Analysis of homozygous deletion of the *p16* gene and correlation with survival in patients with glioblastoma multiforme

TAKANORI KAMIRYO, M.D., KENJI TADA, M.D., SHOJI SHIRAISHI, M.D., NAOKI SHINOJIMA, M.D., HIDEO NAKAMURA, M.D., Ph.D., MASATO KOCHI, M.D., Ph.D., JUN-ICHI KURATSU, M.D., Ph.D., HIDEYUKI SAYA, M.D., Ph.D., and YUKITAKA USHI, M.D., Ph.D.

Departments of Neurosurgery, and Tumor Genetics and Biology, Kumamoto University Medical School, Kumamoto; Department of Neurosurgery, Kagoshima University Medical School, Kagoshima, Japan

Object. One of the most frequent genetic abnormalities found in patients with glioblastoma multiforme (GBM) is homozygous deletion of the *p16* tumor suppressor gene. The authors investigated whether this deletion is associated with prognosis in patients with GBM.

Methods. In 46 adult patients with supratentorial GBM, homozygous deletion of the *p16* gene in tumor DNA was examined using the multiplex polymerase chain reaction assay. The deletion was confirmed in 14 (30.4%) of 46 patients, eight (30.8%) of 26 men and six (30.0%) of 20 women. Cox proportional hazard regression analysis, adjusted for age at surgery, the Karnofsky Performance Scale score, extent of resection, and the MIB-1 labeling index, revealed that homozygous deletion of the *p16* gene was significantly associated with overall survival and progression-free survival in men, but not in women.

Conclusions. The results of this study suggest that *p16* homozygous deletion is a significant unfavorable prognostic factor in male patients with GBM.

Key words • *p16* gene • homozygous deletion • glioblastoma multiforme • prognosis

Glioblastoma multiforme is the most common and most malignant astrocytic tumor of the central nervous system. The patient's age and preoperative KPS score are reliable prognostic factors, as is the extent of tumor resection. The authors of several studies have suggested that genetic alterations of chromosome 10, *p16*, or epidermal growth factor receptor amplification and overexpression are associated with the development and progression of glioma and with disease prognosis.

The *p16* gene, which maps to chromosome 9p21, is a tumor suppressor gene that has been investigated in many human cancers including gliomas. It was frequently inactivated through homozygous deletion in high-grade gliomas. Homozygous deletion of the *p16* gene is often noted in patients with GBM but not in those with medulloblastomas or ependymomas, suggesting a role for *p16* inactivation in the progression of astrocytic gliomas. In fact, introduction of the *p16* gene into *p16*-negative glioma cell lines induced growth suppression, although it had no effect on *p16*-positive cell lines.

The *p16* protein regulates cell cycle control at the G1–S transition and its inactivation leads to loss of cell cycle control and increased proliferation. Although rare in gliomas, other mechanisms that inactivate *p16* include mutation of the gene and transcriptional repression due to hypermethylation of the 5' CpG island in the promoter region.

Among molecular markers predictive of patient outcome, Ki-67 (MIB-1) immunolabeling has been used most widely and is of value in predicting prognosis in patients with astrocytic tumors. Ono, et al., used multiplex PCR assays, reported that gliomas with *p16* homozygous deletion had higher Ki-67 indices than did gliomas without the deletion. Thus, homozygous deletion of the *p16* gene may also affect the prognosis in patients with GBM.

We investigated the correlation between homozygous deletion of *p16* and the duration of survival in 46 patients with GBM. Our study was reviewed and approved by the Human Subjects Review Committee of the University of Kumamoto.

Clinical Material and Methods

Patient Population

The study population consisted of 46 adult patients,
A dose of 45 to 50 Gy was administered to the high intensity region. Patients with more than 50% of the lesion subsumed into the subtotal resection group. Gd-DTPA–enhanced MR imaging as follows: gross-total, no residual lesions (GTR); subtotal, less than 10% of the original tumor mass remained; and partial, more than 10% of the mass remained postoperatively. Subtotal and partial resections were subsumed into the subtotal resection group.

