Jpn. J. Cancer Res. 87, 900–907, September 1996

A Comparative Study of Glioma Cell Lines for *p16*, *p15*, *p53* and *p21* Gene Alterations

Shujing Zhang,[1] Sumio Endo,[1] Hisashi Koga,[1] Tomio Ichikawa,[1] Xuelian Feng,[1] Kiyoshi Onda,[2] Kazuo Washiyama[1] and Toshiro Kumanishi[1,3]

[1]*Department of Molecular Neuropathology, Brain Research Institute, Niigata University, 1 Asahimachi-doori, Niigata 951 and* [2]*Department of Neurosurgery, Brain Research Institute, Niigata University, 1 Asahimachi-doori, Niigata 951*

A total of 10 glioma cell lines were examined for alterations of the *p16*, *p15*, *p53* and *p21* genes, which are tumor suppressor genes or candidates with direct or indirect CDK-inhibitory functions. Genetic alterations (deletions or mutations) were frequently seen in the *p16*, *p15* and *p53* genes in these cell lines, but not in the *p21* gene. When the states of the *p16*, *p15* and *p53* genes were compared among cell lines, all the cell lines showed abnormalities in at least 1 gene, often in 2 or 3 genes coincidentally, suggesting that dysfunction of these genes is closely related to glioma cell growth. Although alteration of all 3 genes was most frequent, there were cell lines having either *p16/p15* or *p53* or *p16* and *p53* gene alterations, suggesting that the time order of these genetic alterations was variable depending on the cell line. Among cell lines examined, one with homozygous *p53* gene deletion seemed of particular practical value, since such a cell line might be useful in various studies, including investigation of the functions of various mutant *p53* genes in the absence of heteromeric protein formation. On examination of the primary tumor tissues, the same alterations of the *p16/p15* and *p53* genes as detected in the cell lines were demonstrated in all 6 cases examined: *p16/p15* gene deletion in 1, *p16* gene mutation in 1 and *p53* gene mutations in 5 cases. This suggested that the *p16/p15* and the *p53* gene alterations and their combinations in at least some glioma cell lines reflected those in the primary glioma tissues.

Key words: Brain tumor — Glioma — Tumor suppressor gene — *p53* gene — *p16* gene

The *p53* tumor suppressor gene is located on chromosome 17p13.1, consists of 11 exons and encodes p53 protein.[1–4] The p53 protein regulates cell proliferation through activation of transcription of the *p21* (Waf1, Cip1, Sdi1, MDA6, CA20) gene,[5] a CDK-inhibitor gene located on chromosome 6p21, the product of which binds to and inactivates various CDKs and PCNA, and ultimately suppresses cell proliferation.[5–7] Various human tumors contain *p53* mutations, mainly in exons 5 through 8, which include the phylogenetically highly conserved domains 2–5.[8] On the other hand, the more recently isolated *p16* (MTS1, INK4, INK4A, CDKN2) gene, a candidate tumor suppressor gene, is located on a different chromosome, 9p21, consists of 3 exons and encodes p16 protein that suppresses cell proliferation during G1 by inhibiting the activities of CDKs 4 and 6.[9–13] *p16* gene alteration (usually deletion) has also been detected commonly in various human malignancies,[9, 10, 14–21] often associated with deletion of the *p15* (MTS2, INK4B) gene, which is another candidate tumor suppressor gene located in the vicinity of the structurally and functionally similar *p16* gene.[9, 12, 13, 21–23] Compared with many other tumor-related genes, the *p53* and the *p16* genes are altered at high frequencies within a given type of tumor. This suggested that inactivation of either gene is very important and might play a major role in carcinogenesis in many tumors. However, genetic alterations of these genes in individual tumors have not been evaluated in many tumors, including gliomas. Analysis from this viewpoint seems essential to gain insight into the mechanism of multistep carcinogenesis, which may vary depending on tumor type and individual tumor.

In this study, we examined and compared 10 glioma cell lines for genetic alterations of the *p16*, *p15*, *p53* and *p21* genes. In some cases, primary glioma tissues from which the cell lines had been derived were also examined for *p16*, *p15* and *p53* gene alterations and the findings were compared with those in the cell lines.

MATERIALS AND METHODS

Glioma cell lines Ten glioma cell lines were used (Table I). Nine (cases 1–6 and 8–10) had been established in our institute,[26–28] and the other was U-251MG (case 7).[29]

DNA isolation DNA was isolated from glioma cell lines and frozen primary glioma tissues by the method of Blin and Stafford[30] and from paraffin-embedded glioma tissue sections by the method of Goelz *et al.*[31]

[3] To whom correspondence should be addressed.

Abbreviations: CDK, cyclin-dependent kinase; GAPDH, glyceraldehydephosphate dehydrogenase; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; SSCP, single strand conformation polymorphism.

