Jpn. J. Cancer Res. **90**, 934–941, September 1999

# Rare-type Mutations of *MMAC1* Tumor Suppressor Gene in Human Glioma Cell Lines and Their Tumors of Origin

Shu-Jing Zhang,[1] Sumio Endo,[1] Tomio Ichikawa,[1] Junichi Yoshimura,[2] Kiyoshi Onda,[2] Ryuichi Tanaka,[2] Kazuo Washiyama[1] and Toshiro Kumanishi[1, 3]

*[1]Department of Molecular Neuropathology and [2]Department of Neurosurgery, Brain Research Institute, Niigata University, 1-757 Asahimachi-doori, Niigata 951-8585*

**A total of 10 glioma cell lines were examined to evaluate the status of the *MMAC1* gene, a candidate tumor suppressor gene. Six cell lines showed mutations with presumed loss of heterozygosity and 1 cell line showed no mRNA expression. The 6 mutations consisted of 3 3-bp deletions (codons 17, 101 or 199), 1 missense mutation (codon 252) and 2 truncation mutations (1 nonsense mutation at codon 233 and 1 2-bp insertion at codon 241). Among them, the 3-bp deletions, which are a rare type of mutation in *MMAC1* gene, were located in the N-terminal half (codons 1–212) of the coding region, which is considered important in *MMAC1* function. The missense mutation was located unusually in the C-terminal half (codons 212–403), but it was in a small region in which some other reported missense mutations are clustered. Thus, these 4 mutations were suggested to have functional effects on the *MMAC1* activity, like the other 2 mutations with predicted protein truncations. By sequence analysis of cDNA clones, we confirmed that all the mutations including these 4 rare ones were in the *MMAC1* gene, not in the *PTH2* pseudogene. In 2 cases, we also examined the primary glioma tissues from which the cell lines had been derived and found the same mutations as in the cell lines in both cases. This suggested that the mutations in these cell lines were derived from the primary glioma tissues, but not from artifacts arising during long-term *in vitro* cultivation.**

Key words: *MMAC1* — *PTEN* — Tumor suppressor gene — Pseudogene — Glioma

The *MMAC1* gene (also called *PTEN* and *TEP1*), a candidate tumor suppressor gene which is located on chromosome 10q23, contains 9 exons and encodes 403 amino acids.[1–3] The proximal half of the protein is homologous to phosphatases and cytoskeleton-associated proteins, tensin and auxilin,[1–3] and its phosphatase activity has been demonstrated in *in vitro* assays.[3–6] *MMAC1* gene alterations have already been examined in various tumors including malignant glioma, prostate carcinoma and endometrial carcinoma.[1, 2, 7–19] Among them, malignant gliomas have revealed frequent alterations in both primary tumor tissues and cell lines,[1, 2, 7–14] consistent with the previous LOH (loss of heterozygosity) studies that showed frequent deletion of regions of chromosome 10 in malignant gliomas.[20–22] *MMAC1* gene alterations in malignant gliomas included small deletions, small insertions, splicing mutations, nonsense mutations and missense mutations. However, despite the considerable number of *MMAC1* gene alterations reported, the entire profile of the alterations in malignant gliomas and other tumors as well has not been fully detailed.

In this study, we examined a total of 10 glioma cell lines for alterations of the *MMAC1* gene and its mRNA and found mutations in 6 cell lines and no mRNA expression in 1 cell line. All the mutations, which included 4 rarely reported ones, were confirmed to be in the *MMAC1* gene itself, but not in the *PTH2* pseudogene (also called *ψPTEN*),[23, 24] and were analyzed in connection with previous results in various malignancies. In 2 cases, we also examined primary glioma tissues from which the cell lines had been derived and detected the same mutations as in the cell lines.

## MATERIALS AND METHODS

**Glioma cell lines** Ten human glioma cell lines were used. Nine (cases 1–6 and 8–10) had been established in our institute[25–28] and the other was U-251MG (case 7).[29] The pathological diagnosis of the primary tumors from which the cell lines were established was anaplastic glioma (WHO grade III) in 8 cases and glioblastoma (WHO grade IV) in 2 cases.[25] Brief summaries of the clinical and pathological data of the patients are given in Table I.

**DNA isolation** DNA was isolated from glioma cell lines and frozen primary glioma tissues as described.[25]

**RNA isolation** Total RNA of glioma cell lines was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method.[30]

[3] To whom correspondence should be addressed.
E-mail: kumanisi@bri.niigata-u.ac.jp

The abbreviations: SSCP, single-strand conformation polymorphism; RT-PCR, reverse transcriptase-polymerase chain reaction; LOH, loss of heterozygosity.

Table I. Summary of *MMAC1* Mutations in Glioma Cell Lines

| Case[a] | Primary tumor | | | *MMAC1* alterations in cell lines | | | | | LOH[g] | *PTH2* expression[h] |
|---|---|---|---|---|---|---|---|---|---|---|
| | Age/sex | Site of tumor[b] | Pa Dx[c] | Exon | Codon | Change | | Predicted effect[f] | | |
| | | | | | | Gene[d] | mRNA[e] | | | |
| 1 | 38/F | T | AG | | | | | | | |
| 2 | 64/M | F | AG | exon 6 | 199 | del ATG* | same | Met del | + | − |
| 3 | 64/F | P | GB | exon 1 | 17 | del CAA | same | Glu del | + | − |
| 4 | 63/M | F | AG | | | | | | | |
| 5 | 27/M | P | AG | exon 7 | 233 | CGA-TGA* | same | stop | + | − |
| 6 | 62/M | T | AG | | | | no mRNA | no protein | | |
| 7 | 75/M | P | GB | exon 7 | 241 | ins TT | same | fs(256) | + | − |
| 8 | 31/F | P | AG | | | | | | | |
| 9 | 51/M | T | AG | exon 7 | 252 | GAT-GGT | same | Asp-Gly | + | − |
| 10 | 58/M | T | AG | exon 5 | 101 | del ATC | same | Ile del | + | − |

