CDKN2 (p16/MTS1) Gene Deletion or CDK4 Amplification Occurs in the Majority of Glioblastomas

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Abstract

Forty-six glioblastomas, 16 anaplastic astrocytomas, and 8 astrocytomas were studied for the loss of the CDKN2 (p16/MTS1) gene on 9p. The CDKN2 locus was homozygously deleted in 19 of 46 glioblastomas (41%) and 1 allele was lost in an additional 13 cases (28%). The deleted regions were limited centromERICally in some cases by the MTS2 locus and telomERICally by the 1063.7 locus. CDKN2 was homozygously deleted in 3 of 16 anaplastic astrocytomas (19%) and 2 further cases showed loss of 1 allele. Amplification of the CDK4 gene was present in 7 of 14 (50%) glioblastomas, 7 of 16 anaplastic astrocytomas, and 3 of 11 (27%) anaplastic astrocytomas with no losses at the CDKN2 locus as well as in 2 of 32 (6%) glioblastomas with CDKN2 losses. Thus one or more of these two genes were shown to be aberrant in 85% of glioblastomas and 59% of anaplastic astrocytomas. None of the 8 astrocytomas showed abnormalities of these genes.

Introduction

Cytogenetic and molecular analyses of anaplastic astrocytomas and glioblastomas [WHO malignancy grades III and IV (1)] indicate consistent loss and amplification of genetic information at several specific genomic locations (2–6). One region of loss found among anaplastic astrocytomas and glioblastomas involves portions of the short arm of chromosome 9. The common region of homozygous deletion has been defined as lying between the D9S171 locus and the IFNc/IFNG gene cluster (4). This region has been reported to be deleted in various tumors suggesting that a tumor suppressor gene common to different malignancies is localized in this limited area. Recently, the CDKN2 (p16/MTS1) gene was identified in this area and shown to be deleted in a series of cell lines (7–9). The CDKN2 gene has been reported to be mutated in some families with inherited melanoma susceptibility (10, 11) and also in a high proportion of esophageal (12), pancreatic (13), and non-small cell lung carcinomas (14).

p16 has been shown to specifically inhibit the binding of the CDK4 protein to cyclin D1 (9). Cyclin D1 and CDK4 form a complex which, among others, is capable of phosphorylating the Rb1 protein and thus p16 is involved in inhibiting cells progressing from G1 into S phase. The MTS2 gene has recently been reported to code for a protein, p15INK4A, which has a similar function (15). We have previously described amplification of the CDK4 gene in approximately 15% of anaplastic astrocytomas and glioblastomas, the MDM2 gene being frequently coamplified (6).

In this study we have carried out an allelic assessment at the CDKN2, MTS2, and surrounding loci in a series of 70 human glioma tissue samples. The findings support a role for the CDKN2 gene as a tumor suppressor gene in glioblastomas and anaplastic astrocytomas; furthermore, we have found that CDK4 gene amplification is a common abnormality in tumors not showing loss of CDKN2 gene alleles. It would appear that aberrations of the genes coding for components of this cell cycle-regulatory system occur in at least 85% of human glioblastomas and 50% of anaplastic astrocytomas.

Materials and Methods

Tumor Material, DNA Extraction, and Southern Blotting Analysis. A total of 70 tumors including 46 glioblastomas, 16 anaplastic astrocytomas, and 8 astrocytomas were included in this study. Almost all of the cases have been reported previously (constant case number) in various studies (4, 6). DNA extraction, Southern blotting, hybridization, exposure on Storage Phosphor Screens, and analysis using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and ImageQuant software were performed as described (4). For Southern blotting, TaqI was used as the restriction enzyme.

DNA Probes. DNA probes were generated by PCR amplification using the following primers and normal human leukocyte DNA as template: CDKN2 (MTS1) (exon 1) (2FU, CCCAGTCCAGCAGTGGATGAGGAGG; reverse, GCCAAGTTGAAACGAGCGG; 1063.7) (exon 2) (42F, CCGAAATGGGAACTTGAAGGC; 551RU, CCGAGTCCAGCAGTGGATGAGGAGG; cDNA from the A431 cell line). A 655-base pair PCR fragment corresponding to bases 158 to 812 of the cyclin D1 cDNA (data not shown). A probe for the cyclin D1 gene was generated by reverse transcription-PCR using the primers TCTCTGGTGTGGCTGAATGTAAC and AAGTGGTTGGCGCTGTTGCT and cDNA from the A431 cell line. A 655-base pair PCR fragment corresponding to bases 158 to 812 of the cyclin D1 cDNA sequence (EMBL Accession No. M64349) was subcloned into the pCR II plasmid was used as a probe. The identity of the probe for cyclin D1 was confirmed by sequencing using the Sequenase Version 2.0 DNA Sequencing Kit. The identity of the probe for cyclin D1 was confirmed by sequencing using the Sequenase Version 2.0 DNA Sequencing Kit.