Table I. Summary of Genetic Alterations in Human Glioma Cell Lines

Case No.	Primary tumors			Genetic alterations in cell lines			
	Age /sex[a]	Site of tumor[b]	Pathological diagnosis[c]	*p16*[d]	*p15*[e]	*p53*[f]	*p21*[g]
4	63/M	lt. F	anaplastic glioma	del	del	237 (Met-Ile)[h]	− (C/C)
5	27/M	rt. P	anaplastic glioma	del[h]	del[h]	273 (Arg-Cys)[h]	− (C/A)
7	75/M	P	glioblastoma	del	del	273 (Arg-His)	− (C/C)
8	62/M	lt. T	anaplastic glioma	del	del	273 (Arg-Cys)	− (C/C)
9	51/M	rt. T	anaplastic glioma	del	del	−	− (C/A)
10	58/M	lt. T	anaplastic glioma	del	del	−	− (C/C)
2	64/M	lt. F	anaplastic glioma	del	−	Intron 9 (Splice)[h]	− (A/A)
1	38/F	lt. T	anaplastic glioma	83 (His-Tyr)[h]	−	del	− (C/A)
3	64/F	rt. P	glioblastoma	−	−	245 (Gly-Ser)[h]	− (C/A)
6	62/M	lt. P	anaplastic glioma	−	−	273 (Arg-Cys)[h]	− (A/A)

a) Age, years; M, male; F, female.
b) rt, right; lt, left; F, frontal; P, parietal; T, temporal.
c) Anaplastic gliomas belong to grade 3 and glioblastomas belong to grade 4 astrocytomas according to the classification proposed by Kernohan *et al.*[24] Based on WHO criteria,[25] the former belong to grade 3 astrocytomas and the latter to grade 4 glioblastomas.
d) del, homozygous deletion; number, number of codon with missense mutation indicated; −, neither homozygous deletion nor detectable mutation.
e) del, homozygous deletion; −, neither homozygous deletion nor detectable mutation.
f) del, homozygous deletion; numbers, numbers of codons with missense mutations indicated; intron 9 (splice), splice mutation at the splice donor site (gt–tt) in intron 9; −, neither homozygous deletion nor detectable mutation. In all the cell lines with mutation, hemizygosity was suggested from the absence of normal bands in SSCP analysis. Data for some cases (cases 2–7) were from our previous study.[4]
g) −, neither homozygous deletion nor detectable mutation. In parentheses, polymorphic changes at codon 31 are shown by C/C (homozygous AGC), A/A (homozygous AGA) and C/A (heterozygous AGC and AGA).
h) The same genetic alteration was detected in the primary glioma tissues from which cell lines were derived, though the state of the other allele was not clear due to possible contamination with normal cell elements.

Southern blot analysis For examination of the *p16* and the *p15* gene deletions, 10 μg of *Eco*R I-digested DNA was electrophoresed and transferred to nitrocellulose membranes.[32] The membranes were prehybridized, hybridized with ^{32}P-labeled probes and autoradiographed as described previously.[33] The probes were PCR-amplified *p16* gene exons 1, 2 and 3 which were prepared using the primers described by Kamb *et al.* (2F and 1108R),[9] Mori *et al.*[34] and Nobori *et al.* (CDK4I3′),[10] respectively. The *p15* gene deletion was examined by the use of the *p16* exon 2 probe which could detect the *p15* gene exon 2 through cross-hybridization.[9,35] S-100β cDNA[36] was used as a control probe.

In cases without homozygous deletion of the *p16* gene, the 5′CpG island of the *p16* gene was examined for methylation according to Merlo *et al.*[37] Briefly, 10 μg of DNA was doubly digested with *Eco*R I and a methylation-sensitive enzyme, *Sma* I or *Sac* II, electrophoresed, blotted, hybridized with ^{32}P-labeled *p16* exon 1 probe and examined for small DNA fragments generated by the methylation-sensitive enzymes (0.65 and 0.4 kbp bands in *Sma* I and 3.3 and 0.3 kbp bands in *Sac* II digestions).

For examination of the *p21* gene, the same membranes as those used for examination of the *p16* and the *p15* gene deletions were hybridized with a *p21* exon 2 probe prepared by PCR using the primers described.[38]

For examination of *p53* gene deletion in 1 case (case 1), DNA was digested with either *Bam*H I or *Eco*R I, electrophoresed, blotted and examined with a *p53* cDNA probe which was prepared from wild-type *p53* mRNA by

RT-PCR and contained sequences corresponding to exons 4–6.

PCR-SSCP analysis For examination of the *p16* gene, two-step PCR was performed as described previously.[33] In the first PCR, the PCR mixture, 50 μl in volume, containing 100 ng of high-molecular-weight DNA or 1 μg of DNA extracted from paraffin sections was subjected to amplification. The primers for *p16* exons 1 and 3 were the same as those described in probe preparation for Southern blot analysis. For examination of *p16* exon 2, regions corresponding roughly to the 5′ half and the 3′ half were separately amplified using the primers described.[33] The PCR products obtained were purified and subjected to a second PCR amplification. In the second PCR, the PCR mixture, 10 μl in volume, contained the same concentrations of reagents as in the first PCR, except that [α-^{32}P]dATP was added and the amount of unlabeled dATP was decreased. Primers were the same as those used in the first PCR. For SSCP analysis, the second PCR products were diluted,[2] denatured at 95°C for 5 min, electrophoresed in 6% non-denaturing polyacrylamide gel at 4°C and exposed to X-ray film.

PCR-SSCP analyses of *p15* exons 1 and 2, *p21* exon 2 and *p53* exons 4–9 were performed under the same experimental conditions as described above except for the primers used. Primers for *p15* exons 1 and 2 were as described by Okuda *et al.*[21] and Kamb *et al.*,[9] respectively. Primers for *p21* exon 2 in the first PCR were as described for probe preparation for Southern blot analysis. In the second PCR, regions corresponding roughly to the 5′ half and the 3′ half of *p21* exon 2 were separately amplified. For the former, the sense primer was the same as that used in the first PCR and the antisense primer was 5′-AGAGCTTGGGCAGGCCAAGG-3′, while for the latter, the sense primer was 5′-CTTCGCCTGGGAGC-GTGTGC-3′ and the antisense primer was the same as that used in the first PCR. Primers for *p53* exons 4–9 were those described in our previous report.[4]

Nucleotide analysis The first PCR products described were ligated to the *Sma* I cloning site of pUC118 plasmid vector. The recombinant plasmids were transfected into competent JM 109 *E. coli* cells, propagated and purified. The clones obtained were sequenced on both strands by the dideoxy chain-termination method[39] using TaqTrack Sequencing Systems (Promega, Madison, WI). In each experiment, sequencing was performed on 4–8 recombinant plasmids derived from different bacterial colonies. To confirm the mutation, a second PCR and subsequent nucleotide analyses were performed.