*a*) All the cell lines except case 7 (U-251MG) were established in our Institute.[25–28] Among them, cases 3, 6 and 10 are Onda 11, 10 and 9 cell lines, respectively.[26, 27]
*b*) Pa Dx, pathological diagnosis; F, frontal; P, parietal; T, temporal.
*c*) AG, anaplastic glioma; GB, glioblastoma.
*d*) Same mutation as in mRNA was detected in all of 2–4 PCR clones examined. In case 6 without mRNA expression, none of the exons revealed any abnormality in SSCP and PCR clone analyses. The finding in case 7, which is U-251MG, is identical to that previously reported[1] for this cell line. del, deletion; ins, insertion; ∗, see below.
*e*) same, same mutation as in gene in all of 4–6 RT-PCR clones examined.
*f*) del, deletion; stop, stop codon formation; fs (256), frameshift followed by premature stop codon formation at codon 256.
*g*) +, LOH was presumed from the absence of normal bands in SSCP of exons (cases 2, 3, 5, 7 and 10) and the absence of normal PCR clones (all cases).
*h*) –, no *PTH2* sequence in all of 4–6 RT-PCR clones examined.
∗ The same genetic alteration was detected in the primary glioma tissue from which cell line was derived (see text).

**Northern blot analysis** Fifteen micrograms of total RNA was electrophoresed and blotted on a nitrocellulose membrane as described.[25] The membrane was hybridized with an *MMAC1* exon 9 probe which was PCR-amplified from placental DNA using the primers 5′-CACCAGATGTTAGTGACAATG-3′ (sense) and 5′-AGTCATTGTTGCTGTGTTTC-3′ (antisense), washed to a final stringency of 0.1× SSC/0.1% SDS at 55°C and autoradiographed as described.[25] *GAPDH* (glyceraldehydephosphate dehydrogenase) cDNA was used as a control probe.[25]

**RT-PCR** Three micrograms of total RNA of glioma cell lines was reverse-transcribed using a commercial kit, "Superscript" Preamplification System (Life Tech. Inc., Rockville, MD) and then almost the entire coding region sequence of *MMAC1* cDNA (about 92%) was PCR-amplified using the primers 5′-GCCATCATCAAAGAGATCGT-3′ (sense) and 5′-TTCTCTGGATCAGAGTCAGT-3′ (antisense).

**SSCP analyses of *MMAC1* cDNA and gene** SSCP analyses were performed on both the *MMAC1* cDNA and gene in all 10 glioma cell lines.

In the analysis of *MMAC1* cDNA, 5 overlapping portions of the coding region, portions 1 to 5 in the 5′ to 3′ direction, were PCR-amplified from the RT-PCR products described above. The primers used for portion 1 were the same sense primer as described for RT-PCR and 5′-GATAAGTTCTAGCTGTGGTG-3′ (antisense), those for 2 were 5′-TGCAGAGTTGCACAATATCC-3′ (sense) and 5′-ACACATAGCGCCTCTGACT-3′ (antisense), those for 3 were 5′-TCTATGGGGAAGTAAGGACC-3′ (sense) and 5′-CTGAGGGAACTCAAAGTACA-3′ (antisense), those for 4 were 5′-TCCAATTCAGGACCCACACG-3′ (sense) and 5′-AAGTATCGGTTGGCTTTGTC-3′ (antisense) and those for 5 were 5′-GACAAGGAATATCTAGTACT-3′ (sense) and the same antisense primer as described for RT-PCR. The target sequences, excluding primer sequences, spanned nucleotide positions (np) 27–283 in portion 1, np 257–506 in 2, np 481–717 in 3, np 699–991 in 4 and np 954–1146 in 5, when the first nucleotide of the coding region was taken as np 1. After PCR, SSCP was performed as described[25] and PCR products showing electrophoretic mobility shifts were cloned and sequenced as described below.

In analysis of the *MMAC1* gene, exons 1 to 9 were PCR-amplified using the primers described by Liaw *et al.*[31] and Wang *et al.*,[7] which corresponded to intronic sequences except for the sense primer for exon 1 and the antisense primer for exon 9, which corresponded to the 5′

and 3′ non-coding region sequences, respectively. After PCR, SSCP was performed as described[25] and PCR products were cloned and sequenced as described below.

**Cloning and sequencing** In the examination of *MMAC1* cDNA, PCR-amplified cDNA portions which showed abnormal mobility in SSCP were ligated to the *Sma*I cloning site of pUC118, transfected into *E. coli* JM109 cells, propagated and sequenced by the dideoxy-termination method. In the sequence analysis, 4 to 6 independent clones were examined for each PCR product. In the examination of the *MMAC1* gene, PCR products were similarly cloned and 2 to 4 independent clones were examined for each exon. In the nucleotide analyses of cDNA portions and exons, special attention was paid not only to mutations, but also to the nucleotide positions in which nucleotides differed between the *MMAC1* gene and the highly homologous pseudogene, *PTH2*.[23, 24] The number of such nucleotide positions was 1 to 5 in each cDNA portion and 1 to 4 in each exon except for exon 9, which had none.

**Examination of primary glioma tissues** In 2 cases, genomic DNA of the primary glioma tissues from which cell lines were derived was also examined by SSCP and nucleotide analyses and the findings obtained were compared with those in the corresponding cell lines.

