Journal

Journal of Neuropathology and Experimental Neurology

Title

Correlation of MTAP Immunohistochemistry with CDKN2A Status Assessed by Fluorescence In Situ Hybridization and Clinicopathological Features in CNS WHO Grade 2 and 3 Meningiomas: A Single Center Cohort Study

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Abstract

CDKN2A homozygous deletion has occasionally been reported in atypical and anaplastic meningiomas, and is considered as one of the genetic alterations commonly involved in their recurrence and malignant progression. MTAP immunohistochemistry has been considered as a promising surrogate marker for *CDKN2A* homozygous deletion in different cancers. In meningiomas, however, this possibility has not been examined. We performed *CDKN2A* FISH and MTAP immunohistochemistry on specimens from 30 patients with CNS WHO grade 2 (n=27) and 3 (n=3) meningiomas, including specimens from primary and recurrent tumors. Then, we evaluated whether MTAP immunohistochemistry correlated with *CDKN2A* homozygous deletion and clinicopathological features. *CDKN2A* homozygous deletion was detected in 12% (3/26) of CNS WHO grade 2 and 67% (2/3) of CNS WHO grade 3 meningiomas, with three cases exhibiting temporal and/or spatial heterogeneity. Of note, MTAP loss was in excellent concordance with *CDKN2A* homozygous deletion (sensitivity; 100%, specificity; 100%). MTAP loss/*CDKN2A* homozygous deletion correlated with cellular proliferation (mitotic rate; P=0.001, Ki-67 labeling index; P=0.03) and poor prognosis (overall survival; P=0.01, progression free survival; P<0.001). In summary, MTAP immunostaining can be a good surrogate marker for *CDKN2A* homozygous deletion in meningiomas, and MTAP loss/*CDKN2A* homozygous deletion may be one of the important prognostic factors for meningiomas.

Key words

MTAP, *CDKN2A* homozygous deletion, FISH, Spatial and temporal heterogeneity, Meningioma

Introduction

Meningiomas are the most common primary tumors of the central nervous system [1]. Meningiomas are usually slow-growing and benign tumors, but a subset develops recurrence even after gross total resection and requires additional surgery or radiotherapy, having the potential for a lethal outcome [1]. The recurrence risk has been evaluated using the Simpson scale, which indicates the extent of surgical resection, and the 2016 WHO classification based on the histological findings [1, 2]. Although the 2016 WHO classification has some prognostic value, some of the grading criteria are subjective and interobserver concordance of assessments of these criteria is not high [3]. Therefore, development of reliable biomarkers has been required for better evaluation of the recurrence risk. Among genetic alterations commonly involved in recurrence and malignant progression of meningiomas, *TERT* promoter mutations and homozygous deletion of the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) have been described in previous reports [4-13].

Although the 2021 fifth edition of the WHO Classification of Tumors of the Central Nervous System (CNS) has not yet been published, the review article written by Louis *et al* summarizes certain changes [14]. It emphasizes the importance of integrated histologic and molecular diagnoses, and establishes some different approaches to both CNS tumor nomenclature and grading. Arabic numerals are employed for CNS tumor grading, neoplasms are graded within tumor types, and the term "CNS WHO grade" is used when assigning grade (In line with the recommendations, we use the updated term "CNS WHO grade" and Arabic numerals instead of Roman numerals in the present study). For meningiomas, morphological classification and grading are done similarly as in the 2016 WHO classification: the criteria defining atypical or anaplastic (CNS WHO grade 2 or 3)

meningioma should be applied regardless of the underlying subtype, while chordoid and clear cell meningioma are assigned to CNS WHO grade 2. In addition, several molecular biomarkers are associated with classification and grading of meningiomas: for example, meningioma with *TERT* promoter mutation and/or homozygous deletion of *CDKN2A/B* will be automatically assigned to CNS WHO grade 3.

In a single relatively large-scale cohort study, *CDKN2A* homozygous deletion was detected in 3% of CNS WHO grade 2 and 27% of CNS WHO grade 3 meningiomas [4], whereas it is rare in CNS WHO grade 1 meningiomas [4-8]. At present, *CDKN2A* homozygous deletion is evaluated using molecular assays such as fluorescence *in situ* hybridization (FISH), multiplex ligation-dependent probe amplification (MLPA), droplet-digital PCR (ddPCR), array comparative genomic hybridization (aCGH), SNP array, next-generation sequencing (NGS) or DNA methylation profiling microarray [15]. However, these molecular assays cannot be performed in all centers because of the long turn-around time, high costs and detailed procedures. Thus, immunohistochemical surrogate markers for *CDKN2A* homozygous deletion will be needed. Although the utility of p16 immunohistochemistry to detect *CDKN2A* homozygous deletion has been examined in different types of cancer, most studies have demonstrated that p16 immunohistochemistry has high sensitivity and low specificity, not being a good surrogate marker [16-19]. In one study of meningiomas, immunohistochemical p16 expression did not correlate with *CDKN2A* alterations [8]. The methylthioadenosine phosphorylase (*MTAP*) gene, located adjacent to *CDKN2A*, is frequently co-deleted with *CDKN2A* in different types of cancer [20]. Several reports have shown that MTAP immunohistochemistry is correlated with *CDKN2A* homozygous deletion and useful in differentiating malignant mesothelioma from reactive mesothelial hyperplasia [21-24].

