Mutation Rate of the CDKN2 Gene in Malignant Gliomas

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Abstract

The CDKN2 gene encodes p16, a protein controlling the cell cycle. CDKN2 is deleted in a relevant number of tumor cell lines, but results of the studies in primary tumors are contradictory. We have investigated by using quantitative polymerase chain reaction and single-strand conformation polymorphism analysis the structure of exon 2 of CDKN2 in 32 malignant gliomas. In 11 tumors the amount of amplified material was 21% of that of controls and in 8 tumors it was 42.3%, suggesting the presence of homozygous and hemizygous deletions of the CDKN2 gene, respectively. However, no abnormality could be detected by single-strand conformation polymorphism analysis. The data confirm in primary gliomas that homozygous deletions are a mechanism of CDKN2 inactivation and suggest that another gene in the vicinity could be targeted by mutations.

Introduction

Recurrent abnormalities of the short arm of chromosome 9 are present in various neoplasias and, in particular, in about 30% of malignant gliomas, the most frequent among brain tumors (1–3). The region on 9p21 that is most frequently deleted in cell lines derived from gliomas and other malignancies contains the CDKN2 gene (previously defined as CDK4 or MTS1) encoding p16, a protein that controls cell proliferation during the G1 by inhibiting the catalytic activity of cyclin-dependent kinase 4 (4–6). The finding of homozygous deletions of CDKN2 in 46–61% of tumor cell lines (4, 5) suggested that p16 is the first component of the cell cycle to be identified as a tumor suppressor, with a mutation rate that could rival that of p53. However the possibility is open that such a mutation rate is somehow skewed by the presence of culture artifacts. Indeed, in primary tumors the frequency of p16 mutations seems to vary considerably according to the tumor type (7–9). Here we present the results of investigations on p16 mutations in primary malignant gliomas using quantitative PCR and SSCP analysis.

Materials and Methods

Tumor samples were obtained during surgery (22 patients) or excised from paraffin-embedded material (10 patients; Ref.10). Exon 2 was amplified by three sets of overlapping primers designed to avoid the amplification of the MTS2 gene (4). PCR mixes (50 μl) contained 250 ng genomic DNA, 0.2 μM of each deoxynucleotide, 5% DMSO, and 2.5 units DNA polymerase from Thermus aquaticus and the buffer was supplied by the manufacturers (Boehringer). Fragment 2A (184 bp) was amplified by primers ACA-CAACCTCTCCTTTCCGT (forward) and AAGCCCTCCCGCGCAGCG (reverse); at 94°C for 60 s, 54°C for 45 s, and 72°C for 30 s, 35 cycles after the initial denaturation in a TRIO-Thermoblock from Biometra; fragment 2B (199 bp) by primers GCCACTCTCACCCGACACC and TGTTTACTGCTCTCG-

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3 The abbreviations used are: PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism; bp, base pairs; HOD, homozygously deleted; HED, hemizygously deleted; CPT, carnitine palmitoyl-transferase.

GTGC (at 94°C for 60 s, 57°C for 45 s, and 72°C for 30 s, 35 cycles); and fragment 2C (130 bp) by primers TGAGCTTGGGAAGCTCTCAG and TCG-CACGCTACCTGCCG (at 94°C for 60 s, 55°C for 90 s, and 72°C for 60 s, 35 cycles).

A fragment of the CPTI gene was amplified by primers AI3S (GGGACAGCATTACATTTTGTATT) and P7AAS (ATACTGGGA-CATATCAGGGGATA), at 94°C for 90 s, 58°C for 90 s, and 72°C for 60 s, using 30 or 35 cycles in the absence of DMSO. Identical volumes of CPT and p16 fragments were loaded in each lane of a 2% agarose gel. The densitometric ratio between p16–2A, p16–2B, and p16–2C, respectively, and CPT was evaluated in controls and tumors using the Bio-Profil software from Vilber-Lourmat. For each tumor a "window" was designed, containing the PCR products from CPTI and CDKN2. The software integrates the area and the intensity of each band and defines the relative size. The statistical analysis of densitometric ratios in tumors and controls was performed using the two-tailed t test and the Statview 512+ software.