## RESULTS

**Analyses of *MMAC1* mRNA expression** Northern blot analysis using the *MMAC1* exon 9 probe revealed several bands, with a major 5.5-kb band in 9 (cases 1–5 and 7–10) of the 10 glioma cell lines (Fig. 1). Signal intensities of the bands varied depending on the cell line. These findings were consistent with previous work.[13] In the 1 remaining cell line (case 6), no distinct band was detected. RT-PCR analysis performed on the coding-region sequence of *MMAC1* mRNA revealed cDNA amplification in the former 9 cell lines, but not in the latter 1 cell line (case 6) (data not shown).

**SSCP analysis of *MMAC1* cDNA** SSCP analysis of *MMAC1* cDNA was performed in the 9 cell lines which revealed mRNA expression in northern blot and RT-PCR analyses. On examination of 5 overlapping portions of the *MMAC1* coding-region cDNA, which consisted of portions 1 to 5 in the 5′ to 3′ direction as described in "Materials and Methods," aberrant bands were seen in one of portions 1 to 4 in 6 cell lines; in portion 1 in case 3, portion 2 in case 10, portion 3 in case 2 and portion 4 in cases 5, 7 and 9 (data not shown). Normal bands were not observed in these 6 cell lines, suggesting the absence of normal transcripts. Subsequent nucleotide analysis of PCR clones revealed mutations in all these 6 cases in all of 4–6 clones examined; 3-bp deletions at codon 17 in case 3, codon 101 in case 10 and codon 199 in case 2, a nonsense mutation at codon 233 in case 5, a missense mutation at codon 252 in case 9 and a 2-bp insertion at codon 241 in case 7 (Table I). The mutation in case 7, U-251MG, has been described.[1] In Fig. 2, representative results of sequencing are shown. In these cases, no normal clones were isolated, consistent with the SSCP profile in which no distinct normal bands were detected.

The nucleotide sequence outside these mutations was identical to that of normal *MMAC1* cDNA in these clones. The nucleotide positions in which the nucleotides differed between *MMAC1* and *PTH2* pseudogene were 3 to 5 in number in cDNA portions 1 to 4. All of these positions in the cDNA portions corresponded to the nucleotides of the *MMAC1*, but not to those of the *PTH2* sequence in all of the 4–6 clones examined in the individual cases. The rep-

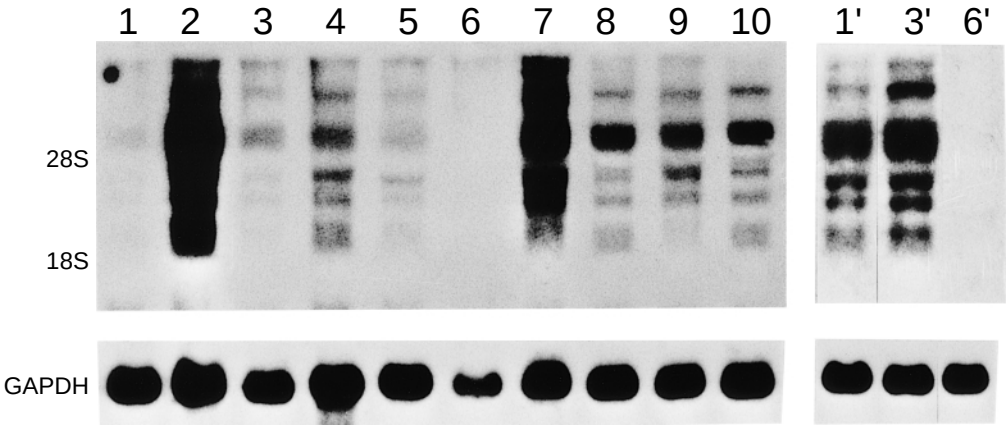


Fig. 1. Northern blot analysis of *MMAC1* mRNA in glioma cell lines. The northern blots of total RNA of glioma cell lines (cases 1–10) were hybridized with *MMAC1* exon 9 probe. Several bands, including a major 5.5-kb band, are seen in 9 cell lines (cases 1–5 and 7–10), but not in 1 cell line (case 6). The rightmost 3 lanes show the results of reexamination of cases 1 (1′), 3 (3′) and 6 (6′). Note the absence of a visible band in case 6 (6′). *GAPDH* cDNA was used as a control probe.

