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Short Communication

MTS1/p16/CDKN2 Lesions in Primary Glioblastoma Multiforme

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The multiple tumor suppressor 1 (MTS1) gene encoding the p16 inhibitor of cyclin-dependent kinase 4 is deleted or mutated in a wide variety of human tumor cell lines, but the importance of this gene as a tumor suppressor in vivo appears to be highly dependent on tumor type. Because MTS1/p16/CDKN2 and the homologous MTS2/p15 gene map to a region of chromosome 9p21, which is frequently deleted in malignant gliomas, we searched for lesions of these genes in primary biopsies of glioblastoma multiforme (GBM). Our analysis confirms a sizable frequency of homozygous deletion of MTS1/p16/CDKN2 (9/27 cases) and also reveals a low but detectable frequency of intragenic DNA lesions (one point mutation in exon 2 leading to premature termination) among GBMs that retain one or both copies of the gene. No mutations were found in exon 2 of MTS2/p15 (12 cases examined), and one GBM showed a DNA deletion breakpoint in the 30 kb between MTS1/p16/CDKN2 and MTS2/p15 resulting in deletion of MTS1/p16/CDKN2 with retention of MTS2/p15. In contrast to the high-grade tumors, none of 12 low-grade gliomas showed MTS1/p16/CDKN2 deletions. These data support a role for MTS1/p16/CDKN2 as a tumor suppressor gene in the in vivo evolution of GBMs. Given that two tumors with hemizygous MTS1/p16/CDKN2 deletions and loss of heterozygosity for chromosome 9p21 did not contain detectable intragenic mutations, there may be one or more additional relevant 9p21 tumor suppressor genes. (Am J Pathol 1995, 146:613–619)

As in other tumor types, the progressive loss or functional inactivation of members of a discrete set of tumor suppressor genes most likely plays a crucial role in the evolution of human gliomas toward increasing malignancy. Because the highest grade of glioma, glioblastoma multiforme (GBM), is also the most common grade at clinical presentation and is associated with mean survival times of <1 year, there is a compelling need for a better understanding of the critical genetic losses that confer the high-grade phenotype. Cytogenetic, loss of heterozygosity (LOH), and deletion analysis in series of gliomas has pinpointed several chromosomal regions including 9p, 10q, 17p, 19q, and 22q that are predicted to harbor relevant tumor suppressor genes, and the 9p, 10q, and possibly 17p losses may be more frequent in high-grade than in low-grade tumors (reviewed in refs. 1 and 2). Deletions of 9p21 were first characterized using probes for the α and β interferon (*IFN*) gene cluster[3–5]. The deletions identified by these probes, which were similar to those observed in a wide variety of other tumor types including lung and bladder carcinomas,[6,7] acute lymphoblastic leukemias,[8] and melanoma cell lines,[9] were often homozygous, suggesting that one or more relevant 9p21 tumor sup-

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pressor genes must lie in close proximity to the *IFN* genes. Subsequently, a search for transcripts arising within a very small minimal deleted region defined by deletion mapping in a series of melanoma cell lines and lying outside of the *IFN* cluster revealed that it contained a gene, *MTS1*, which was identical to a previously identified gene encoding a 16-kd protein inhibitor of the cyclin-dependent kinase 4 (CDK4) kinase.[10,11] Because the CDK4 kinase had been implicated both functionally and by homology as one of a family of cyclin-dependent protein kinases involved in driving progression through the cell cycle,[12] and because tumor cells show abnormal cell cycle regulation, the *MTS1/p16/CDKN2* gene (hereafter abbreviated in the text by the Human Genome Organization designation *CDKN2*) immediately became a strong candidate for the 9p21 tumor suppressor locus. This hypothesis was bolstered by the finding of point mutations within this gene in 14 of 34 melanoma cell lines.[10] Fine mapping of the minimal region of LOH in GBMs[13] is also consistent with the possible involvement of *CDKN2*.

Controversy as to the importance of *CDKN2* as a tumor suppressor gene *in vivo* arose when Cairns et al[14] found a very low frequency of mutations (2/66 cases overall) in the gene in a series of primary tumor biopsies of lung, bladder, kidney, and head and neck carcinomas with LOH at 9p; and Spruck et al[15] found a much lower frequency of deletions and mutations in primary bladder cancers than in bladder cancer cell lines (no mutations in 31 primary tumors from which cell lines had not been derived, although one primary tumor from which a cell line had been derived did show a point mutation). Cairns et al[14] also reported the absence of mutations in each of nine gliomas of unspecified grade that showed 9p LOH. In marked contrast to these findings, Mori et al[16] reported *CDKN2* mutations in 14 of 27 primary esophageal carcinomas, and Caldas et al[17] found mutations in more than half of a large series of pancreatic carcinoma xenografts and short-term explants.