Moreover, it was also suggested that MTAP immunohistochemistry is a reliable surrogate for homozygous *CDKN2A* deletion in adult-type infiltrating astrocytoma by a recent study using two different antibody clones of MTAP [16]. To our knowledge, whether MTAP immunohistochemistry can be a reliable surrogate for *CDKN2A* homozygous deletion in meningiomas has not been examined.

Therefore, the aim of this study was to examine the *CDKN2A* copy number status by FISH in patients with CNS WHO grade 2 and CNS WHO grade 3 meningiomas, including specimens from primary and recurrent meningiomas, and to determine whether MTAP immunohistochemistry is correlated with *CDKN2A* homozygous deletion and clinicopathological features in meningiomas.

Materials and methods

Patients and tumor samples

We collected 264 patients with meningiomas diagnosed and treated between January 2010 and December 2020 at Nara Medical University Hospital, including specimens from primary and recurrent meningiomas. We reviewed hematoxylin-eosin and immunohistochemical specimens of primary and recurrent meningiomas to determine the histological type and grade based on the WHO classification with calculation of the Ki-67 labeling index, and identified 234 patients with CNS WHO grade 1, 27 with CNS WHO grade 2 and 3 with CNS WHO grade 3 meningioma [1, 14]: in the present study, unless otherwise stated, the CNS WHO grade represents the highest grade of primary and recurrent meningiomas in each patient. The subjects of the present study were 30 patients with CNS WHO grade 2 (n=27) and CNS WHO grade 3 (n=3) meningiomas, with a total

of 45 available paraffin-embedded formalin-fixed (FFPE) tissue samples from primary and recurrent meningiomas. In two patients, the histological slide and FFPE tissue sample from the primary meningioma were unavailable because the surgery for the primary meningioma was performed at an outside hospital. For each FFPE tissue sample, a representative FFPE block was selected for MTAP immunohistochemistry and FISH analysis. Clinical characteristics, such as demographic, radiological findings, treatment and follow-up data, were retrospectively obtained. For two patients who underwent surgery for the primary meningioma at an outside hospital, detailed clinical information (e.g., time to first recurrence) was unavailable. Tumor recurrence was defined by radiological progression or reappearance of a tumor mass. This study was approved by the local ethics committee of Nara Medical University Hospital (Approval number: 3036).

Immunohistochemistry

Immunohistochemistry staining was carried out using the whole tissue sections of the representative FFPE blocks. The sections of 4-μm thickness were cut and deparaffinized. After blocking the endogenous peroxidase activity using 3% hydrogen peroxide in methanol for 5 min, heat-induced epitope retrieval was performed at pH 9.0 using Tris-EDTA buffer for 20 min. Then, the sections were incubated for 15 min at room temperature with the primary antibody against MTAP (clone 2G4, 1:200; Abnova, Taiwan).

The sections were immunostained using the Leica Bond-III stainer (Leica, Wetzlar, Germany). Horseradish peroxidase activity was visualized with 3, 3'-diaminovenzidine and the nuclei were counterstained with hematoxylin.

MTAP retention was defined by cytoplasmic expression with or without nuclear staining

in tumor cells, and MTAP loss by the absence of cytoplasmic expression in tumor cells in the presence of positive internal controls, such as inflammatory cells or endothelial cells, as described previously [16, 22]. In each tissue section, any areas lacking cytoplasmic expression in inflammatory cells or endothelial cells were excluded from assessment.

FISH

FISH analysis of *CDKN2A* was performed as described previously [25]. Briefly, dual-color FISH analysis was carried out on 4-μm-thick tissue sections using Vysis LSI CDKN2A Spectrum Orange/CEP9 Spectrum Green probes (Abbott Molecular, Abbott Park, IL, USA). The FISH probe spanned approximately 222 kb in the 9p21 region and contained several genes, including *MTAP*, *CDKN2A* and *CDKN2B*. At least 100 non-overlapping tumor cells were scored for each sample. Homozygous deletion was defined by the loss of two copies of *CDKN2A* in the presence of one or two centromeric signals in >15% tumor cells. Heterozygous deletion was defined by the loss of one copy of *CDKN2A* in the presence of two centromeric signals in >40% tumor cells [26].

Statistical analysis

Quantitative variables between two groups were compared using the Mann-Whitney U test. Categorical variables were compared by the Fisher's test. The Kaplan-Meier method was used to evaluate progression-free survival (PFS) and overall survival (OS), and these were compared by the log-rank test using the statistical software EZR (Easy R) (Saitama Medical Center, Jichi Medical University, Saitama, Japan) [27]. In cases with MTAP retention, PFS or OS was defined as the interval from the day of first surgery until first recurrence or death. In cases with MTAP loss, PFS or OS was defined as the interval from

the day of surgery for the first meningioma with MTAP loss until subsequent recurrence or death.