Northern blot analysis For Northern blot analysis of *p16* mRNA, 10 μg of total RNA was electrophoresed on a 1.3% agarose gel and transferred to nitrocellulose membranes as described by Thomas.[40] The membranes were prehybridized and then hybridized with the ^{32}P-labeled *p16* gene exon 1 probe by the same method as described for Southern blot analysis. After hybridization, the membranes were washed to a final stringency of 0.1× SSC and 0.1% sodium dodecyl sulfate for 30 min twice at 68°C and exposed to X-ray film.

RESULTS

Analysis of *p16* and *p15* gene deletions Southern blot analyses of the *p16* and the *p15* genes were performed on *Eco*R I-digested DNA using the *p16* exon 1, 2 and 3 probes. In the control placenta, the *p16* exon 1 and the *p16* exon 3 probes revealed a single band, 4.3 kbp and 4 kbp, respectively, and the *p16* exon 2 probe revealed two bands, 4 kbp and 5.6 kbp, of which the 4 kbp band was from the *p16* gene (Fig. 1a). In the examination of glioma cell lines, 7-cell lines (cases 2, 4, 5 and 7–10) showed no bands for the *p16* gene with any of the 3

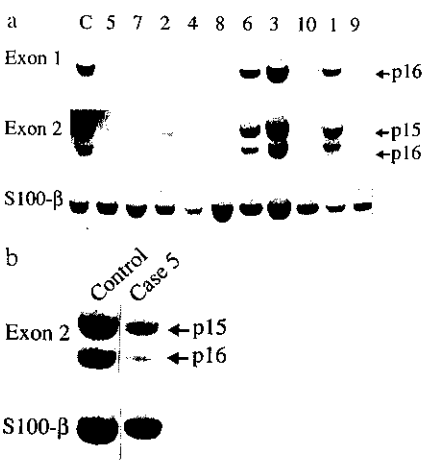

Fig. 1. Southern blot analysis of the *p16* and the *p15* genes. a) Glioma cell lines. Southern blots of *Eco*R I-digested DNAs were hybridized with either the *p16* exon 1 or the *p16* exon 2 probe. With either probe, the band for the *p16* gene is seen in 3-cell lines (cases 1, 3 and 6), but not in the remaining 7-cell lines (cases 2, 4, 5 and 7–10). A similar result was obtained with the *p16* exon 3 probe (data not shown). On examination with the *p16* exon 2 probe, the band representing the *p15* gene which resulted from cross-hybridization is seen distinctly in 3-cell lines (cases 1, 3 and 6) and faintly in 1-cell line (case 2), but not in the remaining 6-cell lines (cases 4, 5 and 7–10). S-100β gene used as an internal control was hybridized in the same membrane. C, control placental DNA. b) Primary glioma tissue of case 5. Southern blot of *Eco*R I-digested DNA was hybridized with the *p16* exon 2 probe. Compared with the control, bands for the *p16* and the *p15* genes are fainter in the primary glioma tissue of case 5. The S-100β gene used as an internal control was hybridized in the same membrane.

probes mentioned above (Fig. 1a), indicating a homozygous deletion of the *p16* gene involving all 3 exons in these cases. The remaining 3-cell lines (cases 1, 3 and 6) showed bands for the *p16* gene with these probes.

Among the 7-cell lines with homozygous deletion of the *p16* gene, 6 (cases 4, 5 and 7–10) also revealed homozygous deletion of the *p15* gene, as could be seen in the examination with the *p16* exon 2 probe (Fig. 1a). While a 5.6 kbp band for the *p15* gene, which resulted from cross-hybridization due to the structural similarity of exons 2 of the *p16* and the *p15* genes,[9, 35] was seen distinctly (cases 1, 3 and 6) or faintly (case 2) in 4-cell lines, it was not seen in these 6-cell lines. Thus, frequent homozygous deletions of the *p16* and the *p15* genes, which were often coincidental, were found in our glioma cell lines. In Table I, these findings are summarized along with other data.

Analysis of *p16* and *p15* gene mutations PCR-SSCP analysis of the *p16* and the *p15* genes was performed on the 4-cell lines (cases 1–3 and 6) in which the *p16* and/or the *p15* genes were detected in Southern blot analysis. Among these 4-cell lines, 3-cell lines (cases 2, 3 and 6) revealed no abnormality in SSCP and nucleotide analyses of the *p16* gene (exons 1–3) and/or the *p15* gene (exons 1 and 2). However, in the case of the remaining 1-cell line (case 1), aberrant bands suggesting an intragenic mutation were detected in SSCP analysis of exon 2 of the *p16* gene (Fig. 2a). From the absence of distinct normal bands, deletion of another allele was suggested. Subsequent nucleotide analysis demonstrated a missense mutation at codon 83 (CAC-TAC, His-Tyr) in exon 2 of the *p16* gene (Fig. 2b) in all 9 plasmid clones derived from two independent PCR products. Although the same mutation has been found only rarely in some tumor tissues,[16, 41] it causes severe functional inactivation as found in *in vitro* assay systems.[42]

Thus, *p16* and *p15* gene inactivations were demonstrated in 8 (7 homozygous deletions and 1 mutation) and 6 (6 homozygous deletions) of the 10 glioma cell lines, respectively (Table I).