Since a highly homologous gene, *MTS2*, encoding a distinct CDK inhibitor (p15) lies about 30 kb upstream of the *CDKN2* gene, this gene must also be considered as a candidate 9p21 tumor suppressor gene. To assess the status of both of these genes in primary gliomas we have tested for deletions and intragenic DNA alterations in a series of GBMs and for DNA deletions in a series of low-grade gliomas. Our findings support a limited tumor suppressor role for *CDKN2* in glioma tumorigenesis *in vivo* but also suggest the existence of one or more additional 9p21 tumor suppressor genes.

Materials and Methods

Tissue Samples and DNA Extraction

Primary tumor biopsies were obtained during surgery at the time of frozen section diagnosis and were from areas immediately adjacent to tissue that was confirmed histologically as predominantly tumor. DNA was extracted from fresh or frozen tissue by proteinase K/SDS treatment and phenol/chloroform extraction. DNA was also extracted from peripheral blood mononuclear cells of each patient for use as a matching normal control.

Southern Blotting and DNA Probes

DNAs from tumor and peripheral blood were digested with the indicated restriction enzymes, resolved on 1% agarose gels, and transferred to nylon membranes. Probes were genomic clones from the human *H19*[18] (exons 3–5), *HRAS*[19] (EJ-Ras), and polymerase chain reaction (PCR) products from the *CDKN2*[10] and *MTS2/p15*[10] second exons, radiolabeled by hexamer priming. To reduce background hybridization due to the high G+C content of the *CDKN2* and *MTS2/p15* sequences, the labeled probes were quenched with human placental DNA. Hybridization was carried out at high stringency using Hybrisol I (Oncor, Gaithersburg, MD). Blots were washed in 0.1 × SSC/0.1% SDS at 65 C for 1 hour. Densitometry was carried out on lightly exposed autoradiograms using a Macintosh flatbed scanner (ScannerOne) and Image 1.43 software (National Institutes of Health, Bethesda, MD).

PCR, Direct DNA Sequencing and LOH Analysis

Amplification of *CDKN2* exon 2 was carried out by 30 cycles of PCR starting with 150–300 ng of genomic DNA. The 5′ primer had the sequence GGCTCTACACAAGCTTCCTT, and the 3′ primer had the sequence TGAGCTTTGGAAGCTCTCAG. Initial denaturation was at 95 C for 5 minutes, with subsequent denaturations at 95 C for 1 minute, annealing at 58 C for 30 seconds, extension at 72 C for 1 minute, and a final extension at 72 C for 5 minutes. Amplification of *CDKN2* exons 1 and 3 and *MTS2/p15* exon 2 was carried out using the primers and conditions described by Kamb et al.[20] Direct sequencing of 30–50 ng of gel-isolated PCR product was carried out using ^{32}P-γ-ATP-labeled primers, dideoxynucleotides, and modified Taq polymerase (Promega, Madison, WI) for 30 cycles with the following cycling parameters: initial

denaturation at 95 C for three minutes, with subsequent denaturations at 95 C for 1 minute, annealing at 50 C for 30 seconds, and extension at 70 C for 1 minute. Some reactions were carried out in the presence of 5% dimethylsulfoxide. The sequence reaction products were analyzed on 6% acrylamide/8 mol/L urea gels. Allelic status at chromosomes 9q and 9p21 was assessed by PCR using primers for the microsatellite markers D9S51 and RPS6, respectively[21–23] (Research Genetics, Hunstville, AL). PCR conditions for both markers were: 30 PCR cycles with an initial denaturation at 94 C for 5 minutes and subsequent denaturations at 94 C for 1.25 minutes, annealing at 55 C for 30 seconds in the first 15 cycles and 15 seconds thereafter, and extension at 72 C for 45 seconds with a final extension for 5 minutes. The PCR products were separated on 3.0 percent Metaphor agarose gels (FMC BioProducts, Rockland, ME) and visualized by ethidium bromide staining.