Results

Patient and tumor characteristics

The current study consisted of 30 patients with CNS WHO grade 2 (n=27) and CNS WHO grade 3 (n=3) meningioma. Twenty-seven CNS WHO grade 2 meningiomas were composed of 26 atypical meningiomas and one clear cell meningioma based on the WHO classification. Of three CNS WHO grade 3 meningiomas, papillary or rhabdoid meningiomas were not included. Of 27 patients with CNS WHO grade 2 meningiomas, 11 underwent more than one surgery: three patients presented initially with CNS WHO grade 1 meningiomas, whereas eight patients had CNS WHO grade 2 meningiomas at the first surgery. Two of three patients with CNS WHO grade 3 meningiomas underwent more than one surgery, and both had CNS WHO grade 3 meningiomas at the first surgery.

Twelve of 30 patients were male and 18 were female. The mean age at the first surgery was 68 years (range, 30-96). The mean follow-up period was 43 months (range, 1-110). The number of surgeries that each patient underwent ranged from one to five. The tumor location was at the convexity in 11 patients, the falx or parasagittal region in 10, the skull base in six, the tentorial region in two, and the infratentorial region in one. In all but one patient, total or subtotal surgical resection was performed with different Simpson grades. One patient (patient #2) underwent surgical biopsy (Simpson grade V). Seventeen of 30 patients received adjuvant radiotherapy. The clinicopathological characteristics of MTAP loss and the MTAP retention groups are shown in Table 1. Patients with meningiomas

with MTAP loss are summarized in Table 2.

MTAP immunohistochemistry

All 45 meningioma samples from 30 patients were appropriately tested and evaluated. In all but one (patient #1) of 13 patients who underwent more than one surgery, there was no difference in MTAP expression status between primary and recurrent meningiomas.

Of 30 cases, 24 (including two in which samples from the primary meningioma were not available), comprising 23 CNS WHO grade 2 and one CNS WHO grade 3 meningiomas, exhibited MTAP retention. In all 24 meningiomas, homogeneous MTAP staining was observed: twenty-two meningiomas had strong staining, which enabled accurate interpretation even with low magnification objectives (2×) (Fig. 1A), whereas two demonstrated faint staining (in some areas, obviously fainter than positive internal controls), which required observation with at least low to intermediate magnification objectives (10, 20×) (Fig. 1B).

Six cases, composed of four CNS WHO grade 2 and two CNS WHO grade 3 meningiomas, exhibited MTAP loss (Fig. 1C). In three of these six cases (patient #2, #3, #4), the loss of cytoplasmic expression was uniformly observed. On the other hand, the remaining three cases (patient #1, #5, #6) had both MTAP loss and retention areas; two areas were somewhat clearly separated rather than intermingled (Fig. 1G). In these three cases with spatial heterogeneity of MTAP loss, FISH analysis was performed on two areas separately.

***CDKN2A* FISH**

All 45 meningioma samples from 30 patients were tested. Although the FISH probe did

not hybridize in one primary meningioma sample from patient #2, FISH analysis was successful for the remaining 44 meningioma samples from 29 patients. In all but one (patient #1) of 13 patients who underwent more than one surgery, there was no difference in *CDKN2A* copy number status between primary and recurrent meningiomas, similar to MTAP expression.

All 24 cases with MTAP retention (strong staining: 22 cases, faint staining: 2 cases), in which FISH analysis was successfully performed, exhibited a normal diploid pattern. Both cases demonstrating uniform loss of MTAP expression (patient #3, #4), in which FISH analysis was able to be performed, harbored *CDKN2A* homozygous deletion. Representative meningiomas with MTAP retention and *CDKN2A* diploid, MTAP loss and *CDKN2A* homozygous deletion are illustrated in Fig. 1. In all three meningiomas with spatial heterogeneity of MTAP loss (patient #1, #5, #6), *CDKN2A* homozygous deletion was identified in the MTAP loss area. On the other hand, the MTAP retention area exhibited a normal diploid pattern (patient #6) or heterozygous deletion (patient #1, 5) (Fig. 1G-J, 2).

In summary, excluding one case (patient #2) in which FISH analysis was unable to be performed, *CDKN2A* homozygous deletion was detected in 12% (3/26) of CNS WHO grade 2 and 67% (2/3) of CNS WHO grade 3 meningiomas. Of note, loss of MTAP expression demonstrated 100% sensitivity (5/5) and 100% specificity (24/24) for detecting *CDKN2A* homozygous deletion.

Temporal and spatial heterogeneity of *CDKN2A* homozygous deletion

As described above, spatial heterogeneity of *CDKN2A* homozygous deletion was detected in three cases (patient #1, #5, #6). Temporal heterogeneity was also observed in

one case (patient #1).

