Vol. 217, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 5, 1995 Pages 195-202

DELETION AND TRANSFECTION ANALYSIS OF THE P15/MTS2 GENE IN MALIGNANT GLIOMAS

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Received October 18, 1995

We have investigated the status of the MTS2 gene, encoding the cyclin-dependent kinase (CDK) inhibitor p15, in 32 glioblastomas. Semi-quantitative PCR identified 7 tumors in which the amplified material was 18.6 % of controls and 7 in which was 48.0 %, suggesting the occurrence of homozygous and hemizygous deletions, respectively. Single strand conformation polymorphism analysis identified one polymorphism but no mutations. We also expressed MTS2 and MTS1, encoding the contiguous and highly homologous CDK inhibitor p16, in U-87 human glioblastoma cells. Both genes, either separately or in combination, inhibit significantly the proliferation rate of U-87 cells but such inhibition is progressively lost. As a whole, the data assign a tumor suppressor role to p15 and confirm homozygous deletions as the favorite mechanism for the inactivation of MTS1 and MTS2 in glioblastomas.

In higher eukaryotes cyclin-dependent kinases (CDK) constitute a family of enzymes that catalyze the reactions required for the progression of the cell cycle. CDKs are the target of a complex network of positive and negative growth signals and CDK-inhibitors, in particular, have emerged in recent years as an important new class of negative CDK regulators (1, 2). The first described gene of this family, encoding p21, is a "general" CDK inhibitor and its expression can be activated by wild-type p53 (3, 4). p27, the gene product of the CDK inhibitor KIP1 gene, shows significant homologies to the N-terminal region of p21 (5). p57, a third component of this group of inhibitors, is encoded by the KIP2 gene and shows a carboxy-terminal domain conserved with p27 (6). While p21, p27 and p57 seem to bind the cyclin or the cyclin-kinase complex, p16, p15 and p18, which belong to the sub-group of D-type CDK inhibitors, seem to interact directly with the kinase (7-9).

The role of mutations of CDK inhibitors in cancer development is under active scrutiny. Alterations of p21 and p27, that are part of the group of the "general inhibitors", seem uncommon (10,11) while the p57 gene, which is located in a chromosomal region involved in sporadic and familiar cancers, has not yet been investigated for mutations. On the contrary,

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0006-291X/95 $12.00

deletions of the p16 gene, located on chromosome 9p21, have been found in multiple human cancers (12-14) and have been particularly studied in human malignant gliomas, where the 9p21 region is frequently affected by structural modifications (15) and homozygous deletions have been mapped by molecular analysis (16).

We previously found evidences for homozygous deletions of the MTS1 gene, encoding p16, in about 34% of primary glioblastomas, the most malignant among gliomas (17). Similar percentages were found by other groups (18, 19) and were twice as high when brain tumor xenografts were analyzed (20). Our data also suggested the occurrence of hemyzygous deletions in 25% of the tumors. However single strand conformation polymorphism (SSCP) analysis of the residual allele did not unveil any mutation, a finding further confirmed when we subsequently cloned and sequenced these PCR products (unpublished data). These results suggested that another gene in the vicinity could be the target for inactivation. The MTS2 gene encodes p15, a CDK inhibitor highly homologous to p16 but regulated by TGF-ß (8), lies about 30 kb upstream of p16 and is therefore a natural candidate to the role of alternative target for deletions.

We are now reporting the results of investigations on the presence of mutations of the p15 gene in the same series of tumors in which we investigated the structure of the p16 gene, as well as data deriving from stable transfections of p16 and p15 cDNAs in a human glioma cell line in which they are not expressed.

MATERIALS AND METHODS

PCR Amplification of the MTS2 gene. A list of the PCR primers employed in this study is presented in table 1. Multiplex PCR were carried out in 50 µl reactions using 250 ng of genomic DNA, 25 picomoles of p15 primers (p15ex2F and p15-3'R), 50 picomoles of

