Rare-type Mutations of MMAC1 TumorSuppressor Gene in Human Glioma Cell
Lines and Their Tumors of Origin

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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.1–4) MMAC1 gene alterations have already been examined in various tumors including malignant glioma, prostate carcinoma and endometrial carcinoma.1,2,7–10) Among them, malignant gliomas have revealed frequent alterations in both primary tumor tissues and cell lines,1,2,7–10) 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 institute25–28) 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)
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 described by Liaw et al.31) and Wang et al.7) which corresponded to intronic sequence in all of 4-6 RT-PCR clones examined. 25, 26)

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'-GCCATCATCACAAGAGAT-CGT-3' (sense) and 5'-TTCTCTGGATCAGAGTCT-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'-GATAAGTTTCTAGCTGTTGTTG-3' (antisense), those for 2 were 5'-TGCGAGGTGTGGCACAATTC-3' (sense) and 5'-ACACATGCGCCTCTGACT-3' (antisense), those for 3 were 5'-AGTCTGTTGCTGTTTTCC-3' (antisense), washed to a final stringency of 0.1x SSC/0.1% SDS at 55°C and autoradiographed as described.25) GAPDH (glyceraldehydephosphate dehydrogenase) cDNA was used as a control probe.25)

Table I. Summary of MMAC1 Mutations in Glioma Cell Lines

| Case | Age/sex | Site of tumor | Pa Dx | Exon | Codon | Change | Predicted effect | LOH | PTH2 expression |
|------|---------|--------------|-------|------|-------|--------|-----------------|------|----------------|
| 1    | 38/F    | T            | AG    | exon 6 | 199 | del ATG | same | Met del | + | - |
| 2    | 64/M    | F            | AG    | exon 1 | 17  | del CAA | same | Glu del | + | - |
| 3    | 64/F    | P            | GB    | exon 7 | 233 | CGA-TGA | same | stop    | + | - |
| 4    | 63/M    | F            | AG    | exon 7 | 241 | ins TT | same | fs(256) | + | - |
| 5    | 62/M    | T            | AG    | exon 7 | 252 | GAT-GGT | same | Asp-Gly | + | - |
| 6    | 31/F    | P            | AG    | exon 7 | 263 | del ATC | same | Ile del | + | - |
| 7    | 51/M    | T            | AG    | exon 7 | 277 | del CAA | same | Glu del | + | - |
| 8    | 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 4-6 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 reported1) for this cell line.  del, deletion; ins, insertion; +, see below.

e) Same mutation as in gene in all of 4-6 RT-PCR clones examined.

f) del, deletion; stop, stop codon formation; fs, 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).
and 3' non-coding region sequences, respectively. After PCR, SSCP was performed as described 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*. 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. In the 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. 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-

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**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.
Mutations in Glioma Cell Lines

Representative 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 \textit{MMAC1} mRNA, but not in the pseudogene-derived mRNA.

\textbf{SSCP analysis of \textit{MMAC1} gene} SSCP analysis of the \textit{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 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.

Fig. 2. Nucleotide analysis of cloned \textit{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 \textit{MMAC1} gene and \textit{PTH2} pseudogene. Note that these nucleotide positions are involved in the nucleotides of the \textit{MMAC1} gene, C' of CGG and the last A' of GAA, but not in those of the \textit{PTH2} pseudogene, in which C' and A' should be replaced with T and G, respectively.

Fig. 3. SSCP analysis of \textit{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.
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 \textit{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 \textit{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...
Among them, 5 of the 6 3-bp deletions were derived from the primary glioma tissues. 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 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. Among them, 5 of the 6 3-bp deletions 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. 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. 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 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. 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 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. 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 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.\textsuperscript{23, 24} The coding sequence of this gene differed only in 18 nucleotides and 10 amino acids from the sequence of the MMAC1 gene.\textsuperscript{14, 23, 24} Its actual expression was noted in some normal and neoplastic tissues and in some cell lines,\textsuperscript{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, -specific nucleotides in sequencing. This suggested that our 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.

Although some previous studies\textsuperscript{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 shift 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)

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