Patient #1 underwent four surgeries. The first surgical specimen was CNS WHO grade 1 meningioma (transitional meningioma), and the three subsequent surgical specimens were CNS WHO grade 2 with increased mitotic activity (four or five mitoses per 10 HPF) and brain invasion (Fig. 2A-C). The first surgical specimen was *CDKN2A* diploid, the second exhibited spatial heterogeneity of *CDKN2A* homozygous deletion, and the third and fourth displayed homogeneity of *CDKN2A* homozygous deletion, consistent with spatial and temporal heterogeneous MTAP immunostaining, by FISH using a representative FFPE block (Fig. 2D-J). In order to confirm that the first surgical specimen had no *CDKN2A* deletion, MTAP immunostaining was additionally carried out using the whole tissue sections of all remaining FFPE blocks of the first surgical specimen. MTAP loss was absent, which confirmed the presence of temporal heterogeneity of *CDKN2A* homozygous deletion. In the second surgical specimen exhibiting spatial heterogeneity of *CDKN2A* homozygous deletion, there was no significant difference in pathomorphological features, including mitotic counts (four mitoses per 10 HPF in both two areas) and Ki-67 labeling indices (MTAP loss: 19%, MTAP retention: 14%), between MTAP loss and MTAP retention areas.

Patients #5 and #6 underwent one surgery for the primary meningioma. In patient #6, like patient #1, there was no significant difference in pathomorphological features, including mitotic counts (five mitoses per 10 HPF in both two areas) and Ki-67 labeling indices (MTAP loss: 12%, MTAP retention: 14%), between MTAP loss and MTAP retention areas. On the other hand, in patient #5, there was a significant difference in pathomorphological features between MTAP loss and MTAP retention areas as follows. The MTAP retention area exhibited a CNS WHO grade 2 appearance with increased

cellularity, small cells with a high nuclear-to-cytoplasmic ratio, sheeting and moderate proliferative activity (Ki-67: 12%), but lacked increased mitotic activity (one mitosis per 10 HPF) and spontaneous necrosis (Fig. 3A-C). On the other hand, the MTAP loss area demonstrated a CNS WHO grade 3 appearance with overtly malignant cytology (characterized by epithelioid and spindle cells with slightly abundant collagenous stroma), increased mitotic activity (15 mitoses per 10 HPF), spontaneous necrosis and high proliferative activity (Ki-67: 35%) (Fig. 3D-F).

Correlation between MTAP loss/*CDKN2A* homozygous deletion and clinicopathological features

Pathologically, MTAP loss (almost the same as *CDKN2A* homozygous deletion) correlated with cellular proliferation. MTAP loss was significantly associated with the mitotic rate (9.8 per 10 HPF versus 2.4 per 10 HPF, P=0.001; Table 1) and the Ki-67 labeling index (20.8% versus 11%, P=0.03; Table 1). All six cases with MTAP loss had four or more mitotic counts per 10 HPF (Table 2).

MTAP loss correlated closely with poor outcomes. Patients with MTAP loss had a significantly shorter OS (median OS: 51 months versus 84 months, P=0.01, Fig. 4A). Across 28 cases, in which the histological slide and the FFPE tissue sample of the primary meningioma and detailed clinical follow-up data were available, there was a strong correlation between MTAP loss and PFS (median PFS: 9 months versus 50 months, P<0.001, Fig. 4B). MTAP loss was not significantly associated with age, sex, tumor location or CNS WHO grade (Table 1).

Discussion

In our series of 29 cases in which FISH analysis was able to be performed, we detected *CDKN2A* homozygous deletion in 12% (3/26) of CNS WHO grade 2 and 67% (2/3) of CNS WHO grade 3 meningiomas. Although the number of cases in our study was small, this frequency was higher than that observed in other series of CNS WHO grade 2 (0-11%) and CNS WHO grade 3 (9-50%) meningiomas [4-8]. This discrepancy may be mainly due to spatial and temporal heterogeneity of *CDKN2A* homozygous deletion as we demonstrated. Regarding spatial heterogeneity, three cases (patient #1, #5, #6) in our study may be determined as heterozygous deletion or diploid by other gene analysis methods due to sample selection bias.

In the present study, we evaluated whether MTAP immunohistochemistry was correlated with *CDKN2A* homozygous deletion in meningiomas. Of note, the loss of MTAP expression was in excellent concordance with *CDKN2A* homozygous deletion by FISH in meningiomas (sensitivity; 100%, specificity; 100%). Although a study of the correlation between MTAP immunohistochemistry and *CDKN2A* homozygous deletion in meningiomas has not yet been published, it has been assessed in previous studies of malignant mesothelioma and adult-type infiltrating astrocytoma, demonstrating good sensitivity (74-88%) and specificity (96-100%) [16, 21-24]. On the other hand, a recent study of 40 pleural mesothelioma cases reported a poor correlation between MTAP immunohistochemistry and *CDKN2A* homozygous deletion by hybrid capture NGS and/or SNP microarray (sensitivity: 46%, specificity: 67%): in their study, however, molecular studies and immunohistochemistry were performed on different surgical specimens or different tissue blocks in 38 of 40 tumors, more likely to limit direct comparison of immunohistochemistry and molecular findings, and affect the result [28]. These authors discussed that a good but incomplete correlation between MTAP

immunohistochemistry and *CDKN2A* homozygous deletion may be due to epigenetic modifications and size of deletion on the chromosome 9p21.3 locus, in addition to subclonal gene deletion [16, 21-24, 28]. For example, epigenetic silencing of *MTAP* through promoter hypermethylation and *MTAP* deletion plays a role in MTAP loss in melanocytic lesions [29]. According to a comprehensive integrative molecular analysis of tumors (composed of approximately 10,000 specimens and 33 types of cancers) in The Cancer Genome Atlas (TCGA) using the cBioPortal database, 69% of tumors with *CDKN2A* homozygous deletion have *MTAP* homozygous deletion, whereas 98.5% of tumors with *MTAP* homozygous deletion demonstrate *CDKN2A* homozygous deletion: only *MTAP* homozygous deletion is rare [30].