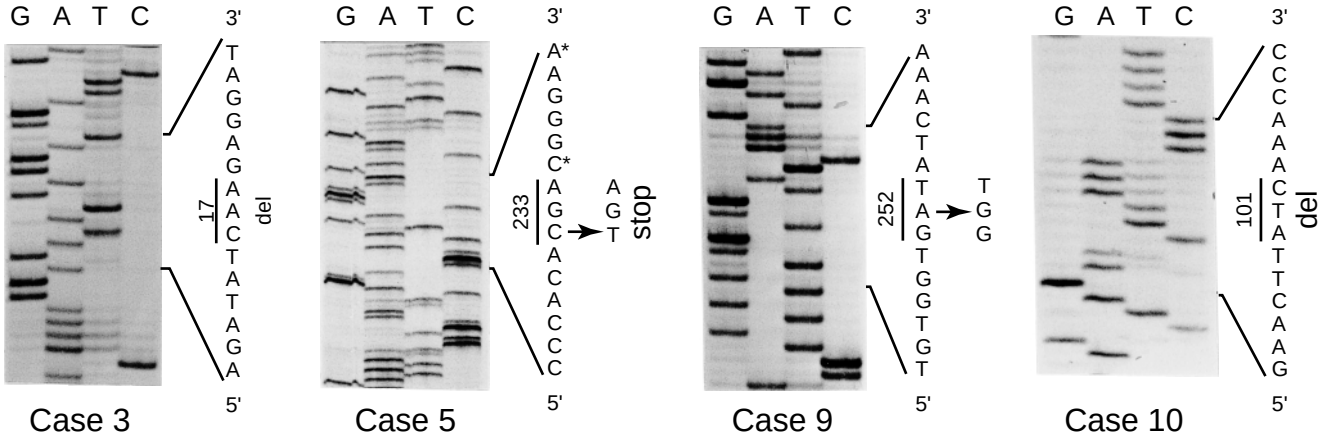


Fig. 2. Nucleotide analysis of cloned *MMAC1* cDNA in glioma cell lines. Nucleotide sequences of cloned cDNA portions of representative cell lines (cases 3, 5, 9 and 10) are shown; 3-bp deletions at codon 17 (cDNA portion 1) in case 3 and codon 101 (cDNA portion 2) in case 10, nonsense mutation at codon 233 (cDNA portion 4) in case 5 and missense mutation at codon 252 (cDNA portion 4) in case 9. ∗ in case 5 indicates the nucleotide positions in which nucleotides are different between the *MMAC1* gene and *PTH2* pseudogene. Note that these nucleotide positions are involved in the nucleotides of the *MMAC1* gene, $C^*$ of CGG and the last $A^*$ of GAA, but not in those of the *PTH2* pseudogene, in which $C^*$ and $A^*$ should be replaced with T and G, respectively.

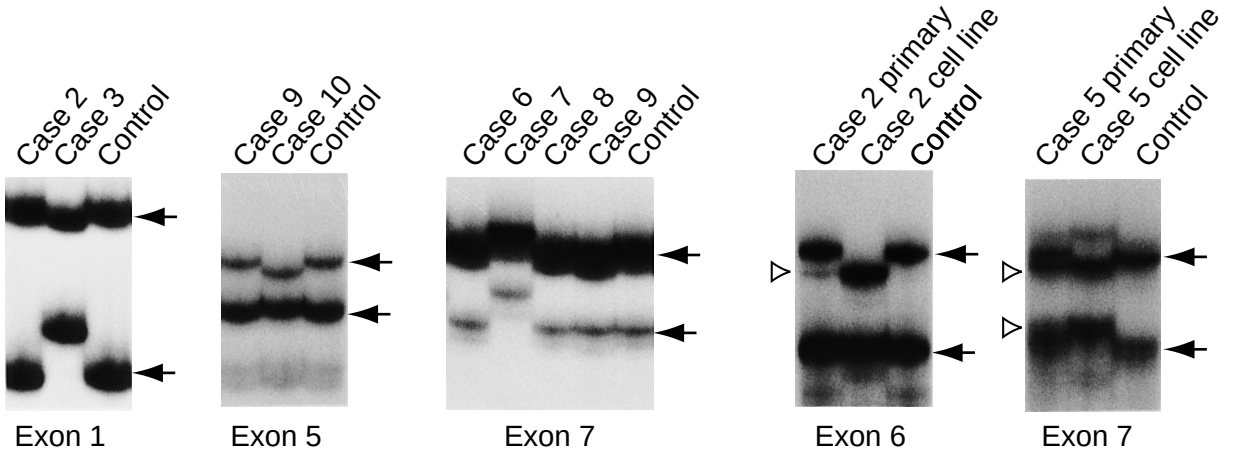


Fig. 3. SSCP analysis of *MMAC1* gene mutations in glioma cell lines. Abnormal electrophoretic patterns in 6 glioma cell lines (cases 2, 3, 5, 7, 9 and 10) and 2 primary tumors (cases 2 and 5) are shown. The positions of normal bands are indicated with arrows. The leftmost 3 panels show aberrant bands in 4 glioma cell lines; in exon 1 in case 3, exon 5 in case 10 and exon 7 in cases 7 and 9. No distinct normal bands were seen in any case except for case 9, in which the aberrant bands show only a slight shift. The rightmost 2 panels show aberrant bands (open arrowheads) in 2 glioma cell lines and their primary tumors; in exon 6 in case 2 and exon 7 in case 5. In both of the 2 cases, the cell line shows only aberrant bands, while the primary tumor (primary) shows both aberrant and normal bands, although the aberrant band in the primary tumor in case 2 is weak and those in case 5 are not clearly separated from the normal bands. In the cell line of case 5, there is an additional weak band with the slowest mobility, but its meaning remains unknown.

resentative sequence is shown in Fig. 2 (see cDNA sequence of case 5). These findings indicated that the mutations detected in these cell lines were in the *MMAC1* mRNA, but not in the pseudogene-derived mRNA.

**SSCP analysis of *MMAC1* gene** SSCP analysis of the *MMAC1* gene was performed in all 9 exons in each cell line. All the abnormalities detected were the same as those detected in cDNA analysis and no additional abnormality was found. In SSCP, the 6 cell lines (cases 2, 3, 5, 7, 9 and 10) which showed mutations in cDNA examination revealed aberrant bands in the corresponding exons (exon 1 in case 3, exon 5 in case 10, exon 6 in case 2 and exon 7

