories) diluted at 1:4000 in 1% BSA/PBS with 10% NGS. The ABC kit was applied for 40 minutes, with DAB used as a chromogen. In control tonsil, nuclear cdk4 immunoreactivity was present in most cells, except for numerous lymphocytes in the mantle zones.

pRb Immunohistochemistry

The pRb immunohistochemical protocol was similar to the above p16 assay, with minor variations. Blocking of endogenous peroxidase activity in H_2O_2/methanol was carried out for 30 minutes. The slides were initially incubated with 10% NHS in 1% BSA/PBS for 30 minutes. The primary mouse monoclonal anti-pRb antibody (G3-245, Pharmingen) was diluted 1:2500 and applied overnight at 4°C. The secondary biotinylated horse anti-mouse antibody was diluted at 1:1000 and applied for 1 hour at room temperature. In the tonsillar tissue, there was distinct nuclear immunohistochemical expression of pRb in the germinal centers and in the basal epithelial layers.

RESULTS

CDKN2A Genetic Analysis and p16 Immunohistochemistry

Homozygous deletion of *CDKN2A* was detected in 12 of 25 (48%) GBM (Fig. 1, Table). Southern blotting confirmed the results of the PCR assay in all cases for which there was sufficient DNA (data not shown). All tumors with *CDKN2A* HD were immunonegative for p16 expression. The remaining 13 cases (52%) lacked *CDKN2A* HD; of these, 6 stained immunohistochemically for p16 (Fig. 2a), but 7 were negative (Fig. 2b). Five of the 6 immunopositive cases showed extensive, diffuse nuclear and cytoplasmic p16 staining (Fig. 2a), while one tumor had relatively discrete areas of stained and unstained neoplastic cells (Fig. 2c). All 13 available blocks for this case were stained for p16 and showed similar heterogeneous positivity. However, to exclude the possibility that immunonegative cases had staining in other regions, we stained at least 4 blocks on all cases, except one tumor