In the present study, of 24 cases with MTAP retention, two exhibited MTAP faint staining. MTAP faint staining has been described with figures by two studies of pleural mesotheliomas [22, 28], and Chapel et al. defined it as tumor cell cytoplasmic staining incontrovertibly fainter than positive internal controls [22]. These two studies, when combined, revealed 18 cases with MTAP faint staining, of which 14 had *CDKN2A* homozygous deletion [22, 28]. In contrast, although only two cases with MTAP faint staining were detected in our study, both were *CDKN2A* diploid. The difference in the association of MTAP faint staining with the *CDKN2A* copy number status between the current study and previous studies may be due to the following: the differences in the pathogenesis or the tumor microenvironment between meningiomas and mesotheliomas, or artefacts in immunohistochemistry. In addition, the current study revealed heterogeneous MTAP immunostaining in three cases (patient #1, #5, #6), similar to previous studies [21, 22, 24, 28]. Although the *CDKN2A* copy number status in each MTAP loss and retention area was not determined separately in these previous studies, all

three cases (patient #1, #5, #6) in our study demonstrated spatial heterogeneity of *CDKN2A* homozygous deletion consistent with heterogeneous MTAP immunostaining. The association of MTAP faint staining with the *CDKN2A* copy number status and the significance of heterogeneous MTAP staining need to be further studied.

In our consecutive series of 30 patients with meningiomas, we aimed to determine whether MTAP immunohistochemistry is correlated with clinicopathological features. MTAP loss (almost the same as *CDKN2A* homozygous deletion) was significantly associated with both the mitotic rate and the Ki-67 labeling index, consistent with a recent study by Guyot et al. [8]: most previous studies did not evaluate or describe the correlation between *CDKN2A* homozygous deletion and cellular proliferation [4-7]. Of note, all five meningiomas with *CDKN2A* deletion in their study had four or more mitotic counts per 10 HPF, similar to our study [8]. In addition, we found pathomorphological changes consistent with temporal or spatial heterogeneity of *CDKN2A* homozygous deletion in two cases (patient #1, #5), which suggested that *CDKN2A* homozygous deletion plays a role in malignant transformation. As for clinical outcomes, patients with MTAP loss in our series had a significantly shorter OS and PFS. Similarly, a single relatively large-scale cohort study demonstrated an independent adverse effect of *CDKN2A* homozygous deletion on the time to progression of patients with meningiomas [4]. Moreover, using DNA methylation-based meningioma classification distinguishing six distinct clinically relevant methylation classes, *CDKN2A* homozygous deletion was essentially observed in the methylation classes intermediate or malignant [4, 13]. Our study and previous studies suggest that *CDKN2A* homozygous deletion is deeply involved in the cellular proliferation and prognosis of meningiomas.

The present study has several limitations. First, we demonstrated the association of

MTAP immunohistochemistry with *CDKN2A* homozygous deletion and clinicopathological features, and temporal and spatial heterogeneity of *CDKN2A* homozygous deletion in meningiomas, but the number of cases was small. These findings (particularly the correlation between MTAP immunohistochemistry and *CDKN2A* homozygous deletion) must be confirmed in a larger series. Second, although the univariate statistical analysis revealed that MTAP loss was significantly prognostic in meningioma cases, the results may be affected to some extent by other prognostic factors such as Simpson grade or CNS WHO grade. Indeed, as for Simpson grade at surgery for the first meningioma with MTAP loss, three of six cases were Grade IV and one was Grade V. Third, we evaluated the *CDKN2A* copy number status solely by FISH using the commercial probe which hybridizes to both *MTAP* and *CDKN2A*. Some studies have shown that FISH is inferior to aCGH or SNP array for detection of *CDKN2A* homozygous deletion in different cancers, emphasizing that focal microdeletions in *CDKN2A* can be missed by FISH using the commercial probe [31-33]. Although only *MTAP* homozygous deletion is generally rare in cancers, as described above, we cannot exclude the possibility that our six cases with MTAP loss included meningiomas with only *MTAP* homozygous deletion: tumors with only *MTAP* homozygous deletion can be determined as those with "*CDKN2A*" homozygous deletion by FISH using the commercial "CDKN2A" probe [16]. Further studies with additional experiments enabling the assessment of copy number at a specific *CDKN2A* locus (e.g., FISH using a specific probe for CDKN2A, MLPA or SNP array) are needed.