All patients received postoperative adjuvant therapy. A dose of 45 to 50 Gy was administered to the high intensity region plus a 2-cm margin demonstrated on T2-weighted MR images; a boost to a total of 55 to 65 Gy was administered to the Gd-DTPA–enhanced area plus a 2-cm margin. Concomitant with radiotherapy, all patients received chemotherapy consisting of nimustine hydrochloride (ACNU) as a single agent or in combination with another agent(s), including procarbazine, vincristine, and interferon. After initial postoperative therapy, all patients were reexamined. The KPS score, tumor recurrence or regrowth, onset of clinical deterioration, and death were recorded.

The survival time was measured as the time from the date of the initial surgery to the date of death. Progression-free survival time was measured from the date of the initial surgery to the onset of clinical deterioration or radiologically confirmed tumor recurrence.

Tumor Samples

Tumor samples were available from all patients. Tumor tissues were frozen immediately after removal and stored at −80°C until the isolation of genomic DNA was performed. Other tumor tissues were placed in 10% formalin for 24 hours and submitted for histopathological examination.

Analysis of p16 for Homozygous Deletions

We used multiplex PCR for the analysis of p16 homozygous deletion. Genomic DNA from the frozen tumors was isolated by using proteinase K digestion and phenolchloroform extraction. The oligonucleotide primer sequences were 5′-ACAAGTCCCTTCCGCCTCAT-3′ (sense) and 5′-GGCGCCGTCGCCCTGCCC-3′ (antisense) for p16 exon 2 and primers 5′-ATTCTCTCCGGAAGACATGG-3′ (sense) and 5′-AGGCTCTTGAGGCCTCAGG-3′ (antisense) for STS reference on the long arm of chromosome 9 (9qSTS). The PCR amplification was performed using a programmable thermal cycler (MJ Research, Boston, MA) in 25-μl reaction volumes, including 150 to 200 ng genomic DNA, 20 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, 200 μM deoxynucleoside triphosphates, 0.19-μM primers for p16, 0.17-μM primers for 9qSTS, 1.25 U Taq polymerase, and 5% dimethyl sulfoxide. After initial 3-minute denaturation at 95°C, the annealing temperature was gradually decreased from 62°C to 55°C for two cycles each at 62°C, 60°C, and 59°C, three cycles each at 58°C, 57°C, and 56°C, and 13 cycles at 55°C (28 total cycles). Each cycle was at 95°C for 1 minute, annealing for 1 minute, and at 72°C for 1 minute. A final extension step of 72°C for 5 minutes was added. The total of 28 cycles was found to extend the linear range under these PCR conditions (data not shown). After PCR, 5 μl of the reaction product was separated by electrophoresis on a 2% agarose gel and stained with 0.5 μg/ml ethidium bromide under ultraviolet illumination. The resulting images were then density scanned. Band intensities for the areas corresponding to the amplimers of p16 and 9qSTS were determined using the 1.62 image program (Wayne Rasband, National Institutes of Health, Bethesda, MD) and the intensity ratio of p16/9qSTS was calculated. The MIB-1 Labeling Index

Immunostaining of the surgical specimens with MIB-1 was performed in all cases. Sections (6 μm) were cut from paraffin-embedded tissues, mounted on glass slides, and dried. Immunostaining was performed using the avidin-biotin-peroxidase complex method described by Hsu et al. After routine deparaffinization, rehydration, and blocking of the endogenous peroxidase activity, the antigen was retrieved as previously described. Slide-mounted sections were incubated in a 0.01-mM sodium citrate retrieval buffer (pH 6) and then microwaved for 10 minutes at maximum power in a 700-W microwave oven. Using a 1:50 dilution, sections were incubated overnight at 4°C in MIB-1 monoclonal antibody (Immunotech S.A., Marseille, France). Antigen–antibody complexes were detected via the 3,3′-diaminobenzidine/H2O2 reaction, which rendered Ki-67–immunopositive nuclei a dark brown color. Slides were lightly counterstained with hematoxylin, dehydrated, cleared, and mounted. Cells were considered positive for Ki-67 when unequivocal diffuse or dotlike brown nuclear staining could be identified. A minimum of 1000 cells within at least 10 adjacent fields were examined, beginning with the area of greatest immunopositivity. Counting was performed by an experienced observer who was blinded to the data in this study. Stromal cell staining involving cytoplasmic positivity was excluded from the counting process. The MIB-1 LI in each case was calculated as the number of positive cells divided by the total number of examined cells in all fields inspected.