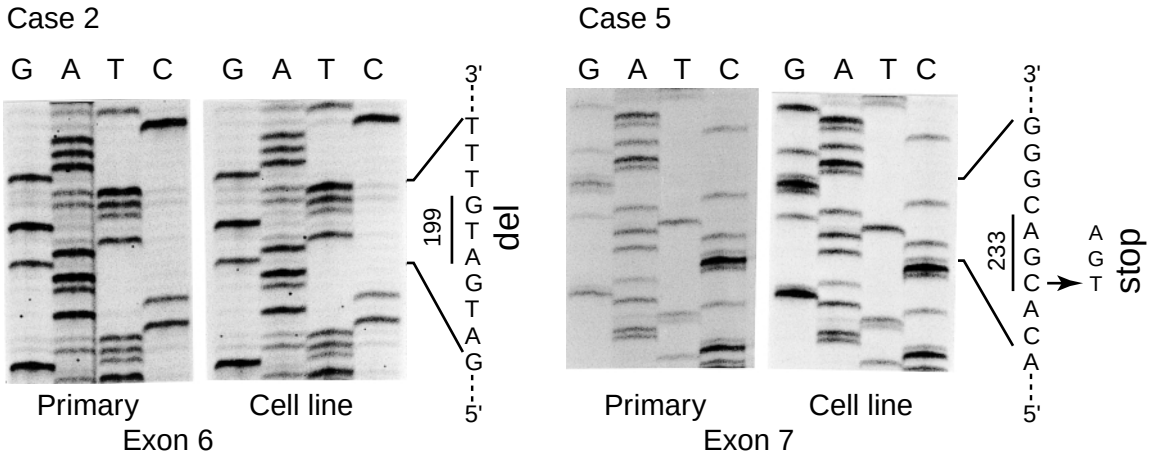


Fig. 4. Comparison of *MMAC1* gene mutations in the cell lines and the primary tumors. Nucleotide sequences of PCR clones of *MMAC1* gene are compared between the cell lines and the primary tumors from which the cell lines had been derived. The same mutations as in the cell lines were detected in the primary tumors (primary) in two cases (cases 2 and 5) examined: 3-bp deletion at codon 199 in exon 6 in case 2 and a nonsense mutation at codon 233 in exon 7 in case 5.

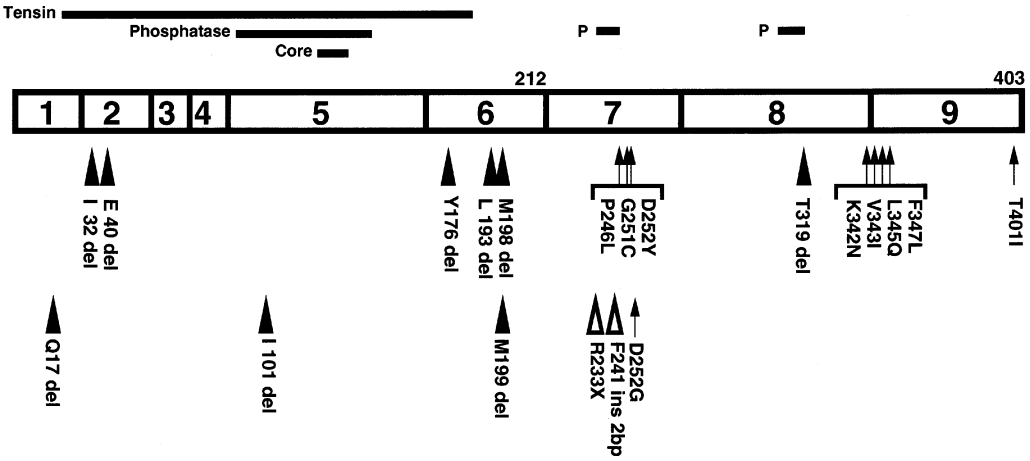


Fig. 5. Schematic illustration showing locations of *MMAC1* mutations identified in glioma cell lines along the *MMAC1* coding sequence. The *MMAC1* coding sequence is shown by an open bar with exon numbers and boundaries. Just beneath the bar, the reported 3-bp deletions (filled arrowheads) in the entire sequence and the reported missense mutations (arrows) which are located in the C-terminal half (exons 7–9, codons 212–403) of the sequence are shown.[7, 8, 10–12, 17, 18, 32–35] At the bottom, the mutations identified in our 6 cell lines, 3 3-bp deletions (filled arrowheads), 1 missense mutation (arrow) and 2 truncation mutations (open arrowheads), are shown. At the top, regions of the coding sequence homologous to tensin/auxilin/phosphatase (Tensin), the phosphatase domain (Phosphatase), the core motif of the phosphatase domain (Core), and the tyrosine phosphorylation acceptor sites (P) are shown with thin, filled bars.

in cases 5, 7 and 9), but not in other exons (Fig. 3). In addition, except for case 9 in which the aberrant bands showed only a slight shift, no distinct normal bands were seen in any case, strongly suggesting the loss of the normal *MMAC1* gene allele (LOH). In subsequent nucleotide analysis, the same mutations as in cDNA were found in all of the 2–4 PCR clones examined in each of these cases, including case 9, in which 4 PCR clones were examined (Table I). Considering these findings together, LOH was suggested to be present in all of the 6 cases. Representative findings from the nucleotide analysis are shown in Fig. 4 and the locations of these mutations in the *MMAC1* sequence are shown in Fig. 5, together with those of some related mutations previously reported.[7, 8, 10–12, 17, 18, 32–35] In Fig. 5, it can be seen that when the coding region (codons 1–403, exons 1–9) was divided into the N-terminal half

(codons 1–212, exons 1–6) and the C-terminal half (codons 212–403, exons 7–9), all of the 3 3-bp deletions were located in the N-terminal half, while the other 3 mutations, including 1 missense mutation, were in the C-terminal half.