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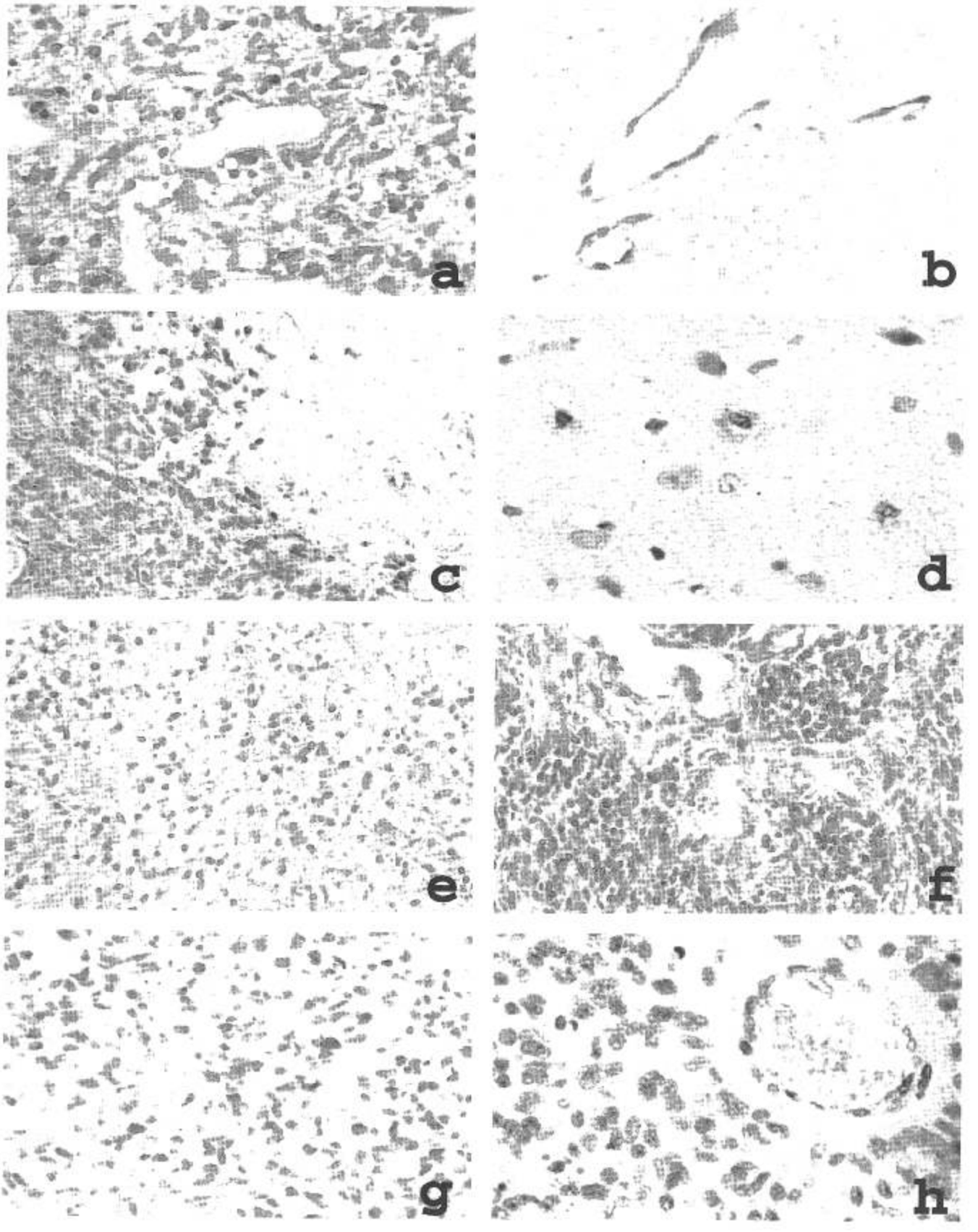
Fig. 2. Immunohistochemistry for p16 (a–d), cdk4 (e, f), and pRb (g, h) (DAB chromogen with hematoxylin counterstain). a: Diffuse nuclear and cytoplasmic staining is present in a GBM (case 1518) with an intact *CDKN2A* gene, but with *CDK4* amplification and *RB* LOH. Note the absence of endothelial staining (×200). b: Case 1634 with *CDKN2A* deletion shows p16-immunonegativity in tumor cells, but striking endothelial positivity (×200). c: Case 1524 showed distinct regions of p16-immunopositivity and p16-immunonegativity, despite identical genetic alterations in the microdissected regions (×200). d: Isolated infiltrating tumor cells stain positively for p16 in case 1644 (which has *RB* LOH), but adjacent neurons and glia are immunonegative for p16 (×400). e: Diffuse cdk4 immunoreactivity is noted in case 1660, which had *CDK4* gene amplification (×200). f: Immunoreactivity similar to that of e is present in case 1662, which lacked *CDK4* gene amplification (×200). g: Strong nuclear staining for pRb is present in case 1532, which had retained both copies of the *RB* gene (×200). h: Only rare vascular cells stain positively for pRb in case 1638, which had allelic loss of the *RB* gene (×400).



TABLE

Summary of results. HD = homozygous deletion; n = normal; 1, 2 = both alleles present; LOH = loss of heterozygosity; nd = no data; ni = noninformative; ns = no shift; * = exon 3 polymorphism; IHC = immunohistochemistry; +/− = discrete p16-immunopositive and p16-immunonegative areas; U = unmethylated; AMP = gene amplification present.