In summary, MTAP immunostaining can be a good surrogate marker for *CDKN2A* homozygous deletion in meningiomas, and heterogeneous staining can reflect the heterogeneity of *CDKN2A* homozygous deletion. MTAP loss/*CDKN2A* homozygous

deletion may be one of the important prognostic factors for meningiomas. Further studies with larger sample sizes, multiple molecular assays and a multivariate analysis are needed for confirmation.

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Figure legends

Fig. 1 MTAP immunohistochemistry and *CDKN2A* FISH (A, D; patient #11, B, E; patient #9, C, F; patient #3, G-J; patient #6).

Most meningiomas with *CDKN2A* diploid showed strong MTAP staining, as in patient #11 (A, D), whereas a few demonstrated MTAP faint staining (obviously fainter than positive internal controls) as in patient #9 (B, E). A subset of meningiomas exhibited MTAP loss and *CDKN2A* homozygous deletion, as in patient #3 (C, F). A few meningiomas had spatial heterogeneity of *CDKN2A* homozygous deletion consistent with heterogeneous MTAP immunostaining, as in patient #6 (G-J). The tumor had a MTAP loss area (lower side) and a MTAP retention area (upper side); the former showed patchy staining due to the presence of some positive internal controls (G). High magnification image of the boxed area in **G**, reveals a clear boundary between these areas (H). The MTAP retention area demonstrated *CDKN2A* diploid (I). The MTAP loss area showed *CDKN2A* homozygous deletion; one or two green signals and no orange signals were seen in tumor cells (upper side), whereas one or two green signals and one or two orange signals were seen in positive internal controls (lower side) such as blood cells or vascular endothelial cells (J).

Fig. 2 The atypical meningioma of patient #1, showing spatial and temporal heterogeneity of MTAP loss and *CDKN2A* homozygous deletion (A, D, G; the first surgical specimen, B, E, H, I; the second surgical specimen, C, F, J; the fourth surgical specimen).

The first surgical specimen was a CNS WHO grade 1 meningioma (transitional meningioma) (A) with uniform MTAP retention/*CDKN2A* diploid (D, G). The second

surgical specimen was a CNS WHO grade 2 meningioma (B, inset shows mitoses) with a MTAP retention/*CDKN2A* heterozygous deletion area (E, H) and a MTAP loss/*CDKN2A* homozygous deletion area (E, I). The fourth surgical specimen was a CNS WHO grade 2 meningioma (with brain invasion) (C, GFAP) with uniform MTAP loss/*CDKN2A* homozygous deletion (F, J). MTAP immunohistochemistry indicated loss of expression in tumor cells in the presence of positive internal controls represented by brain parenchyma (F). Loss of two copies of *CDKN2A* in tumor cells (right side) was noted in the presence of controls represented by brain parenchymal cells (left side) (J).

Fig. 3 The anaplastic meningioma of patient #5, showing spatial heterogeneity of MTAP loss and *CDKN2A* homozygous deletion (A-C; the MTAP retention/*CDKN2A* heterozygous deletion area, D-F; the MTAP loss/*CDKN2A* homozygous deletion area). The MTAP retention/*CDKN2A* heterozygous deletion area (B) had a CNS WHO grade 2 appearance (A) and moderate proliferative activity (C, Ki-67). The MTAP loss/*CDKN2A* homozygous deletion area (E) had a CNS WHO grade 3 appearance, characterized by epithelioid and spindle cells with slightly abundant collagenous stroma (arrows indicate mitotic figures) (D) and high proliferative activity (F, Ki-67).

Fig. 4 Kaplan-Meier curves for progression-free survival (PFS) and overall survival (OS) stratified by MTAP staining status. MTAP loss correlated with both OS (A) and PFS (B) (log-rank test).