Results

MTS1/p16/CDKN2 Deletions

A total of 27 cases of GBM were evaluated for *CDKN2* deletions by Southern blotting using exon 2 as a probe. Normalization for DNA loading was carried out in each case by visual and densitometric comparison to the band intensities obtained in the same lanes with control probes for loci on a different chromosome (chromosome 11; *H19* and *HRAS*) and, in addition, by visual comparison of total DNA loading based on ethidium bromide fluorescence. While none of the cases showed deletions of the control chromosome 11 loci, 9 of the 27 cases (33%) showed homozygous *CDKN2* deletions, and 3 showed apparently hemizygous deletions (Table 1 and Figure 1A). While it is theoretically possible that the apparently hemizygous deletions could reflect contamination of the specimen with normal brain tissue and that they could represent homozygous deletions at the cellular level, the finding of LOH for a 9p21 marker in two of the cases (see below) suggests that these tumors were hemizygous. In any case, it is clear from the combined data that homozygous and hemizygous *CDKN2* deletions together represent a frequent class of genetic lesion in primary GBMs.

To evaluate whether *CDKN2* deletions were specifically associated with high-grade tumors, we carried out an identical Southern blot analysis of 12 low-grade cerebral gliomas. Included in these cases were six pure oligodendrogliomas, three mixed oligoastrocytomas, one pilocytic astrocytoma, and two low-grade gliomas that could not be subclassified. None of these tumors showed deletions (Table 1).

Table 1. *MTS1/p16/CDKN2 Status in 27 Primary GBMs and 12 Low-Grade Gliomas*

Tumor type	Number of cases
Primary GBMs	
Homozygous deletion*	9/27
Hemizygous deletion†	3/27
MTS1/p16/CDKN2 point mutation‡	1/15
MTS2/p15 point mutation§	0/12
No deletion	15/27
Low-grade gliomas	
No deletion	12/12

*Greater than 60% reduction in band intensity on Southern analysis. Eight tumors showed deletion of both *MTS1/p16/CDKN2* and *MTS2/p15*. One tumor showed deletion of *MTS1/p16/CDKN2* with retention of *MTS2/p15*.

†30–50% reduction in band intensity.

‡All cases sequenced through exon 2; two cases with 9p21 LOH and two cases with retention of heterozygosity sequenced through exons 1–3.

§All cases sequenced through exon 2.

Localized MTS1/p16/CDKN2 DNA Lesions

The presence of frequent homozygous deletions encompassing *CDKN2* in GBMs is consistent with the possibility that it could be an important tumor suppressor gene involved in glioma tumor progression; however, given that other genes including the *MTS2/p15* gene and the *IFN* α and β clusters as well as additional genes yet to be identified are encompassed by the majority of 9p21 deletions, a proof will depend on the identification of localizing DNA lesions such as intragenic point mutations or DNA rearrangements. In our Southern blotting analysis we found one tumor (GBM6) that showed a somatic DNA rearrangement on hybridization with the *CDKN2* exon 2 probe, with the generation of new bands on blots of tumor DNA that had been digested with each of several different restriction enzymes (Figure 1, B and C, and data not shown). Rehybridization of the blots with the *MTS2/p15* exon 2 probe, which cross-hybridizes with *CDKN2* sequences, showed that the rearranged bands were again identified but at about twice the previous intensity, normalized to the germline bands in the peripheral blood DNA lane (Figure 1C). Therefore, the rearranged bands contained *MTS2/p15* rather than *CDKN2* sequences. Since digestion with *Sau*3AI restriction enzyme gave a rearrangement at a size of 1 kb (data not shown), the DNA breakpoint maps to within 1 kb of *MTS2/p15* exon 2. Further, the blot obtained after digestion with *Taq*I showed an intact *MTS2/p15* exon 2 and deletion of *CDKN2* exon 2 (Figure 1D). Rehybridization of the blots with probes for *CDKN2* exons 1 and 3 also showed deletions (data