Analysis of the 5′CpG island of the *p16* gene with respect to methylation In 3-cell lines (cases 1, 3 and 6) without homozygous *p16* gene deletion, which included 2-cell lines without mutation (cases 3 and 6) and 1-cell line with a mutation (case 1), the 5′CpG island was examined for methylation by Southern blot analysis using methylation-sensitive enzymes, *Sma* I and *Sac* II, according to Merlo *et al.*[37] In each cell line, the *Eco*R I-digested fragment containing the 5′CpG island was further digested to smaller fragments by these methylation-sensitive enzymes, indicating that the sites for these restriction enzymes in the 5′CpG island were not methylated (Fig. 3a). In agreement with this finding, Northern blot analysis with the use of the *p16* exon 1 probe revealed bands for *p16* mRNA[35] in these 3 cases (Fig. 3b).

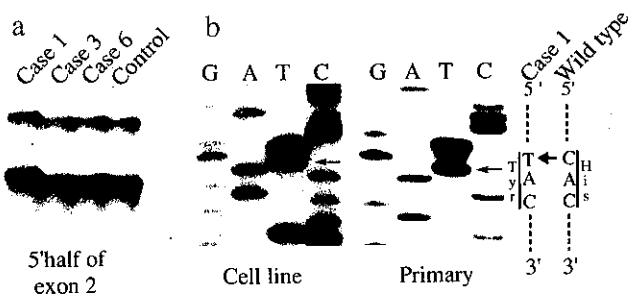

Fig. 2. Detection of *p16* gene mutation at codon 83 in 1-cell line (case 1) and the primary glioma tissue from which it had been derived. a) SSCP analysis of the 5′ half of *p16* exon 2 in 3-cell lines without homozygous deletion of the *p16* gene. Electrophoretic mobility shifts are seen in 1-cell line (case 1). Note that normal bands are not seen in this cell line. Control, placental DNA. b) Nucleotide analysis of the *p16* gene exon 2 in 1-cell line (case 1) and the primary glioma tissue from which it had been derived. A missense mutation at codon 83 (CAC-TAC, His-Tyr) is seen in PCR clones from the cell line and the primary glioma tissue. The same mutation was detected in all 9 PCR clones from the cell line and in 2 of 4 PCR clones from the primary glioma tissue.

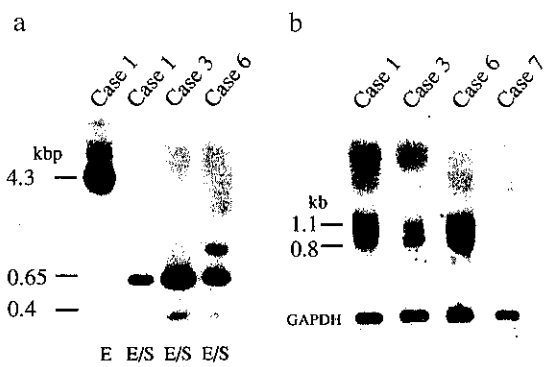

Fig. 3. Analysis of 5′CpG island methylation of the *p16* gene in 3-cell lines without homozygous *p16* gene deletion. a) Southern blots of DNAs doubly digested with *Eco*R I and a methylation-sensitive enzyme, *Sma* I, were hybridized with the *p16* gene exon 1 probe. Each cell line shows only doubly digested small DNA fragments. A band of about 1 kbp in case 6 might be due to insufficient *Sma* I digestion, since the signal density of this band was variable from experiment to experiment. Double digestion with *Eco*R I and *Sac* II also revealed only small DNA fragments (data not shown). E, digested with *Eco*R I alone. E/S, digested with *Eco*R I and *Sma* I. b) Northern blot analysis with the *p16* exon 1 probe shows bands representing *p16* mRNA in these 3-cell lines (cases 1, 3 and 6), but not in 1-cell line (case 7) with homozygous *p16* gene deletion. GAPDH mRNA used as an internal control was hybridized in the same membrane.

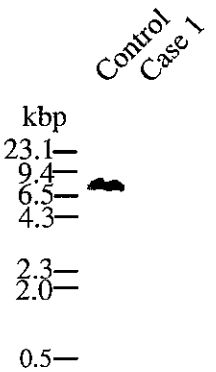

Fig. 4. Southern blot analysis of the *p53* gene in 1-cell line (case 1). The Southern blot of *Bam*H I-digested DNA shows no band hybridized with the *p53* cDNA probe, indicating homozygous *p53* gene deletion. The Southern blot of *Eco*R I-digested DNA gave similar results (data not shown). Control, placental DNA.

Analysis of *p53* gene mutations and deletion Using PCR-SSCP and nucleotide analyses, we detected missense mutations in 6-cell lines (cases 3–8) and a splicing mutation in 1-cell line (case 2). Some of these data have been reported previously.[4] All of the missense mutations were located in the conserved domains, in previously proposed hot spot codons or in their vicinity,[8] suggesting that they were likely to affect the function of the p53 protein. The splicing mutation in 1-cell line was shown to result in abnormal splicing.[4] In another cell line (case 1), our previous study showed no PCR amplification in any of the exons 4–9,[4] suggesting severe alteration of the *p53* gene. In the present study, we performed Southern blot analysis on this cell line using a *p53* cDNA probe which contained a sequence corresponding to exons 4–6. As can be seen in Fig. 4, no hybridized band was detected after either *Bam*H I or *Eco*R I digestion. This finding strongly suggested homozygous deletion of the *p53* gene, which has been described only rarely in cell lines of some tumors,[43] but not in gliomas or glioma cell lines. Thus, *p53* gene alteration with presumed functional inactivation was observed in 8 of the 10 glioma cell lines. In Table I, the states of the *p53* gene in these cell lines detected in the previous[4] and present studies are shown together.