Statistical Analysis

We used the t-test to examine the possible correlation between patient age and the incidence of homozygous deletion of p16 and between the MIB-1 LI and the incidence of homozygous deletion of p16. Actuarial survival curves were generated using the Kaplan–Meier method. The log-rank test was used to estimate differences between survival curves. Cox proportional hazard regression analysis was performed for multivariate analysis. The results of the regression analysis were adjusted for age (≥ 55 compared with > 55 years), preoperative KPS score (≥ 70 compared with < 70), extent of tumor resection (gross total, subtotal, biopsy), and MIB-1 LI (≥ 20 compared with < 20%) as
previously described.\textsuperscript{28,30,67} A probability value of less than 0.05 was considered statistically significant.

\textbf{Results}

\textit{Detection of p16 Homozygous Deletions by Multiplex PCR}

To examine the validity of the multiplex PCR method for the detection of homozygous deletions of the \textit{p16} gene in tissue samples, titration experiments were performed using serial mixtures of normal human leukocyte DNA and DNA from a U251-MG human glioma cell line (Fig. 1). The U251-MG cell line has the \textit{p16} homozygous deletion and no 9q abnormality.\textsuperscript{4,18} Titration experiments were repeated eight times and a standard curve was obtained (Fig. 2). Homozygous deletion of \textit{p16} in the clinical tissue samples was determined according to the slightly modified method of Mochizuki, et al.\textsuperscript{39} Briefly, because histological examination revealed that tumor samples contained less than 20\% nontumor cells, tumor tissues with a \textit{p16}/9qSTS ratio less than 0.46 were judged to contain a homozygous deletion. We reasoned that less than 20\% of total DNA was made up of normal DNA (confidence level, 95\%) based on the \textit{p16}/9qSTS ratio of 20\% normal DNA (0.343 ± 2 standard deviations [0.121]). Figure 3 shows examples of multiplex PCR results in five cases.

\textit{Clinical Characteristics}

Preoperative KPS scores ranged from 50 to 100 (median 80). Of the 46 patients, 15 underwent gross-total resection of their tumor, 26 subtotal resection, and in five patients a biopsy procedure was performed. By the time this analysis was conducted (July 24, 2001), 28 (61\%) of the 46 patients had died of uncontrolled tumor; the remaining 18 patients survived for a median period of 7.6 months (3.3 months–7.2 years) after surgery. Clinical characteristics and prognostic factors are presented in Table 1.

\textit{Homozygous Deletion of the \textit{p16} Gene}

As shown in Table 1, 14 (30.4\%) of 46 patients had the deletion. The incidence was almost the same in both sexes: eight (30.8\%) of 26 men and six (30.0\%) of 20 women. There was no correlation between \textit{p16} deletion and age (p = 0.8646, t-test) or \textit{p16} deletion and MIB-1 LI (p = 0.8563, t-test).

The median survival duration for patients with and without the homozygous deletion was 0.84 and 1.87 years, respectively; the difference was not statistically significant (p = 0.1269, log-rank test; Fig. 4). The median survival of male patients with and without the deletion was 0.75 and 1.34 years, respectively; there was a statistically significant difference between these two groups (p = 0.0030, log-rank test). On the other hand, the deletion did not affect the survival duration for women; it was 1.82 years in those with and 1.98 years in those without the deletion (p = 0.8175, log-rank test).

Progression-free survival data were available in 43 (24 male, 19 female) patients. The median progression-free survival duration of patients with and without the deletion was 0.28 and 0.55 years, respectively; the difference was not statistically significant (p = 0.0977, log-rank test; Fig. 5). Among men with and without the deletion, the difference in
median progression-free survival duration (0.27 and 0.55 years, respectively) was statistically significant (p = 0.0092, log-rank test). This was not the case in women (0.28 compared with 0.5 years; p = 0.7959, log-rank test).

Our statistical analyses suggest that in men with GBM, the p16 homozygous deletion is associated with both median survival and median progression-free survival. To assess the association of survival duration with multiple clinical characteristics, we performed multivariate analysis. As shown in Table 2, the overall survival duration of men was significantly correlated with homozygous deletion of the p16 gene. The overall survival time of women was not associated with the deletion; these results are consistent with the data presented in Fig. 4C. In men, multivariate analysis revealed a correlation between the presence or absence of the deletion and progression-free survival duration (Table 3). Another significant prognostic factor was the patient age.