In another cell line (case 6) which revealed no mRNA expression in northern blot and RT-PCR analyses, no exon revealed any aberrant bands in SSCP or any mutations in 2–6 PCR clones examined. Splicing signals also revealed no abnormality. The remaining 3 cases (cases 1, 4 and 8) which showed no abnormality in cDNA analysis revealed no aberrant bands in SSCP in any of the 9 exons. In the above-described nucleotide analysis of PCR clones, it was also shown that all the cloned sequences were from the *MMAC1* gene, and not from the *PTH2* pseudogene. These findings are summarized in Table I.

**Examination of the primary glioma tissues** The primary glioma tissues from which the cell lines had been derived were also examined in 2 cases (cases 2 and 5) by SSCP followed by nucleotide analysis. In SSCP analysis, the same aberrant bands as those observed in the descendant cell lines were detected in exon 6 in case 2 and in exon 7 in case 5 (Fig. 3). While the cell lines revealed only aberrant bands, the primary tumor tissues revealed both aberrant and normal bands, though the aberrant band in the original tumor was weak in case 2. In subsequent nucleotide analysis of PCR clones, the same mutations as in the cell lines were detected in 3 of 19 clones in case 2 and in 6 of 13 clones in case 5 (Fig. 4, Table I). These findings suggested that the *MMAC1* gene alterations in these cell lines were derived from the primary glioma tissues.

## DISCUSSION

In this study, we examined the status of the *MMAC1* gene in a total of 10 glioma cell lines. In SSCP analysis of cDNA and genomic DNA, we found mutations with presumed LOH in 6 cell lines: 3 in-frame 3-bp deletions, 1 missense mutation and 2 truncation mutations (1 nonsense mutation and 1 2-bp insertion). When the distributions of these mutations were analyzed, all of the 3 3-bp deletions were located in the N-terminal half of the coding region, while the other 3 mutations, including 1 missense mutation, were in the C-terminal half. Regarding in-frame deletions, our review of about 200 mutations reported previously in various malignancies revealed 6 3-bp deletions and 1 each of 9-, 18-, 75- and 204-bp deletions.[1, 2, 9–12, 17, 18, 36] Among them, 5 of the 6 3-bp deletions[10–12, 17, 18] were located in the N-terminal half, as in our 3 cases (see Fig. 5), while other larger deletions were located in either the N-terminal (75-bp deletion) or the C-terminal half (9-, 18- and 204-bp deletions). Thus, it seemed likely that in-frame 3-bp deletions are distributed mainly in the N-terminal half. Although the meaning of this tendency remains unknown, it is possible that small, in-frame deletions in the N-terminal half had effects on the *MMAC1* activity, while those in the C-terminal half usually did not. Since, to our knowledge, cell lines with 3-bp deletion have not been reported, our 3 cell lines might be of particular value in further studies to examine this possibility.

A similar tendency has been pointed out in the case of missense mutations.[1, 14] While mutations which resulted in truncation of the *MMAC1* protein were distributed throughout the entire coding region, missense mutations were usually distributed in its N-terminal half.[1, 14] Our review of about 200 mutations in reported malignancies also revealed the same tendency. Regarding missense mutations, the ratio of mutations in the N-terminal half to those in the C-terminal half was 49:8 in total malignancies, including germ line mutations in Cowden disease. Similarly, it was 27:3 in gliomas. In this respect, our cell line (case 9) which revealed a missense mutation in the C-terminal half seemed of particular interest. To our knowledge, no other such cell line has been reported. When the distributions of the missense mutations in our cell line and the 8 reported ones[7, 8, 32–35] were analyzed, it could be seen that, except for 1 mutation at the 3′-end, all of the remaining 8 mutations, including that in our case, were clustered in either of 2 small regions spanning codons 246–252 and codons 342–347, which were located in the vicinity of a tyrosine phosphorylation acceptor site, or around the junction between exons 8 and 9 (see Fig. 5). Based on the fact that missense mutations were exclusively detected in the N-terminal half in malignancies, Steck *et al.*[1] considered the possibility that, unlike missense mutations in the N-terminal half, those in the C-terminal half had no effect on *MMAC1* activity. However, our observation of the above-described mutation clusters suggested that missense mutations of some particular codons or regions in the C-terminal half have functional effects. This possibility might be supported by the fact that some missense mutations of Cowden disease[32] were located in one of these clusters. However, further studies including *in vitro* functional assays of individual missense mutations would be required to test this possibility.

In another cell line (case 6), we failed to detect mRNA expression in northern blot and RT-PCR analyses. Since none of the exons of the genomic DNA revealed any abnormality in SSCP and PCR clone analyses, some abnormalities in the promoter region, including nucleotide changes or 5′CpG island methylation might be present. Similar transcription blocks have also been observed in some glioma cell lines in previous studies.[13] Including this case, 7 (70%) or 6 (67%, if case 7/U251MG was excluded) of our glioma cell lines revealed abnormalities in the *MMAC1* gene. This frequency of abnormality was consistent with previous results[1, 2, 13] which showed *MMAC1* alteration in about 60–80% of glioma cell lines.

Recently, the presence of a highly homologous pseudogene, *PTH2*, which is a spliced form gene located in a different chromosome (9p), was reported.[23, 24] The coding sequence of this gene differed only in 18 nucleotides and 10 amino acids from the sequence of the *MMAC1* gene.[14, 23, 24] Its actual expression was noted in some normal and neoplastic tissues and in some cell lines,[23] suggesting the need for caution in analysis of *MMAC1* alterations. In the present study, however, none of the PCR clones from cDNA in our 6 mutant cell lines revealed *PTH2*-specific nucleotides in sequencing. This suggested that our glioma cell lines expressed only *MMAC1* mRNA, but no, or only a negligible amount of, *PTH2* mRNA, and that the mutations in these cell lines were in the *MMAC1* gene itself, and not in the pseudogene.