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Kit (USB) (data not shown). The DNA probes for CDK4 and MDM2 have been described previously (6).

**Densitometric Analysis.** Densitometric analysis of allele dosage was performed and calculated as described previously (4). Briefly, hybridization signals were measured using the ImageQuant software, and the "locus of interest" (LOI) hybridization signals were normalized against the signals measured at the control locus D2S44 (CL) on the same blot according to the formula

\[
\text{No. of alleles} = \frac{2 \times N \times \text{LOI(T)}}{\text{LOI(B)}},
\]

with

\[
N = \frac{\text{CL(T@1)}}{\text{CL(T@2)}} \times \frac{\text{CL(B@1)}}{\text{CL(B@2)}}
\]

where T represents tumor, B blood, and A1 and A2 larger and smaller alleles, respectively.

For DNA extraction, tumor pieces containing a minimum of 70% tumor cells were used. On the basis of this principle, we consider an allele value of 0 to 0.6 as representing a homozygous deletion, 0.9 to 1.3 as representing a hemizygous deletion, and values of 1.7 or more as representing retention of both alleles. Only an allele copy number of 5 or more was considered significant amplification.

**Results**

In a previous study we found a common region of homozygous deletion in glioblastomas flanked by the D9S171 and IFNα/ω loci. Nine homozygous deletions involving one or the other or both of these loci were determined among the 47 glioblastomas studied (19%) (4). The incidence of homozygous deletions in this region more than doubled (41%) among the glioblastomas when 4 loci between the D9S171 and the IFNα/ω loci were examined. No homozygous deletions had been identified previously among the anaplastic astrocytomas, yet one case which had previously shown loss of one allele at the IFNα/ω locus and 2 cases in which no losses had been identified at either locus were found to have homozygous deletions located between but not involving the D9S171 or the IFNα/ω loci.

The homozygous deletions in the glioblastomas are frequently large. The smallest region in a single case is found in GB22 and includes the MTS2 and CDKN2 loci (the status of the 1063.7 locus could not be determined in this case) with breakpoints between the c1.b and MTS2 loci centromerically and the CDKN2 and IFNα/ω loci telomerically (Figs. 1 and 2). The homozygous deletions in the anaplastic astrocytomas (AA12, 49, and 18; Fig. 2) all involve a more limited area with AA49 and AA18 (Figs. 1 and 2) limiting the loss of both alleles to the CDKN2 and MTS2 loci with breakpoints centromerically between MTS2 and c1.b and telomerically between CDKN2 and 1063.7. A similar telomeric breakpoint can be found in case GB23 (Figs. 1 and 2) which shows loss of one allele at the CDKN2 locus with retention of both alleles at the 1063.7 locus.

Case GB14 (Figs. 1 and 2) shows a complex pattern of loss with the retention of one allele at the MTS2 locus and loss of both alleles at all surrounding loci suggesting that the MTS2 locus may not always be included in the common region of loss. This is further supported by the deletion mapping of the glioblastomas with losses of one allele. Cases GB25 and GB29 retain both alleles at the MTS2 locus and lose one at the CDKN2 locus and thus have centromeric breakpoints between these loci. Of the loci studied, the common region lost thus always includes only the CDKN2 locus.

When the incidence of losses (of one or both alleles) at all loci are considered, alleles in the region between D9S171 and the IFNα/ω loci were lost in 32 of 46 glioblastomas (70%) with CDKN2 and adjacent loci (MTS2 and 1063.7) showing the highest incidence of loss. The anaplastic astrocytomas showed loss of genetic material from the same region in 10 of 16 cases (63%) but the highest incidence did not occur at the CDKN2 and adjacent loci (5 of 16). The genetic loss was more common at the D9S126 and IFNα/ω loci, often with loss of one allele at both loci, with retention of both alleles at all intervening loci (Fig. 2).

When all tumors were studied for amplification of the CDK4 and MDM2 genes, CDK4 was found to be amplified in 20% of the glioblastomas and 19% of the anaplastic astrocytomas. MDM2 was coamplified in five of the glioblastomas and in two of the anaplastic astrocytomas (Fig. 2). Amplification occurred preferentially among the glioblastomas and the anaplastic astrocytomas which retain both alleles at all loci in the immediate region of the CDKN2 locus. Two glioblastomas, GB28 and GB7, show amplification of CDK4 and MDM2 in the presence of losses of alleles at the CDKN2 and surrounding loci (Fig. 2). GB28 has a homozygous deletion which stretches telomerically from between the D9S126 and the c1.b loci,
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A homzygous deletion of the region encompassing the CDKN2 gene suggests that aberrations of the cell cycle control mechanism are critical for the development of this tumor form. Aberrations of this mechanism are not involved in the development of anaplastic astrocytomas and glioblastomas. They first appear among anaplastic astrocytomas and are most frequent among glioblastomas and thus may be involved in the progression of these tumors. When aberrations of these two genes, the proteins of which are involved in the control of the phosphorylation of Rb1 are included, 85% of the glioblastomas in the series show abnormalities. Cyclin D1, which is also part of this cell cycle-regulatory mechanism, was not found to be amplified in this series. Thus, amplification of cyclin D1 does not appear to be a factor involved in the progression of these tumors.