Fig.1

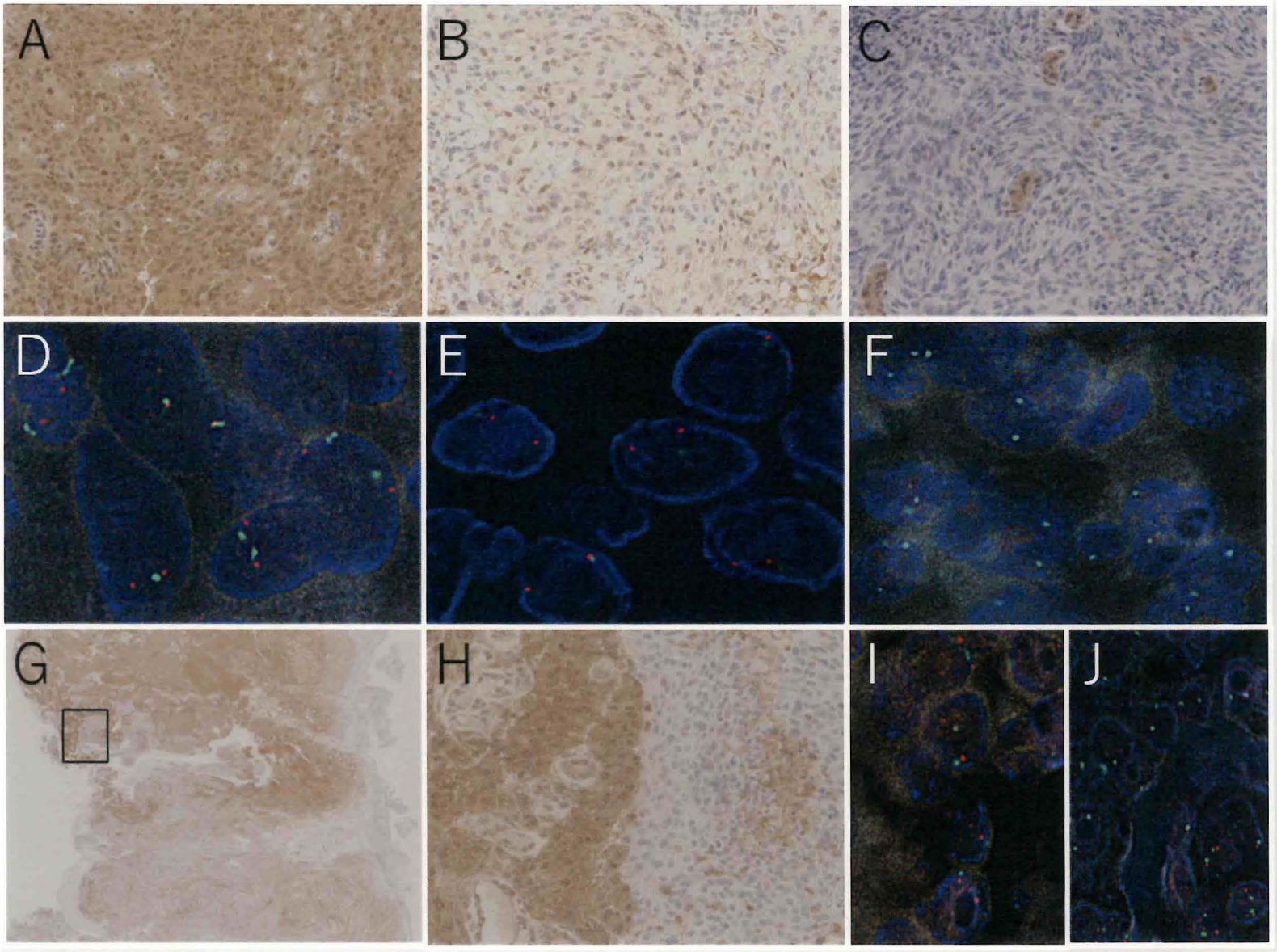

Fig.2

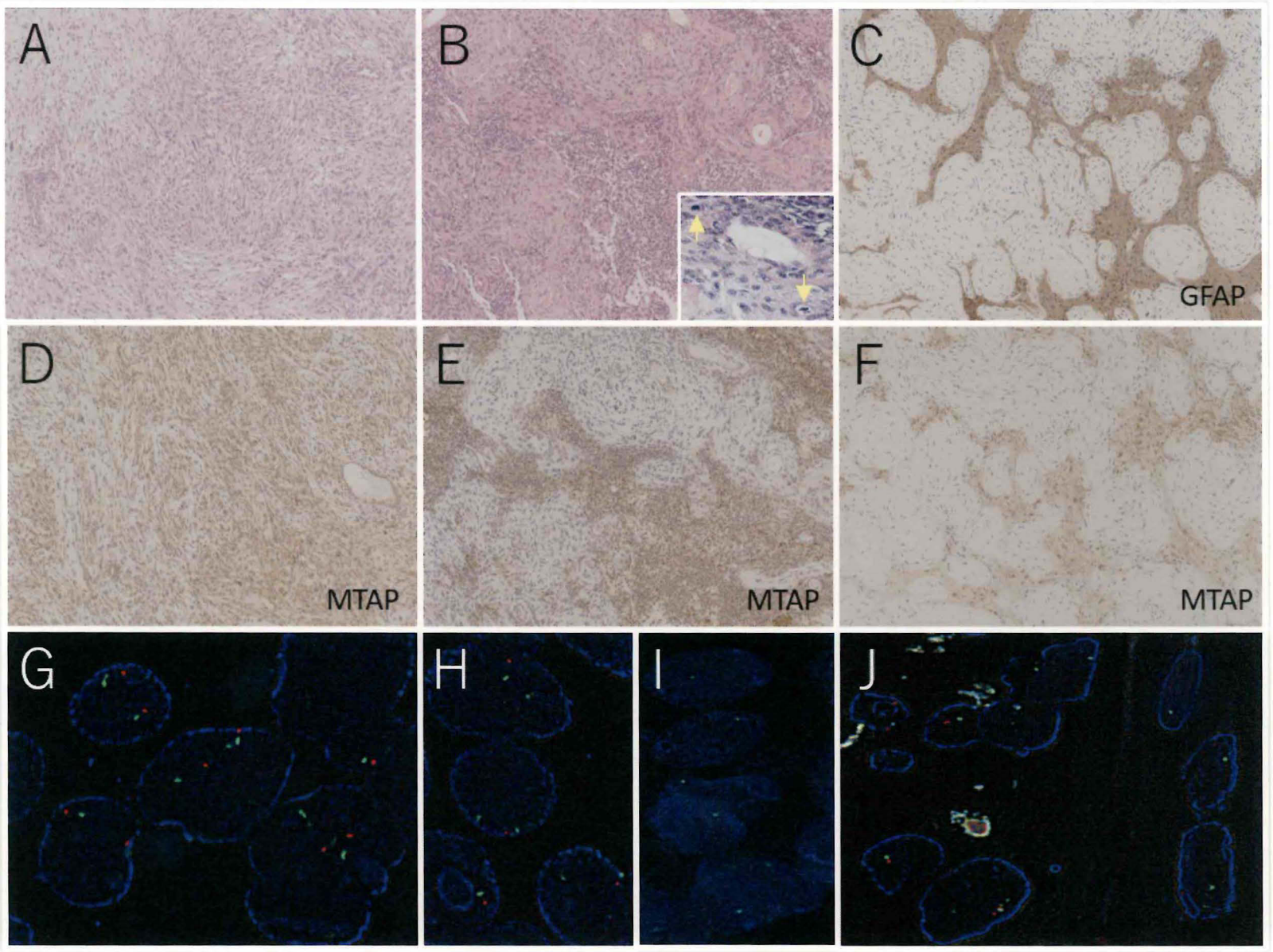