Case #	*CDKN2A* deletion	LOH @ D9S171	LOH @ IFNA	*CDKN2A* SSCP	*CDKN2A* methylation	p16 IHC	LOH @ *RB* 1.20	pRb IHC	*CDK4* amplification	cdk4 IHC
1634	HD					NEG	1, 2	+	n	+
1640	HD					NEG	ni	+	n	+
1646	HD					NEG	1, 2	+	n	+
1648	HD					NEG	ni	+	n	+
1650	HD					NEG	1, 2	+	n	+
1656	HD					NEG	ni	+	AMP	+
1668	HD					NEG	1, 2	+	n	+
1670	HD					NEG	1, 2	+	n	+
1520	HD					NEG	1, 2	NEG	n	+
1534	HD					NEG	LOH	+	n	+
1662	HD					NEG	LOH	+	n	+
1664	HD					NEG	LOH	NEG	n	+
1638	n	1, 2	1, 2	ns		+	LOH	NEG	n	+
1644	n	1, 2	1, 2	ns		+	LOH	NEG	n	+
1504	n	ni	LOH	ns	U	NEG	LOH	NEG	n	+
1514	n	ni	1, 2	ns	U	NEG	LOH	+	n	+
1518	n	1, 2	1, 2	ns		+	LOH	+	AMP	+
1660	n	1, 2	1, 2	ns		+	1, 2	NEG	AMP	+
1532	n	1, 2	1, 2	ns*		+	1, 2	+	AMP	+
1636	n	nd	nd	ns*	U	NEG	1, 2	+	n	+
1642	n	1, 2	1, 2	ns*	U	NEG	1, 2	+	n	+
1500	n	ni	1, 2	ns*	U	NEG	1, 2	+	n	+
1654	n	1, 2	ni	ns	U	NEG	1, 2	+	n	+
1666	n	1, 2	ni	ns	U	NEG	1, 2	+	n	+
1524	n	LOH	LOH	ns		+/−	1, 2	+	n	+

for which no additional blocks were available. No significant staining of neoplastic cells was noted in any of these tumors.

Normal brain did not display staining in any cell type, including neurons, astrocytes, oligodendroglia, and endothelial cells (Fig. 2d). Prominent endothelial staining, however, was noted in the proliferative vasculature of 10 GBM. The neoplastic cells from 8 of these tumors did not express p16, resulting in a striking "vascular pattern" of p16 immunopositivity (Fig. 2b).

The 13 GBM lacking detectable *CDKN2A* HD were studied further for genetic alterations. LOH at D9S171 was detected in 1 of 9 informative cases, and LOH at IFNA in 2 of 10 informative cases (Fig. 3). Interestingly, the one case with LOH at both markers was the tumor that displayed heterogeneous immunohistochemical staining. We therefore extracted DNA from microdissected p16-immunopositive and p16-immunonegative areas. LOH was documented at both D9S171 and IFNA in both immunopositive and immunonegative areas. SSCP on the p16 coding sequences of *CDKN2A* from the 13 GBM showed no migration abnormalities except for identical shifts in the exon 3 amplicon from 4 blood-tumor pairs (Fig. 4). To confirm that these shifts represented a previously described polymorphism, we digested these amplicons with *Msp*I and documented persistence of a 94 bp fragment in addition to the expected 64 bp and 30 bp fragments. Finally, methylation-specific PCR after bisulfite modification of tumor DNA and control normal DNA showed amplification of all studied cases with the unmethylated base-specific primers and no amplification with the methylation-specific primers (data not shown). DNA from the cell lines HT-29 and CaCo2, which have methylation of the *CDKN2A* promoter, amplified with the methylation-specific primers but not with the unmethylated base-specific primers. Notably, therefore, the 7 cases that failed to express p16 despite the absence of *CDKN2A* HD had no evidence of *CDKN2A* gene mutations or methylation of the *CDKN2A* promoter, and only one case had chromosome 9p21 LOH (Table).

CDK4 Gene Amplification and cdk4 Immunohistochemistry

CDK4 amplification was present in 4 (16%) GBM (Fig. 5, Table). Southern blotting confirmed the results of the PCR assay in all cases for which there was sufficient

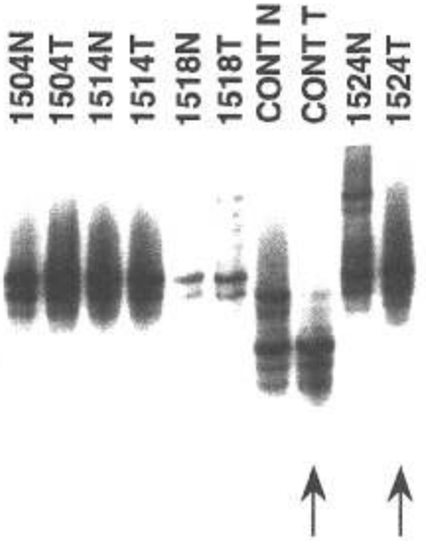


Fig. 3. Chromosome 9p21 LOH at the D9S171 polymorphism. Allelic loss (arrow, upper band) is evident in tumor DNA (T) from case 1524 when compared with constitutional DNA (N) from the same case. A control case (CONT) also shows LOH.