Analysis of *p21* gene In Southern blot analysis with the *p21* exon 2 probe, each cell line revealed a single 9 kbp band in *Eco*R I digestion, suggesting the absence of major rearrangement (data not shown). PCR-SSCP and nucleotide analyses of the *p21* gene were performed on exon 2 which contains about 90% of the coding sequence. No abnormality was detected in any of the 10-cell lines including 2-cell lines without detectable *p53* gene alteration (Table I). However, a polymorphism[44,45] was seen at codon 31, which was homozygous AGC in 4, homozygous AGA in 2 and heterozygous AGC and AGA in 4-cell lines (Table I).

Examination of primary glioma tissues In some cases, the *p16* and *p53* genes in the primary glioma tissues from which the cell lines had been derived were also examined. The *p16* gene was examined in 2 cases (cases 1 and 5), in which the cell lines showed homozygous *p16* and *p15* gene deletions (case 5) or a missense mutation at codon 83 of the *p16* gene (case 1). In the former case (case 5), Southern blot analysis of the primary tumor revealed only faint bands of the *p16* and the *p15* genes in examination with the *p16* exon 2 probe (Fig. 1b, Table I). In the latter case (case 1), the same missense mutation (CAC-TAC, His-Tyr) at codon 83 of the *p16* gene as in its cell line was detected in 2 of 4 PCR clones from paraffin-embedded tumor tissue (Fig. 2b, Table I). Examination of the *p53* gene in the primary tumor tissues was performed in our previous study,[4] in which we detected in 5 cases (cases 2–6) the same missense (cases 3–6) or splicing (case 2) mutations as in the cell lines (Table I). Thus, in all cases examined, the same genetic alterations as in the cell lines were detected in the *p16* and the *p53* genes in the primary gliomas, although the states of other alleles were not clear due to possible contamination of normal cell elements in the tumor tissues. These findings strongly suggested that these gene alterations detected in the cell lines had occurred in the primary tumors.

DISCUSSION

In this study, we examined the states of the *p16*, *p15*, *p53* and *p21* genes in 10 human glioma cell lines which had been derived from grade 3 or 4 malignant gliomas.[26] On examination of the *p16*, *p15* and *p53* genes, we detected frequent alterations in each gene in our glioma cell lines. In addition, when the states of the *p16*, *p15* and *p53* genes were compared among cell lines, all the cell lines showed abnormalities in at least 1 gene, and often in 2 or 3 genes coincidentally (Table I). Although coincident *p16* and *p15* gene alterations were often observed in various cell lines and tumors,[9,12,13,21–23] their coincidence with *p53* gene alterations seemed less common.[15,46] Thus, the frequent alteration of each of these genes and the frequent coincidence of alterations seemed to be a feature of glioma cell lines, suggesting that alterations of these 3 genes are closely related with glioma cell growth. Arap *et al.*[35] obtained similar findings in *p16* and *p53* genes in their glioma cell lines. Although coincidental alteration of all 3 genes was most frequent in our cell lines, some showed either *p16/p15* or *p53* or *p16* and *p53* gene alterations (Table I). Thus, it was also suggested that the

time order of genetic alteration of these genes was variable from case to case.

During examination of the *p16* gene, we detected homozygous deletion at a high frequency, in agreement with previous results on glioma cell lines[9, 10, 18, 35] and primary[17, 21, 47, 48] and xenografted[17] malignant glioma tissues. In addition, we detected a missense mutation (CAC-TAC, His-Tyr) at codon 83 of the *p16* gene in 1-cell line (case 1), in which the lack of another allele was suggested by SSCP and sequence analyses. Moreover, the same mutation was found in the primary glioma from which this cell line had been derived. Except for some types of tumors,[16, 34, 41, 49] mutation of the *p16* gene is uncommon in many malignancies and, to our knowledge, it has not been reported in gliomas. Although at present the functional effect of individual mutations of the *p16* gene is largely unknown, some mutations have been examined with respect to their effect on CDK-inhibitory activity, using *in vitro* assay systems.[42, 50] Yang *et al.*[42] examined several mutations, including the same mutation (His-Tyr) at codon 83 as in our cell line (case 1). They showed that the mutational effect was variable, from negligible to severe, depending on the mutation; and it was rather severe in the case of the mutation (His-Tyr) at codon 83, which reduced the CDK-inhibitory activity to about 8% of the wild-type level. A decreased half-life of the *p16* protein with this mutation has also been reported.[51] Thus, it was strongly suggested that the *p16* gene was severely inactivated in our mutant cell line and that the inactivated gene was derived from the primary glioma.

In 3-cell lines without homozygous *p16* gene deletion, 5′CpG island methylation was also examined. 5′CpG island methylation was shown to be an important alternative mechanism of *p16* gene inactivation in recent studies.[37, 52] Merlo *et al.*[37] reported that transcription of the *p16* gene was blocked when the 5′CpG islands of this gene were methylated. They found methylated 5′CpG islands associated with blocked *p16* mRNA expression in about 40–80% of carcinoma cell lines. Though it was less frequent, about 20–30% of cases examined also showed 5′CpG island methylation in primary tumors, including glioma tissues. However, in our 3 glioma cell lines without homozygous deletion which included 2-cell lines without mutation (cases 3 and 6) and 1-cell line with a mutation (case 1), dysfunction of the *p16* gene due to 5′CpG island methylation was not suggested in Southern and Northern blot analyses.