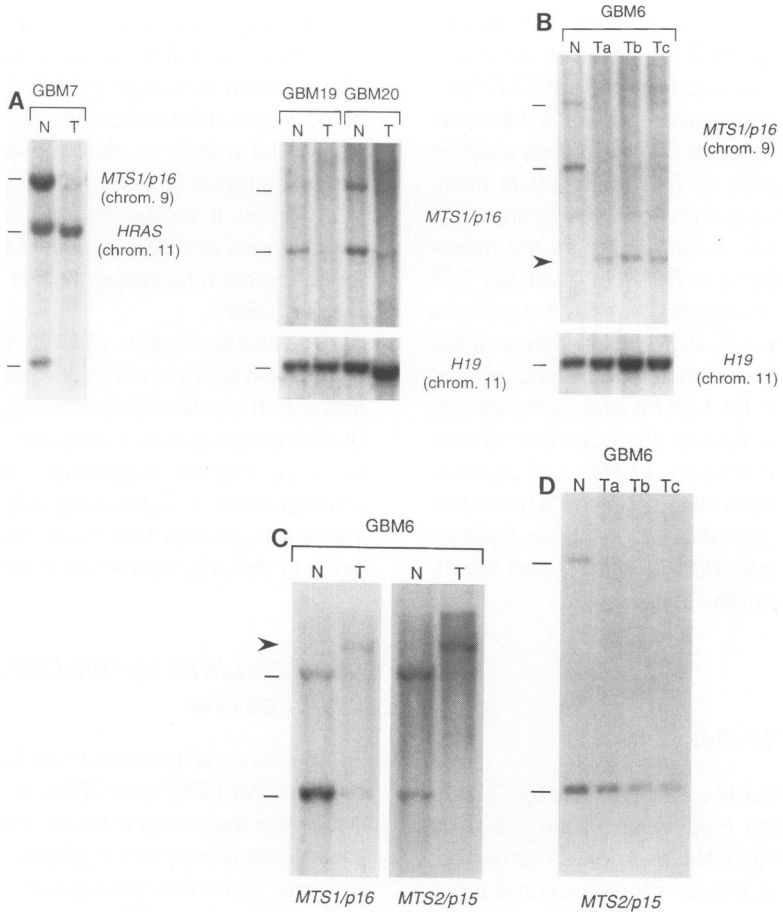

Figure 1. *Southern blot analysis of the* MTS1/p16/CDKN2-MTS2/p15 *locus in GBMs.* (**A**) *Examples of homozygous deletions in GBMs. Genomic DNAs from tumor tissue* (*T*) *and corresponding peripheral blood mononuclear cells* (*N*) *were digested with* Taq *I* (left panel) *or* Eco*RI* (right panel). *DNA loading in each lane was quantitated by reference to the band signals obtained with the control chromosome 11 probes. The* MTS1/p16/CDKN2 *probe detects germline bands at ~4.5 and 1.5 kb with* Taq *I digestion, and 7.5 and 5 kb with* Eco*RI digestion; the more intense bands in each digest represent the* MTS1/p16/CDKN2 *gene, while the less intense bands* (*1.5-kb band on* Taq *I digestion and 7.5-kb band on* Eco*RI digestion*) *represent cross-hybridization to the* MTS2/p15 *gene.* (**B**) *DNA rearrangement detected by the* MTS1/p16/CDKN2 *probe in GBM6 with* Eco*RI digestion. The germline bands are indicated by the dashes and a prominent lower rearranged band at 3 kb is indicated by the arrowhead. There is a marked loss of germline band intensity in the tumor lanes. Three fragments of tumor tissue are indicated as Ta, Tb, and Tc.* (**C**) *Localization of the DNA breakpoint in GBM6 to the vicinity of* MTS2/p15 *by comparison of band intensities after hybridization with* MTS1/p16/CDKN2 *and* MTS2/p15 *probes. With* Bgl*II digestion the* MTS1/p16/CDKN2 *and* MTS2/p15 *germline bands are at 5 and 9 kb, respectively; the rearranged band at 12 kb is less intense than the* MTS1/p16/CDKN2 *germline band on hybridization with the* MTS1/p16/CDKN2 *probe but is more intense than the* MTS1/p16/CDKN2 germline band on hybridization with the MTS2/p15 *exon 2 probe, indicating that it contains* MTS2/p15 *rather than* MTS1/p16/CDKN2 *sequences. Preferential hybridization of the rearranged band with the* MTS2/p15 *probe was also seen on* Eco*RI and* Sau*3AI blots* (*data not shown*). (**D**) *Retention of an intact* MTS2/p15 *exon 2* (lower band) *and homozygous loss of* MTS1/p16/CDKN2 (upper band) *in GBM6 shown by* Taq *I digestion and hybridization with the* MTS2/p15 *probe.*

not shown). By reference to the published map[10] we can therefore conclude that the DNA deletion in this tumor extends from the 3′ border of *MTS2/p15* through the entire *CDKN2* gene, resulting in deletion of *CDKN2* with hemizygous retention of an intact *MTS2/p15* gene. Barring long-range effects on transcription, this suggests that one or more chromosome 9p21 GBM tumor suppressor genes map to a region that includes *CDKN2* and 3′ (downstream) sequences but that excludes *MTS2/p15*.