Table 1. LIST OF THE PRIMERS USED IN THIS STUDY FOR p15 AND p16 AMPLIFICATION

Name	Sequence	Use
P15-5'	5' GGAAGAGTGTCGTTAAGTTTACG 3'	cDNA amplification
P15-3'	5' GTTGGCAGCCTTCATCGAAT 3'	cDNA amplification
P15-5'F	5' GCGCGTCTGGGGGCTGCGGAA 3'	cDNA-exon 1 amplification
P15-3'R	5' GCGGCTGGGGAACCTGGCGTC 3'	cDNA-exon 2 amplification
P15ex1R	5' CTGGATCGCGCGCCTCCC 3'	exon 1 amplification
P15ex2F	5' TGCCCCGGCCGGCATCTC 3'	exon 2-exon 2I amplification
P15aR	5' GCCCTCCCGGGCAGCATCA 3'	exon 2I amplification
P15aF	5' CTGCCACTCTCACCCGACCGG 3'	exon 2II amplification
P15 IM	5' CTGCCAGAGAGAGCAGAGTGGTC GGAGCC 3'	polymorphism detection
P16-5'	5' CGGAGAGGGGGAGAACAGAC 3'	cDNA amplification
P16-3'	5' TACGAAAGCGGGGTGGGTT 3'	cDNA amplification
P16Fs	5' GAACAGACAACGGGCGGC 3'	cDNA amplification
P16Rs	5' GGCAGTTGTGGCCCTGTAG 3'	cDNA amplification

CPT primers (AI1AS and GGS, ref. 21), 1.5 mM Tris pH 8, 200 μM dNTPs, 1.5 mM $MgCl_2$, 10 % DMSO. 4 min of initial denaturation at 94°C were followed by 35 cycles at 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min. A final extension of 5 min was performed at 72°C. PCR products were separeted by electrophoresis on 2% agarose gels. Densitometric analysis was performed using the Bio-Profil software from Vilber-Lourmat as previously described (17). For each sample (tumor or control DNA) the result was the average of at least two amplifications.

Amplification of the polymorphic fragment of intron 1 was performed with primers p15ex2F and p15IM using the same conditions as for exon 1 with 1 min of denaturation for each cycle. The PCR product was digested with BamHI and loaded on a 15 % polyacrylamide gel.

Exon 1 and 2 of p15 were amplified from DNA of the human glioblastoma cell line U-87 (American Type Culture Collection), using the same conditions described for exon 1 but increasing of 30 sec both the denaturation and the annealing/extension time. Fragments 2A and 2B of exon 2 of p16 were amplified from U-87 DNA as described previously (17), except for a 15 sec increase in the annealing and the extension time.

SSCP analysis. Amplification of exon 1 of p15 was carried out in a 50 μl reaction using 250 ng of genomic DNA, 50 picomoles of primers P15-5'F and P15ex1R (these and other primers employed for PCR experiments are reported in table 1), 1.5 mM Tris pH 8, 200 μM dNTPs, 1.5 mM $MgCl_2$, 5 % DMSO. After an initial denaturation of 4 min at 94°C, 35 cycles were performed at 94°C for 30 sec and 70°C for 1 min. A final extension of 5 min at 72°C was then performed.

Amplification of fragment I and II of exon 2 were carried out with primers p15ex2F-p15aR and p15aF-p15-3'R respectively, using 3 μl of purified PCR of exon 2 (QIAquick Spin PCR purification Kit) and same conditions as for exon 1. 3-10 μl of PCR products were loaded on a 10% acrylamide (49:1), 10% glicerol gel. After running at 30 W in TBE 1X DNA strands were evidenced by silver staining as described (11).

Sequencing of PCR products was performed with the automatic sequencer 373A using the suggested protocols (Applied Biosystems).

RT-PCR of MTS1 and MTS2 and cloning into a eukaryotic expression plasmid. RT-PCR from U-87 total RNA (4 μg) was performed using the Invitrogen cDNA cycle kit with oligo-dT and random primers. Amplification of p15 or p16 cDNA was performed with primers p15-5'/ p15-3' and p16-5'-p16-3', respectively. About 20% of the reaction mixture was examined by agarose gel electrophoresis, while the remaining sample was purified by QIAquick Spin columns (Qiagen) and subsequently re-amplified with nested primers p15-5'F/ p15-3'R and p16Fs/ p16Rs, respectively.

RT-PCR was performed using myoblast poly-A+ RNA (0.75 μg) for p15 and total fibroblast RNA (4 μg) for p16 cDNA amplification, respectively. The two-step procedure described for amplification from U-87 cDNA was used in both cases. The amplification of actin cDNA was used as a control in all RT-PCR reactions. The resulting cDNAs were both subcloned in the pCR 3 vector using the eukaryotic TA cloning kit (Invitrogen).