So far, *p53* gene alterations in a variety of human malignancies have been reported and the most frequent alteration has been shown to be intragenic mutations with or without hemizygosity. In accordance with this, our glioma cell lines also revealed frequent mutations with hemizygosity. In addition, we observed homozygous deletion of the *p53* gene in 1-cell line (case 1). From the present Southern blot and the previous PCR analyses,[4] the deleted region was suggested to be extensive. Homozygous deletion involving an extensive region of the *p53* gene is extremely rare in various tumors[43] and, to our knowledge, it has not been reported in gliomas or glioma cell lines. By introducing a mutant *p53* gene in such a cell line, it should be possible to analyze in detail the function of the expressed *p53* in the absence of heteromeric p53 proteins.

Since cell growth control by the *p53* gene is mediated by the *p21* gene,[5] genetic alteration of the *p21* gene might have the same effect as that of the *p53* gene. However, except for a polymorphic change at codon 31, we detected no *p21* gene alterations in our cell lines, including 2-cell lines without *p53* gene alteration. Similar findings have been reported in various tumors.[44, 45]

In this study, we also examined *p16*, *p15* and *p53* gene alterations in some of the primary tumor tissues from which cell lines had been derived. In the cases of the *p16* and *p15* genes, we examined 2 primary glioma tissues (cases 1 and 5). Consistent with the observations in the cell lines, *p16* and *p15* gene deletions were presumed to have occurred in one primary tumor (case 5) and the same missense mutation of the *p16* gene was detected in another (case 1). Regarding the *p53* gene, we examined 5 primary tumors and demonstrated the same mutations as those detected in the cell lines in all of these cases.[4] Thus, although the states of other alleles in the primary tumors were not clear due to possible contamination with normal cell elements, it seemed likely that the *p16*, *p15* and *p53* gene alterations and their combinations in at least some of the glioma cell lines reflected those in the primary tumors. Therefore, further detailed analyses of glioma cell lines, including correlation of *p16*, *p15* and *p53* gene inactivation with abnormalities of other tumor-related genes,[53] through which we might gain insight into the details of multistep carcinogenesis or progression of primary gliomas, are warranted.

ACKNOWLEDGMENTS

We are grateful to Drs. R. Tanaka, K. Morii and I. Tsumanuma of the Department of Neurosurgery of our Institute and Y. Takahashi, S. Abe, H. Usui of our laboratory for their constant interest and support. Moreover, we wish to thank Ms. C. Honma, Mr. K. Kobayashi and Mr. S. Oyanagi and Ms. H. Kato of our department for their technical assistance. This study was supported by the Ministry of Education, Science, Sports and Culture of Japan, the Japanese Cancer Research Resources Bank, the Fujisawa Foundation, the Inoue Foundation for Science and the Sasagawa Health Science Foundation.

(Received April 5, 1996/Accepted June 3, 1996)