To search for intragenic mutations, we next subjected *CDKN2* exon 2 PCR products from each of the 15 cases that lacked homozygous deletions to direct sequencing. Among these cases we found one (GBM2) with a G-to-A transversion, which resulted in the conversion of codon 102 from trp (TGG) to a premature stop codon (TGA) (Figure 2A). We note that an identical mutation affecting the same codon was reported by Kamb et al[10] in one melanoma cell line and Caldas et al[17] in two pancreatic carcinoma xenografts. This mutation generated a new *Dde*I restriction enzyme site, and digestion of the exon 2 PCR product with *Dde*I clearly showed that the mutation was absent from the germline of this patient and therefore had occurred as a somatic event in the tumor (Figure 2B). The detection of the mutation by restriction analysis starting from two independent PCR reactions excluded sequencing and PCR artifacts.

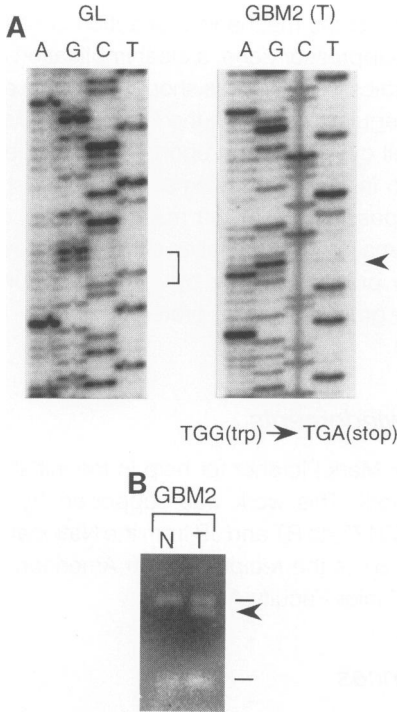

Figure 2. *Mutational analysis of the* MTS1/p16/CDKN2 *gene.* (**A**) *Direct PCR sequencing of exon 2 in GBM2. The mutation is indicated by the arrowhead; the germline sequence (GL) is shown for comparison, with the codon corresponding to the mutation bracketed.* (**B**) Dde *I restriction analysis of exon 2 PCR products from tumor tissue and the peripheral blood mononuclear cells. The mutation creates a unique* Dde *I site in the tumor.*

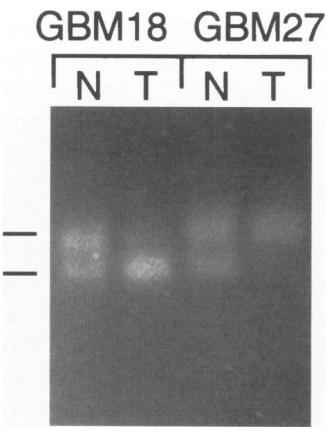

Figure 3. *LOH analysis in two GBMs using the 9pRPS6 microsatellite marker. PCR products from blood mononuclear cell (N) and tumor (T) DNAs were separated by high-resolution Metaphor agarose gel electrophoresis. There is a loss of one of the two allelic bands in each of the tumors. Both of these cases were negative for* MTS1/p16/CDKN2 *(exons 1–3) and* MTS2/p15 *(exon 2) mutations.*

While germline and mutated bands of roughly equal intensity were reproducibly seen on *Dde*I analysis of PCR reactions from this case and while there was no evidence of hemizygous *CDKN2* deletion on Southern analysis (data not shown), we nevertheless cannot conclude that the mutation was heterozygous, because we cannot rule out contamination with non-neoplastic brain tissue in the biopsy of this case (which also did not clearly show LOH at 9p21 microsatellite markers; data not shown).