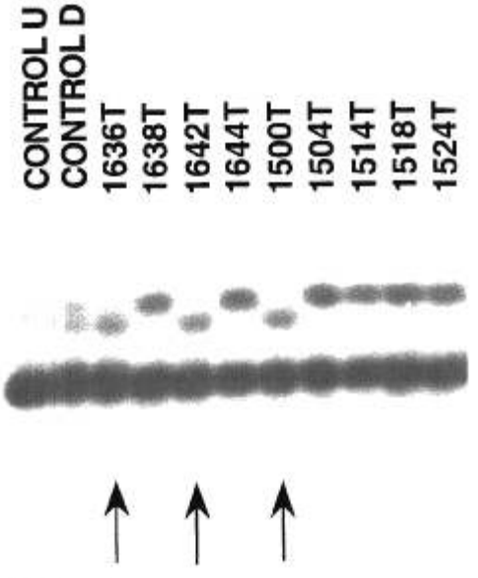


Fig. 4. SSCP analysis of the *CDKN2A* gene. Mobility shifts (arrows) are noted in the exon 3 amplicons from cases 1636, 1642, and 1500. Identical shifts were present in the constitutional DNA from these cases (not shown). Control U = undenatured normal DNA; control D = denatured normal DNA.

DNA (data not shown). By immunohistochemistry, cdk4 was widely expressed in all tumors, with almost all cases staining strongly. Immunopositivity was predominantly nuclear, although some cytoplasmic staining was present as well (Figs. 2e, f). Those cases with *CDK4* gene amplification (Fig. 2e) appeared immunohistochemically similar to those without gene amplification (Fig. 2f).

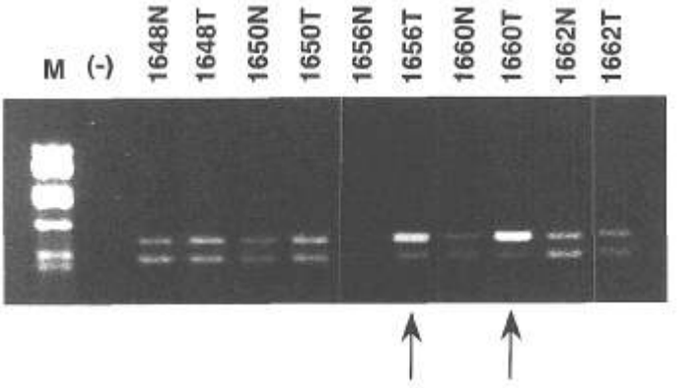


Fig. 5. Differential PCR analysis of the *CDK4* gene. Amplification of the *CDK4* gene is present in tumor DNA (T) from cases 1656 and 1660 (arrows), as evidenced by preferential amplification of the *CDK4* amplicon (top band) relative to the control amplicon (lower band). Constitutional DNA (N) from the same cases does not show amplification, although 1656N amplified poorly. M = pUC18/*Hae*III digest size marker; (−) = "no DNA" lane.

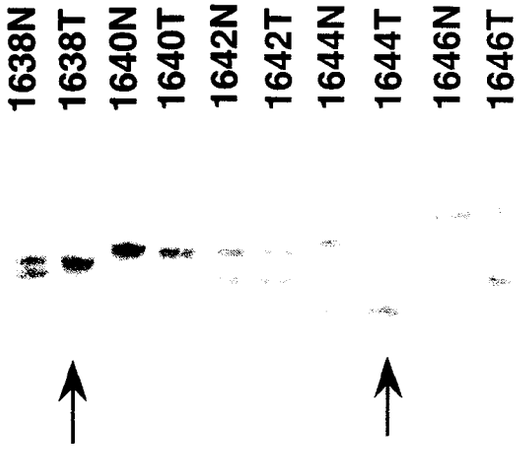


Fig. 6. *RB* gene LOH. Allelic losses (arrows) at the RB 1.20 polymorphism are noted in the tumor DNA (T) in cases 1638 (light bottom band) and 1644 (light top band) when compared with constitutional DNA (N) from the same cases.