REFERENCES

1) Batra, S. K., Rasheed, B. K. A., Bigner, S. H. and Bigner, D. D. Oncogenes and anti-oncogenes in human central nervous system tumors. *Lab. Invest.*, **71**, 621–637 (1994).
2) Zhang, S. J., Feng, X. L., Koga, H., Ichikawa, T., Abe, S. and Kumanishi, T. p53 gene mutations in pontine gliomas of juvenile onset. *Biochem. Biophys. Res. Commun.*, **196**, 851–857 (1993).
3) Koga, H., Zhang, S. J., Kumanishi, T., Washiyama, K., Ichikawa, T., Tanaka, R. and Mukawa, J. Analysis of p53 gene mutations in low- and high-grade astrocytomas by polymerase chain reaction-assisted single-strand conformation polymorphism and immunohistochemistry. *Acta Neuropathol.*, **87**, 225–232 (1994).
4) Koga, H., Zhang, S. J., Washiyama, K., Ichikawa, T., Onda, K. and Kumanishi, T. Analysis of p53 gene mutations in human glioma cell lines. *Brain Tumor Pathol.*, **13**, 1–10 (1996).
5) El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W. and Vogelstein, B. WAF_1, a potential mediator of p53 tumor suppression. *Cell*, **75**, 817–825 (1993).
6) Waga, S., Hannon, G. J., Beach, D. and Stillman, B. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature*, **369**, 574–578 (1994).
7) Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R. and Beach, D. p21 is a universal inhibitor of cyclin kinases. *Nature*, **366**, 701–704 (1993).
8) Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C. C. p53 mutations in human cancers. *Science*, **253**, 49–53 (1991).
9) Kamb, A., Gruis, N. A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavtigian, S. V., Stockert, E., Day, R. S., III, Johnson, B. E. and Skolnick, M. H. A cell cycle regulator potentially involved in genesis of many tumor types. *Science*, **264**, 436–440 (1994).
10) Nobori, T., Miura, K., Wu, D. J., Lois, A., Takabayashi, K. and Carson, D. A. Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature*, **368**, 753–756 (1994).
11) Serrano, M., Hannon, G. J. and Beach, D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature*, **366**, 704–707 (1993).
12) Hannon, G. J. and Beach, D. $p15^{INK4B}$ is a potential effector of TGF-β-induced cell cycle arrest. *Nature*, **371**, 257–261 (1994).
13) Guan, K.-L., Jenkins, C. W., Li, Y., Nichols, M. A., Wu, X., O'Keefe, C. L., Matera, A. G. and Xiong, Y. Growth suppression by p18, a $p16^{INK4/MTS1}$- and $p14^{INK4B/MTS2}$-related CDK6 inhibitor, correlates with wild-type pRb function. *Genes Dev.*, **8**, 2939–2952 (1994).
14) Lo, K.-W., Huang, D. P. and Lau, K.-M. p16 gene alterations in nasopharyngeal carcinoma. *Cancer Res.*, **55**, 2039–2043 (1995).
15) Okamoto, A., Demetrick, D. J., Spillare, E. A., Hagiwara, K., Hussain, S. P., Bennett, W. P., Forrester, K., Gerwin, B., Serrano, M., Beach, D. H. and Harris, C. C. Mutations and altered expression of $p16^{INK4}$ in human cancer. *Proc. Natl. Acad. Sci. USA*, **91**, 11045–11049 (1994).
16) Caldas, C., Hahn, S. A., da Costa, L. T., Redston, M. S., Schutte, M., Seymour, A. B., Weinstein, C. L., Hruban, R. H., Yeo, C. J. and Kern, S. E. Frequent somatic mutations and homozygous deletions of the p16 (MTS1) gene in pancreatic adenocarcinoma. *Nat. Genet.*, **8**, 27–32 (1994).
17) Jen, J., Harper, J. W., Bigner, S. H., Bigner, D. D., Papadopoulos, N., Markowitz, S., Willson, J. K. V., Kinzler, K. W. and Vogelstein, B. Deletion of p16 and p15 genes in brain tumors. *Cancer Res.*, **54**, 6353–6358 (1994).
18) Gomi, A., Sakai, R., Ogawa, S., Shinoda, S., Hirai, H. and Masuzawa, T. Frequent loss of the cyclin-dependent kinase-4 inhibitor gene in human gliomas. *Jpn. J. Cancer Res.*, **86**, 342–346 (1995).
19) Giani, C. and Finocchiaro, G. Mutation rate of the CDKN2 gene in malignant gliomas. *Cancer Res.*, **54**, 6338–6339 (1994).
20) Schmidt, E. E., Ichimura, K., Reifenberger, G. and Collins, V. P. CDKN2 (p16/MTS1) gene deletion or CDK4 amplification occurs in the majority of glioblastomas. *Cancer Res.*, **54**, 6321–6324 (1994).
21) Okuda, T., Shurtleff, S. A., Valentine, M. B., Raimondi, S. C., Head, D. R., Behm, F., Curcio-Brint, A. M., Liu, Q., Pui, C.-H., Sherr, C. J., Beach, D., Look, A. T. and Downing, J. R. Frequent deletion of $p16^{INK4a/MTS1}$ and $p15^{INK4b/MTS2}$ in pediatric acute lymphoblastic leukemia. *Blood*, **85**, 2321–2330 (1995).
22) Okamoto, A., Hussain, S. P., Hagiwara, K., Spillare, E. A., Rusin, M. R., Demetrick, D. J., Serrano, M., Hannon, G. J., Shiseki, M., Zariwala, M., Xiong, Y., Beach, D. H., Yokota, J. and Harris, C. C. Mutations in the $p16^{INK4}$/MTS1/CDKN2, $p15^{INK4B}$/MTS2, and p18 genes in primary and metastatic lung cancer. *Cancer Res.*, **55**, 1448–1451 (1995).
23) Otsuki, T., Clark, H. M., Wellmann, A., Jaffe, E. S. and Raffeld, M. Involvement of CDKN2 ($p16^{INK4A}$/MTS1) and $p15^{INK4B}$/ MTS2 in human leukemias and lymphomas. *Cancer Res.*, **55**, 1436–1440 (1995).
24) Kernohan, J. W., Mabon, R. F., Svien, H. J. and Adson, A. W. Symposium on a new and simplified concept of gliomas; simplified classification of gliomas. *Proc. Mayo Clin.*, **24**, 71–75 (1949).
25) Kleihues, P., Burger, P. C. and Scheithauer, B. W. "Histological Typing of Tumours of the Central Nervous System," 2nd Ed., pp. 11–15 (1993). Springer-Verlag, Berlin.
26) Onda, K., Tanaka, R., Washiyama, K., Takeda, N., and Kumanishi, T. Correlation of DNA ploidy and morphological features of human glioma cell cultures with the establishment of cell lines. *Acta Neuropathol.*, **76**, 433–440 (1988).