Transfection and proliferation assays. U-87 cells (80% confluent) were transfected using 5 μg of plasmid DNA purified by Qiagen-tip 100 (Qiagen) and cationic liposomes (DOTAP, Boehringer). Triplicate experiments were performed for each transfection. Forty-eight hours after the lipofection, cells were selected with G418 (300 μg of active drug per ml of culture medium).

The proliferation assay was performed on 1.5 x 10^4 cells plated in triplicate in a 96 well culture plate. 1 μCi of [^{3}H]-thymidine in a 100 μl volume was added to each well three-four hours after seeding and the incorporation was measured 48-72 h later.

RESULTS

We first assessed the amount of tumor DNA that could be amplified by primers encompassing the second exon of the p15 gene. Figure 1 shows several examples of this semi-quantitative analysis. Control DNA, a fragment of the CPT gene located on chromosome 1 (21), was co-amplified in the same tube and the average p15/ CPT densitometric ratio was 1.017 ± 0.274 in 18 control DNA. The densitometric ratio in 18 of the 32 tumors examined

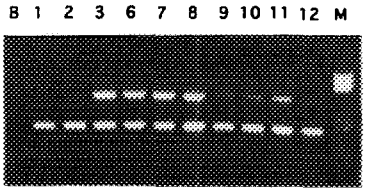

Figure 1. Multiplex PCR of the p15 and the CPT gene in glioblastomas. Electrophoresis on a 2% agarose gel of DNA resulting from the contemporary amplification on 10 tumors of exon 2 of the p15 gene (upper band) and of part of the control CPT gene (lower band). p15 amplification is normal in samples 3 to 8 and deficient in the others. Lane B, blank. Lane M, markers V (Boheringer).

was similar to controls (1.087 ± 0.274; figure 2A). In the other 14 cases the p15/ CPT ratio was lower than the control range: 7 of these tumors had values that were 18.6 % than controls (average ratio 0.189 ± 0.082; p=0.0001 vs. controls) while in the remaining 7 the average ratio was 48 % than controls (0.488 ± 0.081; p=0.0003 vs. controls). Thus, the two groups seem representative of malignant gliomas harboring, respectively, homozygous or hemizygous deletions of the p15 gene. In 47 % of the cases similar patterns of deletion were obtained with the analysis of the p16 gene (figure 2B). In particular, homozygous deletions of both genes were present in four cases while deletions of either of them were present in 10.

In order to look for intragenic mutations, SSCP analysis was performed for both exon 1 and exon 2 of the p15 gene in all tumor DNAs. Shifted bands could only be detected in two cases, but a similar pattern was found in the corresponding lymphocyte DNA (figure 3A). The sequence of tumor DNA fragments showed a C to A transversion at position -27 with respect

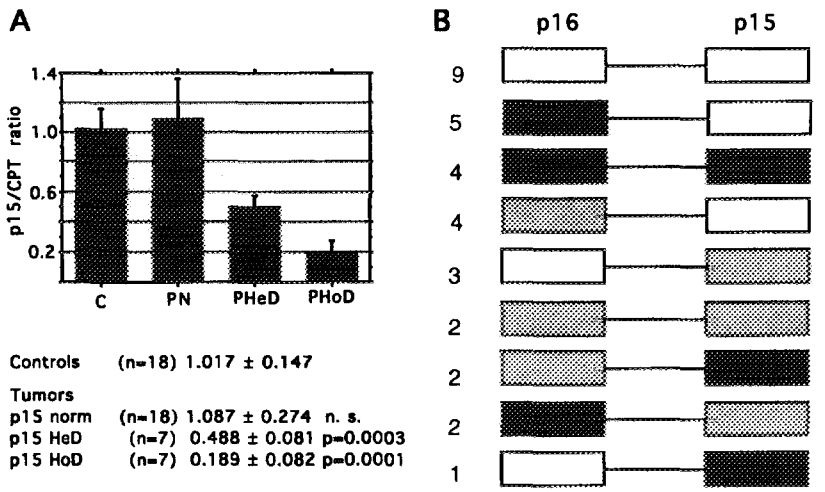

Figure 2. Semi-quantitative analysis of the p15 gene amplification and comparison with p16. Part A shows the average p15/CPT (the gene used as internal control) densitometric ratios in 18 controls and in 32 glioblastomas. 18 tumors had normal ratios (P15 norm) while in the other 14 the amplification pattern suggested the presence of hemyzygous (p15 HeD, n=7) or homozygous (p15 HoD, n=7) deletions. Part B summarizes the status of the p16 and the p15 gene in the same tumors: empty squares indicate a normal pattern of amplification, gray squares hemyzygous deletions and black squares homozygous deletions. Results for p16 derive from our previous work (17).