Although some previous studies[1, 2, 13, 14] have examined glioma cell lines for *MMAC1* gene alterations, to our knowledge, analysis of the primary glioma tissues from which cell lines were derived has not been performed. This seemed true also in the cases of many other tumors. In this study, we examined 2 primary glioma tissues from which our cell lines (cases 2 and 5) had been derived and detected the same electrophoretic shifts in SSCP and the same mutations in PCR clones as those in the cell lines in both cases. However, in 1 case (case 2), the primary glioma tissue revealed a weak aberrant band in SSCP and infrequent mutant PCR clones. Whether these findings were due to contaminating normal tissue or to the existence of neoplastic cells without a mutant *MMAC1* gene remains unknown. In either case, however, it was indicated that the *MMAC1* alterations in these cell lines were derived from the primary glioma tissues, but not from artifacts arising during long-term *in vitro* cultivation.

**ACKNOWLEDGMENTS**

We are grateful to Drs. I. Takeshita, M. Fumita, Y. Kotani and the late Drs. H. Kosaka and K. Nishida of our laboratory for their interest and support. This work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan, the Fujisawa Foundation, the Inoue Foundation for Science, the Sasagawa Health, Science Foundation, the Yoneyama Foundation and the Association of International Education, Japan.

*(Received March 2, 1999/Revised June 22, 1999/Accepted June 24, 1999)*

**REFERENCES**

1) Steck, P. A., Pershouse, M. A., Jasser, S. A., Yung, W. K. A., Lin, H., Ligon, A. H., Langford, L. A., Baumgard, M. L., Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D. H. F. and Tavtigian, S. V. Identification of a candidate tumour suppressor gene, *MMAC1*, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat. Genet.*, **15**, 356–362 (1997).
2) Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S. H., Giovanella, B. C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M. H. and Parsons, R. *PTEN*, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science*, **275**, 1943–1947 (1997).
3) Li, D.-M. and Sun, H. *TEP1*, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor β. *Cancer Res.*, **57**, 2124–2129 (1997).
4) Myers, M. P., Stolarov, J. P., Eng, C., Li, J., Wang, S. I., Wigler, M. H., Parsons, R. and Tonks, N. K. *P-TEN*, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase. *Proc. Natl. Acad. Sci. USA*, **94**, 9052–9057 (1997).
5) Maehama, T. and Dixon, J. E. The tumor suppressor, *PTEN/MMAC1*, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.*, **273**, 13375–13378 (1998).
6) Furnari, F. B., Huang, H.-J. S. and Cavenee, W. K. The phosphoinositol phosphatase activity of *PTEN* mediates a serum-sensitive G1 growth arrest in glioma cells. *Cancer Res.*, **58**, 5002–5008 (1998).
7) Wang, S. I., Puc, J., Li, J., Bruce, J. N., Cairns, P., Sidransky, D. and Parsons, R. Somatic mutations of *PTEN* in glioblastoma multiforme. *Cancer Res.*, **57**, 4183–4186 (1997).
8) Liu, W., James, C. D., Frederick, L., Alderete, B. E. and Jenkins, R. B. *PTEN/MMAC1* mutations and *EGFR* amplification in glioblastomas. *Cancer Res.*, **57**, 5254–5257 (1997).
9) Chiariello, E., Roz, L., Albarosa, R., Magnani, I. and Finocchiaro, G. *PTEN/MMAC1* mutations in primary glioblastomas and short-term cultures of malignant gliomas. *Oncogene*, **16**, 541–545 (1998).
10) Rasheed, B. K. A., Stenzel, T. T., McLendon, R. E., Parsons, R., Friedman, A. H., Friedman, H. S., Bigner, D. D. and Bigner, S. H. *PTEN* gene mutations are seen in high-grade but not in low-grade gliomas. *Cancer Res.*, **57**, 4187–4190 (1997).
11) Duerr, E.-M., Rollbrocker, B., Hayashi, Y., Peters, N., Meyer-Puttlitz, B., Louis, D. N., Schramm, J., Wiestler, O. D., Parsons, R., Eng, C. and von Deimling, A. *PTEN* mutations in gliomas and glioneuronal tumors. *Oncogene*, **16**, 2259–2264 (1998).
12) Maier, D., Zhang, Z., Taylor, E., Hamou, M.-F., Gratzl, O., Van Meir, E. G., Scott, R. J. and Merlo, A. Somatic deletion mapping on chromosome 10 and sequence analysis of *PTEN/MMAC1* point to the 10q25–26 region as the primary target in low-grade and high-grade gliomas. *Onco-*