27) Onda, K., Washiyama, K., Tanaka, R., Nishiyama, A., Usui, H. and Kumanishi, T. Establishment of human glioma cell lines with stable and unstable GFAP expression. *Biomed. Res.*, **13**, 327–334 (1992).
28) Yamazaki, K. Tumorigenicity of established human glioma cell lines in lasat and nude mice . *Neuropathology*, **3**, 29–38 (1982) (in Japanese).
29) Bigner, D. D., Bigner, S. H., Ponten, J., Westermark, B., Mahaley, M. S. Jr., Ruoslahti, E., Herschman, H., Eng, L. F. and Wikstrand, C. J. Heterogeneity of genotypic and phenotypic characteristics of fifteen permanent cell lines derived from human gliomas. *J. Neuropathol. Exp. Neurol.*, **40**, 201–229 (1981).
30) Blin, N. and Stafford, D. W. A general method for isolation of high molecular weight DNA from eukaryotes. *Nucleic Acids Res.*, **3**, 2303–2308 (1976).
31) Goelz, S. E., Hamilton, S. R. and Vogelstein, B. Purification of DNA from formaldehyde fixed and paraffin embedded human tissue. *Biochem. Biophys. Res. Commun.*, **130**, 118–126 (1985).
32) Southern, E. M. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.*, **98**, 503–517 (1975).
33) Kumanishi, T., Zhang, S.-J., Ichikawa, T., Endo, S. and Washiyama, K. Primary malignant lymphoma of the brain: demonstration of frequent p16 and p15 gene deletions. *Jpn. J. Cancer Res.*, **87**, 691–695 (1996).
34) Mori, T., Miura, K., Aoki, T., Nishihira, T., Mori, S. and Nakamura, Y. Frequent somatic mutation of the MTS1/CDK4I (multiple tumor suppressor/cyclin-dependent kinase inhibitor) gene in esophageal squamous cell carcinoma. *Cancer Res.*, **54**, 3396–3397 (1994).
35) Arap, W., Nishikawa, R., Furnari, F. B., Cavenee, W. K. and Huang, H.-J. S. Replacement of the p16/CDKN2 gene suppresses human glioma cell growth. *Cancer Res.*, **55**, 1351–1354 (1995).
36) Morii, K., Tanaka, R., Takahashi, Y., Minoshima, S., Fukuyama, R., Shimizu, N. and Kuwano, R. Structure and chromosome assignment of human S100 α and β subunit genes. *Biochem. Biophys. Res. Commun.*, **175**, 185–191 (1991).
37) Merlo, A., Herman, J. G., Mao, L., Lee, D. J., Gabrielson, E., Burger, P. C., Baylin, S. B. and Sidransky, D. 5′ CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. *Nat. Med.*, **1**, 686–692 (1995).
38) Michieli, P., Chedid, M., Lin, D., Pierce, J. H., Mercer, W. E. and Givol, D. Induction of WAF1/CIP1 by a p53-independent pathway. *Cancer Res.*, **54**, 3391–3395 (1994).
39) Sanger, F., Nicklen, S. and Coulson, A. R. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467 (1977).
40) Thomas, P. S. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA*, **77**, 5201–5205 (1980).
41) Zhang, S.-Y., Klein-Szanto, A. J. P., Sauter, E. R., Shafarenko, M., Mitsunaga, S., Nobori, T., Carson, D. A., Ridge, J. A. and Goodrow, T. L. Higher frequency of alterations in the p16/CDKN2 gene in squamous cell carcinoma cell lines than in primary tumors of the head and neck. *Cancer Res.*, **54**, 5050–5053 (1994).
42) Yang, R., Gombart, A. F., Serrano, M. and Koeffler, H. P. Mutational effects on the $p16^{INK4a}$ tumor suppressor protein. *Cancer Res.*, **55**, 2503–2506 (1995).
43) Masuda, H., Miller, C., Koeffler, H. P., Battifora, H. and Cline, M. J. Rearrangement of the p53 gene in human osteogenic sarcomas. *Proc. Natl. Acad. Sci. USA*, **84**, 7716–7719 (1987).
44) Shiohara, M., El-Deiry, W. S., Wada, M., Nakamaki, T., Takeuchi, S., Yang, R., Chen, D.-L., Vogelstein, B. and Koeffler, H. P. Absence of WAF1 mutation in a variety of human malignancies. *Blood*, **84**, 3781–3784 (1994).
45) Koopmann, J., Maintz, D., Schild, S., Schramm, J., Louis, D. N., Wiestler, O. D. and von Deimling, A. Multiple polymorphisms, but no mutations, in the WAF1/CIP1 gene in human brain tumors. *Br. J. Cancer*, **72**, 1230–1233 (1995).
46) Spruck, C. H. III, Gonzalez-Zulueta, M., Shibata, A., Simoneau, A. R., Lin, M.-F., Gonzales, F., Tsai, Y. C. and Jones, P. A. p16 gene in uncultured tumours. *Nature*, **370**, 183–184 (1994).
47) Dreyling, M. H., Bohlander, S. K., Adeyanju, M. O. and Olopade, O. I. Detection of CDKN2 deletions in tumor cell lines and primary glioma by interphase fluorescence *in situ* hybridization. *Cancer Res.*, **55**, 984–988 (1995).
48) Walker, D. G., Duan, W., Popovic, E. A., Kaye, A. H., Tomlinson, F. H. and Lavin, M. Homozygous deletions of the multiple tumor suppressor gene 1 in the progression of human astrocytomas. *Cancer Res.*, **55**, 20–23 (1995).
49) Yoshida, S., Todoroki, T., Ichikawa, Y., Hanai, S., Suzuki, H., Hori, M., Fukao, K., Miwa, M. and Uchida, K. Mutations of $p16^{Ink4}$/CDKN2 and $p15^{Ink4B}$/MTS2 genes in biliary tract cancers. *Cancer Res.*, **55**, 2756–2760 (1995).
50) Ranade, K., Hussussian, C. J., Sikorski, R. S., Varmus, H. E., Goldstein, A. M., Tucker, M. A., Serrano, M., Hannon, G. J., Beach, D. and Dracopoli, N. C. Mutations associated with familial melanoma impair $p16^{INK4}$ function. *Nat. Genet.*, **10**, 114–116 (1995).
51) Shapiro, G. I., Park, J. E., Edwards, C. D., Mao, L., Merlo, A., Sidransky, D., Ewen, M. E. and Rollins, B. J. Multiple mechanisms of $p16^{INK4A}$ inactivation in non-small cell lung cancer cell lines. *Cancer Res.*, **55**, 6200–6208 (1995).
52) Gonzalez-Zulueta, M., Bender, C. M., Yang, A. S., Nguyen, T. D., Beart, R. W., van Tornout, J. M. and Jones, P. A. Methylation of 5′CpG island of p16/CDKN 2 tumor suppressor gene in normal and transformed human tissues correlates with gene silencing. *Cancer Res.*, **55**, 4531–4535 (1995).
53) Hesketh, R. "The Oncogene Facts Book," (1995). Academic Press, London.