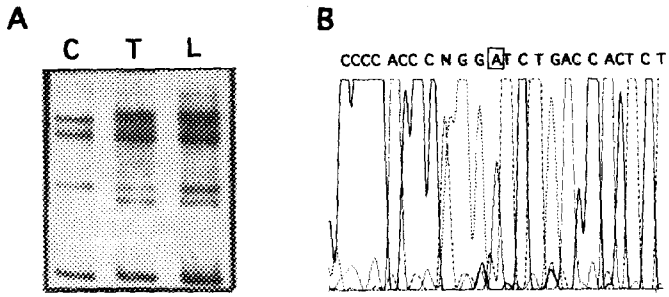

Figure 3. Identification of a polymorphism in intron 1 of the p15 gene. Part A shows the SSCP analysis of fragment 21 (see table 1) of the p15 gene in control DNA (lane C, homozygous C:C) and in tumor and lymphocyte DNA of a patient with glioblastoma multiforme (lane T and L, respectively; heterozygous A:C). Part B shows part of the p15 sequence containing the polymorphism. The variable nucleotide is squared.

to the 3' end of the intervening sequence between the two exons (figure 3B). This base change was investigated by amplifying genomic DNA of 17 control DNAs with primers creating a BamHI site if nucleotide A is present. This study demonstrated the C/A polymorphism in the heterozygous form in 6/17 controls (35 %). Therefore, the PCR-SSCP analysis of the p15 gene failed to detect intragenic mutations but confirmed that, as in the case of p16, deletions of one or both copies of the gene do occur in primary tumors.

To assess the relevance of alterations of these two cognate genes in glioma tumorigenesis we also cloned their corresponding cDNAs into a eukaryotic expression plasmid. In these constructs the expression of the foreign gene was controlled by the CMV promoter/enhancer and the selection in eukaryotic cells was provided by the neo gene. The U-87 recipient cell line, derived from a human GBM, was characterized for the expression of the p15 and p16 genes by RT-PCR and by PCR on genomic DNA. Exons 1 and 2 could be amplified from the MTS2 but not from the MTS1 gene. However, the expression of neither gene was detectable even after two subsequent RT-PCR with nested primers (data not shown).

We then transfected the p15 and p16 cDNAs either separately or in combination and started G418 selection of transfected U-87 cells. In order to define the consequences of CMV-driven expression of these genes on cell cycle progression we studied the incorporation of [^{3}H]-thymidine at different time points after the transfection. The results, shown in table 2, indicate a relevant decrease of [^{3}H]-thymidine incorporation 20 days after transfection. Eleven days later, however, the difference is abolished in p15-transfected cells and is significantly decreased in the other two cell populations. The third determination, performed after one week, confirmed this tendency and demonstrated that the [^{3}H]-thymidine incorporation rate is even higher in cells that were previously expressing p15 and/or p16. Control PCR were performed on DNA extracted at different time points, using a forward primer in the CMV promoter/ enhancer and a reverse primer in the p15 or p16 region.While the neomycin resistance gene could always be amplified, p15 or p16 cDNAs were only detectable during the initial weeks after the transfection, when the U-87 proliferation rate was inhibited (data not

Table 2. EFFECTS OF STABLE TRANSFECTIONS OF THE p15 AND p16 GENES INTO U-87 GLIOMA CELLS AT DIFFERENT TIME POINTS.

	Day 20	Day 31	Day 37
pRC-CMV mock	8591 ± 1583 100 %	18211 ± 3365 100 %	9264 ± 1301 100 %
pRC-p15	3349 ± 374 39 % **	20874 ± 2854 114.6 %	12996 ± 1829 140.3 %
pRC-p16	2582 ± 530 30 % **	15326 ± 1928 84.2 %	13738 ± 1235 148.3 %*
pRC-p15.16	3430 ± 251 39.9 % *	14488 ± 3993 79.6 %	11608 ± 823 125.3 %

Lipofections, G 418 selection and the assay of [^{3}H] -thymidine incorporation were performed as indicated in Methods.
Each value indicates the number of cpm ± the standard error and results from the average of three transfections, each evaluated in triplicate (for each point n=9).
The asterisk indicates that the difference versus the control of the same day is statistically significant (* $p \leq 0.02$; ** $p \leq 0.01$; t- test, two tails).

shown). Furthermore, nude mice inoculated with these cells developed tumors that were similar, both in number and in size, to those obtained with mock-transfected U-87 cells.