RB LOH and pRb Immunohistochemistry

LOH was documented at the intragenic RB 1.20 locus in 8 of 22 (36%) informative cases (Fig. 6, Table). Nineteen of 25 cases (76%) exhibited extensive nuclear immunohistochemical expression of pRb (Fig. 2g) and 6 cases (24%) failed to stain or stained very weakly (Fig. 2h). Of the 22 informative cases, 4 tumors had *RB* LOH and were immunonegative and 12 maintained both *RB* alleles and were immunopositive (Table). However, 4 cases with *RB* LOH were immunopositive and 2 cases that maintained both *RB* alleles lacked significant pRb staining. The trend toward correlation between *RB* LOH and pRb immunohistochemical results did not reach statistical significance.

Regional heterogeneity for pRb staining was not noted, including in the single case that displayed distinct p16-positive and negative areas. However, in those GBM with conspicuous areas of pseudopalisading around necrosis, the viable cells immediately adjacent to the necrotic tissue tended to be immunonegative for pRb, whereas the surrounding palisading cells were often strongly pRb-positive. Nonetheless, this phenomenon was not universal, as one case with obvious pseudopalisading failed to stain for pRb. Nuclear pRb staining was present in proliferating tumor endothelium in 12 cases, 9 with pRb-positive neoplastic cells and 3 with pRb-negative tumor cells.

DISCUSSION

The present study sought to determine the molecular genetic correlates of p16, cdk4 and pRb immunohistochemical analysis. We detected HD of the *CDKN2A* gene in 48% of cases, in agreement with the range observed in previous studies (2, 3, 5, 6, 8, 9). As expected, those GBM with HD of the *CDKN2A* gene did not have immunohistochemically detectable p16 protein in neoplastic cells; conversely, GBM that displayed p16 immunoreactivity in neoplastic cells did not have *CDKN2A* HD. These results suggest that p16 immunohistochemistry may be a useful initial screening procedure, subsequently eliminating the need for detailed *CDKN2A* gene analysis of those tumors that are p16-immunopositive.

On the other hand, the significance of p16 immunonegativity is less clear. Over one-third of p16-immunonegative GBMs did not have HD of the *CDKN2A* gene. Furthermore, detailed mutation, allelic loss, and promoter methylation analyses failed to reveal other mechanisms for gene inactivation in these cases, in concordance with the observed rarity of such alternative *CDKN2A* events in GBM (2, 5, 6, 9–14, 27–29). Thus, although we cannot completely exclude inactivation of the *CDKN2A* gene in these tumors, the gene is not inactivated by mechanisms previously described for *CDKN2A*.

It is possible, however, that lack of p16 immunoreactivity may not necessarily imply loss of p16 expression; in other words, lack of p16 immunoreactivity may not reflect gene inactivation, but may represent a physiologically normal state. Indeed, we did not observe p16 immunoreactivity in normal brain, including in normal astrocytes, and p16 expression is low in most normal cell types at both the mRNA and protein levels (2, 30, 31). In this regard, elevated expression of p16 to a level where it is detectable immunohistochemically might occur only when p16 is physiologically upregulated. For instance, p16 is upregulated in cells that lack functional pRb (4, 32–38) or overexpress cdk4 (4, 20). Significantly, in the present study, all 5 GBM with diffuse immunohistochemical staining for p16 had *CDK4* amplification, *RB* LOH, or both. Such data support the speculation that p16 immunohistochemical positivity represents a response to other alterations in the p16-cdk4-pRb pathway. p16 immunohistochemistry may therefore provide a useful screening procedure in this respect as well, with p16-immunopositivity strongly suggesting either *CDK4* amplification or *RB* LOH.