*gene*, **16**, 3331–3335 (1998).
13) Furnari, F. B., Lin, H., Huang, H.-J. S. and Cavenee, W. K. Growth suppression of glioma cells by *PTEN* requires a functional phosphatase catalytic domain. *Proc. Natl. Acad. Sci. USA*, **94**, 12479–12484 (1997).
14) Teng, D. H.-F., Hu, R., Lin, H., Davis, T., Iliev, D., Frye, C., Swedlund, B., Hansen, K. L., Vinson, V. L., Gumpper, K. L., Ellis, L., El-Naggar, A., Frazier, M., Jasser, S., Langford, L. A., Lee, J., Mills, G. B., Pershouse, M. A., Pollack, R. E., Tornos, C., Troncoso, P., Yung, W. K. A., Fujii, G., Berson, A., Bookstein, R., Bolen, J. B., Tavtigian, S. V. and Steck, P. A. *MMAC1/PTEN* mutations in primary tumor specimens and tumor cell lines. *Cancer Res.*, **57**, 5221–5225 (1997).
15) Cairns, P., Okami, K., Halachmi, S., Halachmi, N., Esteller, M., Herman, J. G., Jen, J., Isaacs, W. B., Bova, G. S. and Sidransky, D. Frequent inactivation of *PTEN/MMAC1* in primary prostate cancer. *Cancer Res.*, **57**, 4997–5000 (1997).
16) Suzuki, H., Freije, D., Nusskern, D. R., Okami, K., Cairns, P., Sidransky, D., Isaacs, W. B. and Bova, G. S. Interfocal heterogeneity of *PTEN/MMAC1* gene alterations in multiple metastatic prostate cancer tissues. *Cancer Res.*, **58**, 204–209 (1998).
17) Kong, D., Suzuki, A., Zou, T.-T., Sakurada, A., Kemp, L. W., Wakatsuki, S., Yokoyama, T., Yamakawa, H., Furukawa, T., Sato, M., Ohuchi, N., Sato, S., Yin, J., Wang, S., Abraham, J. M., Souza, R. F., Smolinski, K. N., Meltzer, S. J. and Horii, A. *PTEN1* is frequently mutated in primary endometrial carcinomas. *Nat. Genet.*, **17**, 143–144 (1997).
18) Risinger, J. I., Hayes, A. K., Berchuck, A. and Barrett, J. C. *PTEN/MMAC1* mutations in endometrial cancers. *Cancer Res.*, **57**, 4736–4738 (1997).
19) Tashiro, H., Blazes, M. S., Wu, R., Cho, K. R., Bose, S., Wang, S. I., Li, J., Parsons, R. and Ellenson, L. H. Mutations in *PTEN* are frequent in endometrial carcinoma but rare in other common gynecological malignancies. *Cancer Res.*, **57**, 3935–3940 (1997).
20) James, C. D., Carlbom, E., Dumanski, J. P., Hansen, M., Nordenskjold, M., Collins, V. P. and Cavenee, W. K. Clonal genomic alterations in glioma malignancy stages. *Cancer Res.*, **48**, 5546–5551 (1988).
21) Fults, D., Pedone, C. A., Thomas, G. A. and White, R. Allelotype of human malignant astrocytoma. *Cancer Res.*, **50**, 5784–5789 (1990).
22) Rasheed, B. K. A., McLendon, R. E., Friedman, H. S., Friedman, A. H., Fuchs, H. E., Bigner, D. D. and Bigner, S. H. Chromosome 10 deletion mapping in human gliomas: a common deletion region in 10q25. *Oncogene*, **10**, 2243–2246 (1995).
23) Kim, S. K., Su, L.-K., Oh, Y., Kemp, B. L., Hong, W. K. and Mao, L. Alterations of *PTEN/MMAC1*, a candidate tumour suppressor gene, and its homologue, *PTH2*, in small cell lung cancer cell lines. *Oncogene*, **16**, 89–93 (1998).
24) Dahia, P. L. M., FitzGerald, M. G., Zhang, X., Marsh, D. J., Zheng, Z., Pietsch, T., von Deimling, A., Haluska, F. G., Haber, D. A. and Eng, C. A highly conserved processed *PTEN* pseudogene is located on chromosome band 9p21. *Oncogene*, **16**, 2403–2406 (1998).
25) Zhang, S.-J., Endo, S., Koga, H., Ichikawa, T., Feng, X.-L., Onda, K., Washiyama, K. and Kumanishi, T. A comparative study of glioma cell lines for *p16*, *p15*, *p53* and *p21* gene alterations. *Jpn. J. Cancer Res.*, **87**, 900–907 (1996).
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) Chomczynski, P. and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156–159 (1987).
31) Liaw, D., Marsh, D. J., Li, J., Dahia, P. L. M., Wang, S. I., Zheng, Z., Bose, S., Call, K. M., Tsou, H. C., Peacocke, M., Eng, C. and Parsons, R. Germline mutations of the *PTEN* gene in Cowden disease, an inherited breast and thyroid cancer syndrome. *Nat. Genet.*, **16**, 64–67 (1997).
32) Lynch, E. D., Ostermeyer, E. A., Lee, M. K., Arena, J. F., Ji, H., Dann, J., Swisshelm, K., Suchard, D., MacLeod, P. M., Kvinnsland, S., Gjertsen, B. T., Heimdal, K., Lubs, H., Moller, P. and King, M.-C. Inherited mutations in *PTEN* that are associated with breast cancer, Cowden disease, and juvenile polyposis. *Am. J. Hum. Genet.*, **61**, 1254–1260 (1997).
33) Kohno, T., Takahashi, M., Manda, R. and Yokota, J. Inactivation of the *PTEN/MMAC1/TEP1* gene in human lung cancers. *Genes Chromosom. Cancer*, **22**, 152–156 (1998).
34) Gronbaek, K., Zeuthen, J., Guldberg, P., Ralfkiaer, E. and Hou-Jensen, K. Alterations of the *MMAC1/PTEN* gene in lymphoid malignancies. *Blood*, **19**, 4388–4390 (1998).
35) FitzGerald, M. G., Marsh, D. J., Wahrer, D., Bell, D., Caron, S., Shannon, K. E., Ishioka, C., Isselbacher, K. J., Garber, J. E., Eng, C. and Haber, D. A. Germline mutations in *PTEN* are an infrequent cause of genetic predisposition to breast cancer. *Oncogene*, **17**, 727–731 (1998).
36) Guldberg, P., thor Straten, P., Birck, A., Ahrenkiel, V., Kirkin, A. F. and Zeuthen, J. Disruption of the *MMAC1/PTEN* gene by deletion or mutation is a frequent event in malignant melanoma. *Cancer Res.*, **57**, 3660–3663 (1997).