**Discussion**

We investigated whether homozygous deletion of the p16 tumor suppressor gene is associated with prognosis in patients with GBM. To make the patient cohort as uniform as possible, we selected patients with supratentorial GBM who were treated with surgery and postoperative radiotherapy and chemotherapy. We excluded patients older than 70 years of age because in these individuals chemotherapy is contraindicated.

Multiplex PCR is most widely used to detect p16 homozygous deletions in clinical samples because this method is simple, fast, and reliable. Multiplex PCR was highly concordant with data obtained by fluorescence in situ hybridization and comparative genomic hybridization. Therefore, we used multiplex PCR in our study of the possible correlation between p16 homozygous deletions and the prognosis in patients with GBM.

The prognostic value of p16 abrogation in GBM remains controversial because data from most previous studies have failed to demonstrate an association between p16 abrogation and clinical outcome. James, et al., who analyzed homozygous deletions of the p16 gene in 135 glioma samples by using multiplex PCR, found no significant association with survival in 60 patients with Grade 4 tumors. Alleyne, et al., performed Western blot analysis and compared progression-free survival between patients with and without p16 protein expression; they found no significant difference. Kraus, et al., compared two age- and sex-matched groups of patients with GBMs and found that the frequency of p16 homozygous deletions was not significantly different between patients with shorter and longer postoperative time to tumor progression. Their findings also suggested that in patients with GBM, the presence or absence of the deletion was of no prognostic significance for overall patient survival. Paduvali, et al., examined p16

---

**TABLE 1**

Characteristics of and prognostic factors in 46 patients*

<table>
<thead>
<tr>
<th>Factor</th>
<th>Overall Survival</th>
<th>Progression-Free Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of Cases</td>
<td>Median (yrs)</td>
</tr>
<tr>
<td>age (yrs)</td>
<td>0.0430</td>
<td>9.201</td>
</tr>
<tr>
<td>≥55</td>
<td>30 1.86</td>
<td>30 0.42</td>
</tr>
<tr>
<td>&gt;55</td>
<td>16 0.89</td>
<td>13 0.38</td>
</tr>
<tr>
<td>sex</td>
<td>0.0890</td>
<td>0.1532</td>
</tr>
<tr>
<td>male</td>
<td>26 0.90</td>
<td>24 0.38</td>
</tr>
<tr>
<td>female</td>
<td>20 1.98</td>
<td>19 0.50</td>
</tr>
<tr>
<td>preop KPS score (%)</td>
<td>0.6694</td>
<td>0.4369</td>
</tr>
<tr>
<td>&lt;70</td>
<td>8 1.86</td>
<td>8 0.27</td>
</tr>
<tr>
<td>≥70</td>
<td>38 1.34</td>
<td>35 0.42</td>
</tr>
<tr>
<td>extent of resection</td>
<td>0.6209</td>
<td>0.5229</td>
</tr>
<tr>
<td>gross-total</td>
<td>15 1.34</td>
<td>14 0.39</td>
</tr>
<tr>
<td>subtotal</td>
<td>26 1.34</td>
<td>24 0.42</td>
</tr>
<tr>
<td>biopsy</td>
<td>5 —</td>
<td>5 0.33</td>
</tr>
<tr>
<td>MIB-1 LI (%)</td>
<td>0.4718</td>
<td>0.8249</td>
</tr>
<tr>
<td>&lt;20</td>
<td>26 1.86</td>
<td>25 0.44</td>
</tr>
<tr>
<td>≥20</td>
<td>20 1.34</td>
<td>18 0.35</td>
</tr>
<tr>
<td>homozygous deletion of p16</td>
<td>0.1269</td>
<td>0.0977</td>
</tr>
<tr>
<td>yes</td>
<td>14 0.84</td>
<td>13 0.28</td>
</tr>
<tr>
<td>no</td>
<td>32 1.87</td>
<td>30 0.55</td>
</tr>
</tbody>
</table>

* — = not available.
protein expression in 130 GBMs by using immunohistochemical methods and found no significant correlation between immunoreactivity and patient survival. In a series of 77 high-grade astrocytomas, Kirli, et al.,28 used univariate and multivariate analyses to examine the association of the expression of \(p16\), \(p21\), and \(p53\) with patient survival and the clinical and proliferative characteristics of the tumor. Their parameters included patient age, tumor histology, extent of surgery, and Ki-67 LI. They found that \(p16\) immunonegativity and Ki-67 LI were independent indicators of a poor prognosis. Although the reasons for these divergent findings remain unclear, the results may be affected by the methods used for the detection of homozygous deletions and the statistical analyses.