Two additional findings from the present study support the assertion that p16 immunohistochemical results may reflect physiological regulation in addition to structural genomic alteration. One, GBMs showed marked variation in intratumoral endothelial p16 expression. This expression was not surprisingly independent of both *CDKN2A* genomic status and the p16 expression status of the tumor. Furthermore, there was regional heterogeneity, with some blood vessels demonstrating strong immunoreactivity while nearby vessels were immunonegative. Two, GBM tumor cells may themselves display striking regional variation in p16 expression (as in case 1524) despite identical genomic status of the *CDKN2A* locus. Such findings indicate physiological regulation of p16 expression in GBMs that is independent of structural genetic changes.

A recent study reported a closer correlation between *CDKN2A* gene status and immunohistochemical p16 expression, with all immunonegative cases showing *CDKN2A* HD (39). However, western blot analyses and another immunohistochemical study have shown that a significant number of GBMs that lack *CDKN2A* deletions do not express p16 protein (13, 14, 40). In combination with our results, these latter findings support the conclusion that p16 immunohistochemistry (or western blotting) cannot replace molecular genetic analysis of the *CDKN2A* gene. Instead, p16 immunohistochemistry provides useful complementary information and may be a valuable and simple screening assay. As such, the results of p16 immunohistochemistry could direct detailed molecular genetic studies toward the *CDKN2A* gene if p16-immunonegative, or toward the *CDK4* and *RB* genes if p16-immunopositive.

CDK4 amplification occurs in approximately 10–20% of GBM (3, 4, 6, 16–20), as confirmed by our PCR and Southern blot studies. Prior studies of cdk4 expression have shown good correlation between gene amplification and level of expression on western blotting; only those cases with *CDK4* gene amplification have markedly elevated cdk4 levels (19). However, cdk4 immunohistochemistry showed little variation between cases with and without *CDK4* gene amplification, suggesting that immunohistochemistry fails to distinguish between low and high cdk4 levels. The role of cdk4 immunohistochemistry in the assessment of GBM, therefore, remains dubious, and molecular genetic analysis or western blotting must remain the mainstays for evaluation of this component of the pathway.

Ready analysis of the *RB* gene in primary tumor samples has traditionally been hampered by the large size of the gene, which makes mutation analysis cumbersome. Only 2 studies have analyzed the coding region of *RB* in gliomas in detail, both demonstrating mutations in approximately 5–10% of GBMs (6, 15). LOH of the *RB* region on chromosome 13q14, however, provides a convenient albeit indirect measure of *RB* gene status. As in the present study, which detected *RB* LOH in 36% of informative cases, *RB* LOH has been reported in 35–45% of GBM (6, 15). Assessment of pRb directly in primary tumor tissues has also been hampered, primarily because anti-pRb antibodies that perform well on formalin-fixed, paraffin-embedded tissues have not been widely available until recently. In a prior study using a noncommercial polyclonal antibody directed against a peptide encoded by exon 10, we demonstrated moderate correlation between molecular genetic and immunohistochemical findings (15). Those findings agree with the present study, utilizing a different antibody. Of 16 informative pRb-immunopositive tumors, 12 had maintained heterozygosity as expected, but 4 showed *RB* LOH. Such discrepant cases could arise from the antibody recognizing a mutant protein truncated carboxy-terminal to the epitope site (31). Alternatively, such cases could represent retention of one wild-type copy of *RB*. Detailed mutation analysis and western blot analyses could sort out these possibilities, but were not conclusive in our previous study (15). Of 6 informative pRb-immunonegative GBMs, 4 had *RB* LOH with presumed inactivation of the remaining allele, but 2 pRb-immunonegative cases retained both *RB* alleles. These latter discrepant cases could represent biallelic point mutations or alternative mechanisms for gene inactivation. Regardless of the mechanisms, however, it would seem prudent to combine *RB* LOH and pRb immunohistochemistry in the assessment of GBMs. Even with such combined analysis, selected cases may require detailed mutation analysis and/or western blotting to implicate this component of the pathway.

In summary, with the exception of cdk4 immunohistochemistry, immunohistochemical analyses of the p16-cdk4-pRb pathway provide useful information that can be used in a complementary manner to that provided by molecular genetic analyses. In particular, p16 immunohistochemistry may be a practical screening procedure to direct further molecular genetic studies either toward the *CDKN2A* gene or toward the *CDK4* and *RB* genes.