Results and Discussion

We have investigated the presence of abnormalities in the structure of the second exon of the CDKN2 gene, encoding 68% of the p16 protein, in genomic DNA of 32 malignant gliomas. We initially amplified by PCR three overlapping fragments (p16–2A, -2B, and -2C) that encompass the entire exon in tumor and controls. A portion of CPTI, a housekeeping gene located on chromosome 1 (11), was also amplified and used as an internal standard. Examples of amplifications of p16–2A and CPT are shown in Fig. 1. Densitometric analysis of the PCR products identified a pool of 11 tumors with p16/CPT ratios 1 (or >1) SD lower than that of controls. These tumors were named HOD and showed 21% residual amplification of the CDKN2 gene (as an average of the three fragments), very likely representing the area and the intensity of each band and defines the relative size. The statistical analysis of densitometric ratios in tumors and controls was performed using the two-tailed t test and the Statview 512+ software.

A second series of eight tumors showed lower p16/CPT densitometric ratios than controls (average, 42.3%): these tumors were named HED. In both groups tumors were selected only if results were consistent for each of the three p16 fragments. Both HOD and HED tumor/CPT ratios were significantly lower than those of controls (Table 1).

We subsequently investigated the presence of point mutations in these same p16 fragments by SSCP analysis: no abnormality was found, in particular among the HED tumors. Fig. 2 shows the typical result of one such analysis.

Therefore we have found quantitative alterations of the CDKN2 gene in 59% of malignant gliomas. According to densitometric scanning, alterations in 34% of the patients could be caused by the homozygous deletion of the gene, while in the glioma cell lines 71–87.5% of such deletions have been reported (4, 5). This discrepancy can be hardly accounted for by the presence of normal cells in...
the primary tumor. Rather, it suggests that CDKN2 alterations can favor the establishment of cell lines and are consequently highly represented in cultured gliomas. Similar observations have recently developed in the case of primary bladder cancers and deriving cell lines (9). Nevertheless the finding of CDKN2 homozygous deletions in one third of primary malignant gliomas clearly indicate that this is a major mechanism for the inactivation of the gene. While our work was in progress Cairns et al. (8) reported 10–20% of deletions in different primary tumors, including brain tumors. The number of observations and the histological diagnosis were not provided, and it is possible that the lower percentage of homozygous deletions in their study is due to the inclusion of low-grade gliomas which show a low number of deletions in the short arm of chromosome 9 (1–3).

It is also of relevance that 25% of the gliomas that we analyzed showed amplification patterns consistent with the hemizygous deletion of the CDKN2 gene. This is in agreement with results of Southern blot hybridization studies in primary gliomas using probes derived from the IFN genes, lying in the proximity of the CDKN2 gene (1–3). In these studies the IFNBI probe, for example, detected 12–23% of hemizygous deletions. The higher rate that we found could reflect the fact that we have studied the actual target of deletions or a region closer than IFNBI. Therefore, different sets of experiments indicate that the loss of one copy of the CDKN2 gene in gliomas is a consistent finding. This loss is one part of the mechanism for inactivation of tumor suppressor genes (12); if CDKN2 is one such gene, in malignant gliomas the remaining allele should be inactivated by other genetic alterations.

However when we looked for point mutations or microdeletions by SSCP analysis no such alteration could be found, in particular among the four HED tumors. SSCP analysis should detect 70–90% of mutations (13). Furthermore, we have only analyzed exon 2 of the CDKN2 gene, which encodes two thirds of the p16 coding sequence. However, while investigating the entire coding sequence, 17 (85%) of 20 point mutations were found in this exon (4, 8, 9). Even with these limitations the number of tumors that we have examined should be sufficient to unveil the presence of point mutations. Therefore, unless hemizygous deletions are a mechanism restraining per se CDKN2 activity, that is unless CDKN2 acts dominantly, it is possible that another gene in the proximity is involved in the pathogenesis of gliomas by genetic alterations affecting the short arm of chromosome 9. The investigation of this possibility, along with the definition of the role of CDKN2 inactivation by homozygous deletions in the development of gliomas, will certainly be the subject of active research in the near future.

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References

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