Our multiplex PCR assays revealed that homozygous deletion of the \(p16\) gene was associated with shorter survival in men, but not in women with GBMs, suggesting a sex difference. In addition, men with the deletion had the shortest median overall survival duration and the shortest median progression-free survival time. Authors of several epidemiological studies documented a lower risk for GBM in female populations.3,13,32,37,68 Although most investigators have not been able to identify sex as a prognostic factor, some claim that women have a better prognosis.12,26,36,46,53,56 The possibility of hormonal involvement has been suggested.26,53 In their population-based case-control study, Schlehofer, et al.,57 demonstrated that menopause is significantly associated with the development of glioma, indicating a role of female hormones. In animal models, estrogen appears to confer a survival advantage and to play a role in the lower incidence of GBM.35,51 Furthermore, the possibility cannot be ruled out that in patients with GBM, sex hormones or sex differences, that is, differences in the susceptibility of X and Y chromosomes to tumorigenesis, play a role in both pathogenesis and prognosis.37 Although in our study the number of patients with homozygous deletions

**Fig. 4.** Graphs showing the correlation of \(p16\) homozygous deletion with survival time in patients with GBM. Kaplan–Meier curves depict survival time from initial surgery for 46 patients (A), 26 men (B) and 20 women (C). The median survival time of each group is shown in brackets. Probability values are shown.

**Fig. 5.** Graphs showing correlation of \(p16\) homozygous deletion with progression-free survival time in patients with GBM. Kaplan–Meier curves show time to progression from the initial surgery for 43 patients (A), 24 men (B) and 19 women (C). The median survival time of each group is shown in brackets. Probability values are shown.
of p16 was too small to draw firm conclusions regarding differences in survival between men and women, a larger study will disclose whether such differences exist.

The frequency of homozygous deletions of p16 in our study (30.4%) was lower than the 33 to 74% reported by others.11,17,25,41,59,64 To our knowledge, there are four reports in which Japanese patients39,48,65 were analyzed; in three of those multiplex PCR was used to detect the deletions. The reported frequency of homozygous deletions of p16 detected by this method was 17.1 to 46.2%. Mochizuki, et al.39 noted a lower frequency in Japanese than in caucasian patients. The frequency of homozygous deletions of the p16 gene in our study (30.4%) falls within the range reported in the other four studies undertaken in Japan.

We did not examine hypermethylation of the 5' CpG island or mutation of the p16 gene; however, these abnormalities have been reported to be rare in gliomas.8,14,19,33,34,38,58 In our study, homozygous deletion of the p16 gene was associated with prognosis. This is presumably because the homozygous deletion is the most effective means of inactivating the INK4a-ARF locus where three tumor suppressor genes, p16, p15, and p14ARF, are located, resulting in abrogation of both Rb and p53 pathways.16,21 A systematic investigation is needed of the components of these pathways in relation to the prognosis of patients with GBM.22 Although we do not have enough data on anaplastic astrocytomas, the significance of the p16 homozygous deletion in these tumors will be made clear by the study of large populations. Systematic large-scale studies will provide more information regarding various factors affecting the prognosis and response to adjuvant therapy in patients with tumors of the nervous system and will facilitate the development of better management strategies.

### Conclusions

Our results suggest that homozygous deletion of the p16 gene is an unfavorable prognostic factor in men but not in women with GBM.

### Acknowledgments

We thank Dr. Akira Kitagawa, Department of Pharmaceutics, Kumamoto University Medical School Hospital, for help with the statistical analyses; Dr. Koji Oka, Department of Neurosurgery, Nakamura Memorial Hospital, Sapporo, for providing DNA samples from patients with glioma that were used in this study; and Masayo Obata for technical assistance.

### References

Homozygous deletion of p16 in glioblastomas multiforme


Manuscript received March 27, 2001.
Accepted in final form November 20, 2001.

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Sports, Science and Culture of Japan and by Grants-in-Aid from the Pacific Rim Research Program (USA).

Address reprint requests to: Takahari Kamiryo, M.D., Department of Neurosurgery, Kumamoto University Medical School, 1-1-1 Honjo, Kumamoto 860-8556, Japan. email: tkamiryo-nsu@umin.ac.jp.