REFERENCES

1. Cordon-Cardo C. Mutation of cell cycle regulators: Biological and clinical implications for human neoplasia. Am J Pathol 1995;147: 545–60
2. Jen J, Harper W, Bigner SH, et al. Deletion of p16 and p15 genes in brain tumors. Cancer Res 1994;54:6353–58
3. Schmidt EE, Ichimura K, Reifenberger G, Collins VP. CDKN2 (p16/MTS1) gene deletion or CDK4 amplification occurs in the majority of glioblastomas. Cancer Res 1994;54:6321–24
4. He J, Olson JJ, James CD. Lack of $p16^{INK4}$ or retinoblastoma protein (pRb), or amplification-associated overexpression of cdk4 is observed in distinct subsets of malignant glial tumors and cell lines. Cancer Res 1995;55:4833–36
5. Ueki K, Ono Y, Henson JW, von Deimling A, Louis DN. CDKN2/p16 or RB alterations occur in the majority of glioblastomas and are inversely correlated. Cancer Res 1996;56:150–53
6. Ichimura K, Schmidt EE, Goike HM, Collins VP. Human glioblastomas with no alterations of the CDKN2 ($p16^{INK4A}$, MTS1) and CDK4 genes have frequent mutations of the retinoblastoma gene. Oncogene 1996;13:1065–72
7. Louis DN. A molecular genetic model of astrocytoma histopathology. Brain Pathol 1997;7:755–64
8. Walker DG, Duan W, Popovic EA, Kaye AH, Tomlinson FH, Lavin M. Homozygous deletions of the multiple tumor suppressor gene 1 in the progression of human astrocytomas. Cancer Res 1995;55: 20–23
9. Schmidt EE, Ichimura K, Messerle KR, Goike HM, Collins VP. Infrequent methylation of CDKN2A (MTS1/p16) and rare mutation of both CDKN2A and CDKN2B (MTS2/p15) in primary astrocytic tumours. Br J Cancer 1997;75:2–8
10. Moulton T, Samara G, Chung W, et al. MTS1/p16/CDKN2 lesions in primary glioblastoma multiforme. Am J Pathol 1995;146:613–19
11. Ueki K, Rubio M-P, Ramesh V, et al. MTS1/CDKN2 gene mutations are rare in primary human astrocytomas with allelic loss of chromosome 9p. Hum Molec Genet 1994;3:1841–45
12. Merlo A, Herman JG, Mao L, et al. 5'CpG island methylation is associated with transcriptional silencing of the tumor suppressor p16/CDKN2/MTS1. Nature Med 1995;1:686–92
13. Fueyo J, Gomez-Manzano C, Bruner JM, et al. Hypermethylation of the CpG island of p16/CDKN2 correlates with gene inactivation in gliomas. Oncogene 1996;13:1615–19
14. Costello JF, Berger MS, Su Huang H-J, Cavanee WK. Silencing of p16/CDKN2 expression in human gliomas by methylation and chromatin condensation. Cancer Res 1996;56:2405–10
15. Henson JW, Schnitker BL, Correa KM, et al. The retinoblastoma gene is involved in malignant progression of astrocytomas. Ann Neurol 1994;36:714–21
16. Reifenberger G, Reifenberger J, Ichimura K, Melzter PS, Collins VP. 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 1994;54:4299–4303
17. He J, Allen JR, Collins VP, et al. CDK4 amplification is an alternative mechanism to p16 gene homozygous deletion in glioma cell lines. Cancer Res 1994;54:5804–7
18. Reifenberger G, Ichimura K, Reifenberger J, Elkahloun AG, Meltzer PS, Collins VP. Refined mapping of 12q13-q15 amplicons in malignant gliomas suggests CDK4/SAS and MDM2 as independent amplification targets. Cancer Res 1996;56:5141–45
19. Rollbrocker B, Waha A, Louis DN, Wiestler OD, von Deimling A. Amplification of the cyclin dependent kinase 4 (CDK4) gene is associated with high cdk4 protein levels in glioblastoma multiforme. Acta Neuropathol 1996;92:70–74
20. Nishikawa R, Furnari F, Lin H, et al. Loss of $p16^{INK4}$ expression is frequent in high grade gliomas. Cancer Res 1995;55:1941–45
21. Louis DN. The p53 gene and protein in human brain tumors. J Neuropathol Exp Neurol 1994;53:11–21
22. Kleihues P, Burger PC, Scheithauer BW. The new WHO classification of brain tumours. Brain Pathol 1993;3:255–68
23. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning. A laboratory manual. Cold Spring Harbor: Cold Spring Harbor Press; 1989