These experiments demonstrate that p15 and/or p16 overexpression, either in combination or separately, can significantly restrain the growth-rate of glioma cells and indicate that the expression of these two genes is selected against during the culture of tumor cells.

DISCUSSION

We have studied the sequence of the p15 gene in 32 malignant gliomas previously investigated for mutations of the contiguous p16 gene (17). Alterations were found in 44 % of the cases and were characterized by deletions of either one or both copies of the p15 gene and by the absence of intragenic alterations detectable by SSCP analysis, a pattern of alterations resembling closely that of the p16 gene in gliomas (17-19).

In particular, the presence of homozygous deletions of p15 was suggested in 7 of 32 tumors. Similar deletion rates were found in T-cell malignancies (22, 23) and in non small cell lung cancers (24, 25). In malignant gliomas homozygous deletions were found with higher frequency (27/ 38 gliomas examined) by RT-PCR performed on RNA from tumors xenografted in nude mice (20). Since non neoplastic cells do not grow in the mice, the elimination of this source of contamination of normal DNA might have contributed to the higher frequency of detectable deletions but other factors should probably be considered. First, other mutations could have selectively affected the first exon or the regulatory regions of the p15 gene. These alterations would escape our analysis but could be detected by RT-PCR. At the same time it is possible that some over-representation of deletions is due to genetic alterations occurring or amplified during xenografting. This possibility was ruled out in four of the 27 tumors with homozygous deletions (see figure 1B in ref. 20), but it cannot be

excluded that in few other cases such secondary phenomenons had occurred. Finally, in the report by Jen et al (20), hemyzygous deletions were not considered and this might also contribute to the different mutation rates.

Nevertheless, our data support a tumor suppressor role for the p15 gene in glioma tumorigenesis. This concept is also sustained by the results of the transfection of this gene in the U-87 glioma cell line, where no expression of p15 can be detected by PCR analysis. Following the transfection, the growth rate of tumor cells goes below 40% of controls, even if one month after the transfection this value goes back to the levels of mock-transfected cells. Thus, these experiments demonstrate that p15 expression can significantly inhibit the tumor phenotype, even if p15 expressing cells are subsequently selected against during the culture.

We obtained similar results by transfecting the p16 gene alone or in combination with p15. These observations can provide a possible explanation for differences found in the levels of p16 expression after transfection into tumor cells. Okamoto et al (14) could not detect p16 by immunoblot analysis of the cell population expanded after the transfection while Arap et al (26) were able to detect p16 protein expression in transfected glioma cells and attributed the discrepancy to the transfection of an incomplete cDNA by Okamoto et al or to cell-specific differences. However their immunoblots were performed 7-10 days after transfection: according to our results the selection against p15 clones becomes relevant after about 20 days, so that the time of the analysis might very well explain differences in the results.

Also for p15, as for p16, homozygous deletions are the favorite, and possibly the only mechanism of gene inactivation. Jen et al. have proposed that since such deletions can allow the contemporary inactivation of p15 and p16 they are selected for the advantage conferred to the cell clone that initially harbors them (20). Indeed, the data we obtained 20 days after the transfection of p15 and/or p16 indicate that both genes may act independently as tumor suppressors, so that the inactivation of both by a unique hit could be of advantage for tumor cells. When compared, the alterations of these two genes indicate that homozygous deletions inactivating one or both of them are present in 14 of 32 tumors (see figure 2B). The densitometric analysis of PCR products from the two genes strongly suggests that the homozygous inactivation of both takes place in four of 32 cases, but this figure could be underestimated because of the presence of non-neoplastic cells in the tumor specimen obtained during surgery.

In conclusion, our data indicate that the inactivation of the p15 gene plays a role in the malignant evolution of human gliomas. These results, in combination with the previous data obtained on mutations of the p16 gene, indicate that alterations of the 9p21 region, together with p53 mutations and with alterations of an as yet uncharacterized tumor suppressor gene on chromosome 10q, are among the main factors responsible for glioma tumorigenesis.

ACKNOWLEDGMENTS

We thank Stefano DiDonato for his support to this project, Ruth Albarosa, Franco Carrara, Bruno M. Colombo and Luca Roz for making available tumor and control DNA and Lidia De Filippis for the help in sequence analysis. This work was partially supported by AIRC with a fellowship to M. T. and a research grant to G. F.

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