Some of the findings suggest that CDKN2 may not be the only tumor suppressor gene of interest on 9p. The interstitial homozygous deletion in GB7 encompasses areas centromeric to the c.1b locus. In addition GB10, AA14, AA50, AA34, and AA4 show interstitial losses of one allele in the same region. Another area telomeric to the 1063.7 locus and including the IFNa/loci is frequently involved in interstitial deletions in GB10, AA14, AA30, AA34, and AA19. These two interstitial deletions are commonly found in the same tumor. In the light of reports that suggest that mutation of the CDKN2 gene occurs more frequently in cell lines than in tumors (16, 17), the present data document that the region containing the CDKN2 gene is at which the case was not informative or where quantitative analysis gave intermediate values, not permitting a determination of the state of the alleles. B, summary of the status of amplification at the CDK4 and MDM2 loci. Copy numbers are indicated.

Fig. 2. A, summary of the status of alleles at all loci studied between D9S126 and IFNa/loci in all glioblastomas and anaplastic astrocytomas of the series. The results at the CDKN2 locus represent a combination of the findings using both the CDKN2 exon 1 and exon 2 probes, which gave similar allele values. Loci for which no data are given are loci to a position telomERICally between the 1063.7 and the IFNa/loci. GB28 shows an allele copy number of just over 5 for the CDK4 gene. GB7, which shows loss of one allele at all loci between the c.1b and the 1063.7 loci has a high level of CDK4 and MDM2 amplification (Fig. 2). None of the three anaplastic astrocytomas with losses in the region of the CDKN2 gene showed evidence of amplification of CDK4 or MDM2.

All of the low-grade astrocytomas were assessable at the CDKN2, MTS2, CDK4, and MDM2 loci and no evidence of any losses of alleles or amplification of genes was found. All 70 tumors were additionally screened for amplification of the cyclin D1 gene. However, no amplification was found in this series.

Discussion

The results of this study implicate the CDKN2 gene to be involved in the development of anaplastic astrocytomas and glioblastomas. Homozygous deletion of the region encompassing the CDKN2 gene was found in a high percentage of glioblastomas (41%). In addition, we found the same region homozgyously lost in 19% of the anaplastic astrocytomas. The latter deletions could not be detected in a previous study (4) inasmuch as they are restricted to a region located inbetween the loci examined formerly.

The distance between the CDKN2 gene and its flanking loci, MTS2 and 1063.7 has been estimated to be between 20 and 30 kilobases (7). In this study, breakpoints have been demonstrated within this relatively short distance in a number of the tumors. Breakpoints occurred between the MTS2 locus and the CDKN2 locus in GB25, GB29, and GB14, and between CDKN2 and 1063.7 in GB23, AA18, and AA49. The common region of loss, when data from both the homozygous deletions and losses of one allele are combined, is thus between the MTS2 locus centromERICally and the 1063.7 locus telomERICally and encompasses a region, estimated to be 40–50 kilobases, within which the CDKN2 gene lies.

The finding that amplification of the CDK4 gene occurs in up to 50% of the glioblastomas which show no abnormalities in the region of the CDKN2 gene suggests that aberrations of the cell cycle control mechanism are critical for the development of this tumor form. Aberrations of this mechanism are not involved in the development of astrocytomas. They first appear among anaplastic astrocytomas and are most frequent among glioblastomas and thus may be involved in the progression of these tumors. When aberrations of these two genes, the proteins of which are involved in the control of the phosphorylation of Rb1 are included, 85% of the glioblastomas in the series show abnormalities. Cyclin D1, which is also part of this cell cycle-regulatory mechanism, was not found to be amplified in this series. Thus, amplification of cyclin D1 does not appear to be a factor involved in the progression of these tumors.

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definitely involved in the majority of glioblastomas and some anaplastic astrocytomas. It remains to be seen whether there are any other genes in this limited, commonly deleted region. Recently, MTS2 has been shown to code for another inhibitor of CDK4 and CDK6 (15). Although MTS2 is not included in our commonly deleted region, loss of this gene could also contribute to tumorigenesis, because MTS2 was found to be deleted in addition to CDKN2 in a high percentage of cases studied. Mutational analysis of the CDKN2 gene in the cases retaining one or both alleles is ongoing. Such studies and the effects of the expression of the wild type gene on the phenotype of tumor cells will confirm or exclude the CDKN2 gene as a tumor suppressor gene involved in the progression of gliomas.

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References

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