24. Louis DN, von Deimling A, Seizinger BR. A (CA)n dinucleotide repeat assay for evaluating loss of allelic heterozygosity in small and archival human brain tumor specimens. Am J Pathol 1992;141: 777–82
25. Louis DN, Rubio M-P, Correa K, Gusella JF, von Deimling A. Molecular genetics of pediatric brain stem gliomas. Application of PCR techniques to small and archival brain tumor specimens. J Neuropath Exp Neurol 1993;52:507–15
26. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands. Proc Nat Acad Sci USA 1996;93:9821–26
27. Giani C, Finocchiaro G. Mutation rate of the CDKN2 gene in malignant gliomas. Cancer Res 1994;54:6338–39
28. Sonoda Y, Yoshimoto T, Sekiya T. Homozygous deletion of the *MTS1/p16* and *MTS2/p15* genes and amplification of the *CDK4* gene in glioma. Oncogene 1995;11:2145–49
29. Kyritsis AP, Zhang B, Zhang W, et al. Mutations of the p16 gene in gliomas. Oncogene 1996;12:63–67
30. Shapiro GI, Edwards CD, Kobzik L, et al. Reciprocal Rb inactivation and $p16^{INK4}$ expression in primary lung cancers and cell lines. Cancer Res 1995;55:505–9
31. Geradts J, Kratzke RA, Niehans GA, Lincoln CE. Immunohistochemical detection of the cyclin-dependent kinase inhibitor 2/multiple tumor suppressor gene 1 (CDKN2/MTS1) product $p16^{INK4A}$ in archival human solid tumors: Correlation with retinoblastoma protein expression. Cancer Res 1995;55:6006–11
32. Otterson GA, Kratzke RA, Coxon A, Kim YW, Kaye F. Absence of $p16^{INK4}$ protein is restricted to the subset of lung cancer lines that retains wildtype RB. Oncogene 1994;9:3375–78
33. Tam SW, Shay JW, Pagano M. Differential expression and cell cycle regulation of the cyclin-dependent kinase inhibitor $p16^{INK4}$. Cancer Res 1994;54:5816–20
34. Li Y, Nichols MA, Shay JW, Xiong Y. Transcriptional repression of the D-type cyclin-dependent kinase inhibitor p16 by the retinoblastoma susceptibility gene Product pRb. Cancer Res 1994;54: 6078–82
35. Stone S, Jiang P, Dayananth P, et al. Complex structure and regulation of the *p16 (MTS1)* locus. Cancer Res 1995;55:2988–94
36. Yeager T, Stadler W, Belair C, Puthenveettil J, Olopade O, Reznikoff C. Increased p16 levels correlate with pRb alterations in human urothelial cells. Cancer Res 1995;55:493–97
37. Parry D, Bates S, Mann DJ, Peters G. Lack of cyclin D-Cdk complexes in Rb-negative cells correlates with high levels of $p16^{INK4/MTS1}$ tumor suppressor gene product. EMBO J 1995;14:503–11
38. Aagaard L, Lukas J, Bartkova J, Kjerulff A-A, Strauss M, Bartek J. Aberrations of $p16^{Ink4}$ and retinoblastoma tumour-suppressor genes occur in distinct sub-sets of human cancer cell lines. Int J Cancer 1995;61:115–20
39. Rao LS, Miller DC, Newcomb EW. Correlative immunohistochemistry and molecular genetic study of the inactivation of the $p16^{INK4A}$ genes in astrocytomas. Diagn Mol Pathol 1997;6:115–22
40. Nakamura M, Konishi N, Hiasa Y, et al. Immunohistochemical detection of CDKN2, retinoblastoma and p53 gene products in primary astrocytic tumors. Int J Oncol 1996;8:889–93

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