If *CDKN2* is the only relevant 9p21 tumor suppressor gene, then the classical "two-hit" model for tumor suppressor gene inactivation would predict that all GBMs with 9p21 LOH would show mutations in this gene. Two GBMs with hemizygous *CDKN2* deletions in our series showed LOH at a 9p21 microsatellite marker (RPS6) as indicated by a marked reduction of one of the two allelic bands (Figure 3). Both of these cases, together with two cases that retained both alleles of the gene, were subjected to direct PCR sequencing through all three exons of the *CDKN2* gene, and no mutations were found in the exons and splice donor and acceptor sites (our sequence analysis also confirmed the nucleotide change, T to G at nucleotide 98 in exon 1, previously reported by Hussussian et al[24] as a correction to the originally published sequence and revealed a second change, G to C at nucleotide 494 in the 3′ untranslated region of exon 3, which was present homozygously in both tumor and normal DNA of all three cases and which we interpret as a common polymorphism). To search for possible *MTS2/p15* mutations we sequenced the second exon of this gene (containing 63% of the coding capacity) in 12 tumors, including three cases with hemizygous deletions; no mutations were found.

Discussion

As one of the most clinically malignant human tumors, GBM has been the focus of increasingly intense molecular investigation. This has led to the recognition that several specific chromosomal regions are frequently subject to LOH and/or gross deletion in these tumors, and it is felt that each of these regions must contain one or more relevant tumor suppressor genes. Since chromosome 9p21 is clearly one of the most commonly deleted regions in GBMs as well as in a variety of other high-grade adult malignancies, the discovery that the *CDKN2* gene, encoding an inhibitor of a cyclin-dependent protein kinase, maps to this region and is frequently deleted and mutated in tumor cell lines[10,11] provoked the immediate expectation that a critical tumor suppressor gene had been identified. Enthusiasm was subsequently dampened by reports that *CDKN2* lesions in carcinomas[14,15] and possibly in gliomas[14] were much more frequent in cell lines than in primary tumors, suggesting that the loss of this gene might be an artifact of selection in cell culture, but the opposite conclusion was reached in

studies of primary esophageal cancers and pancreatic carcinomas, which found a high frequency of intragenic point mutations.[16,17] The current study was designed to address the *in vivo* relevance of *CDKN2* as a tumor suppressor gene in human malignant gliomas. Our finding of a high frequency of homozygous deletions of this gene in primary GBMs was not unexpected in view of similar data obtained previously with the closely linked *IFN* probes,[4,5] but our data confirm that *CDKN2* is included in a large proportion of GBM-associated 9p21 deletions. This type of genetic lesion seems to be specifically associated with the malignant phenotype, since it was not observed in our series of low-grade gliomas.

More definitive in terms of support for the *CDKN2* tumor suppressor hypothesis is our observation that rare GBMs without homozygous deletions can show intragenic *CDKN2* DNA lesions. While we were able to detect a *CDKN2* point mutation in only one case in our series, we cannot exclude inactivating genetic or epigenetic lesions at this locus in some proportion of the remaining cases. Our sequence analysis in the non-deletion cases was focused on exon 2, containing 69% of the coding sequence of the gene, but four cases were also completely sequenced through the remaining two exons. Nevertheless, even in the absence of any coding mutations or DNA rearrangements, the gene could be functionally inactivated by transcriptional or post-transcriptional mechanisms. In this regard, studies of p16 protein expression in GBMs and other human neoplasms will clearly be of great interest. Certainly, however, the low frequency of intragenic lesions that we have observed and the lack of such lesions in two cases with 9p21 LOH suggests the possibility that there might exist one or more additional tumor suppressor genes in the 9p21 region. A study by Ueki et al,[25] which appeared while this paper was under review, reached similar conclusions.

The *MTS2/p15* gene, situated within 30 kb of *CDKN2*,[10] is one candidate for the second putative tumor suppressor activity.[26] While eight of nine homozygous deletions of *CDKN2* in our series also encompassed *MTS2/p15INK4B*, one case (GBM6) clearly showed selective deletion of *CDKN2* with a breakpoint of the deletion mapping between *MTS2/p15* and *CDKN2*. This observation, together with the fact that no *MTS2/p15* exon 2 mutations were observed in our series of GBMs, suggests the existence of an as yet unidentified tumor suppressor gene, perhaps situated downstream of *CDKN2*. Mapping of the downstream breakpoint of the GBM6 deletion may be useful in narrowing the region in which this gene is likely to reside.

In terms of the mechanism of action of *CDKN2* as a tumor suppressor gene, a clear implication of functional studies is that its loss should affect the ability of cells to regulate entry into the DNA synthesis phase of the cell cycle. Recent reports of *CDK4* gene amplification in malignant gliomas[27,28] suggest the intriguing possibility that the malignant cells can escape normal growth regulation either by removal of an inhibitory protein (p16) or by overexpression of the cognate growth-positive protein complex (CDK4-cyclin D).

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