Fig.3

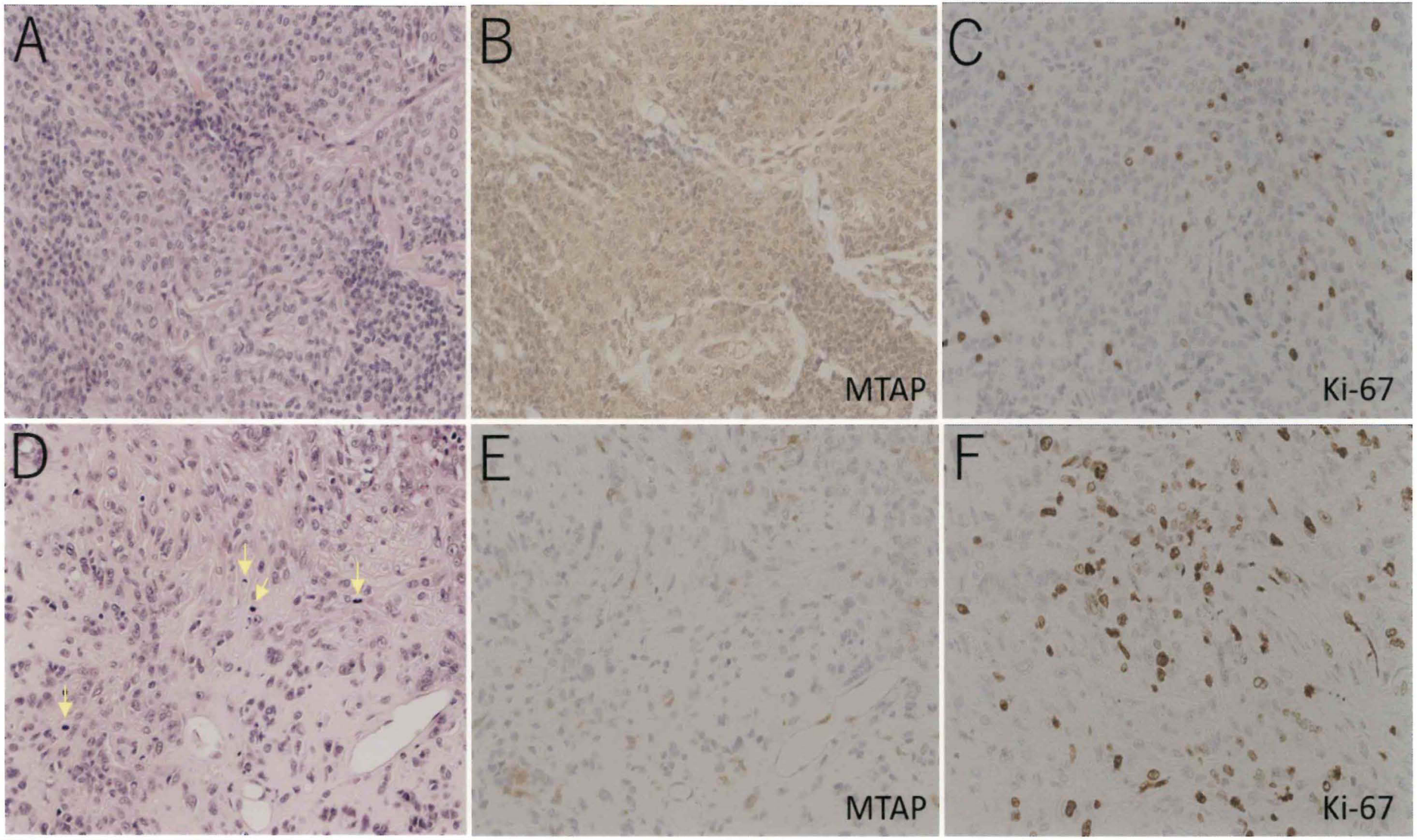

Fig.4

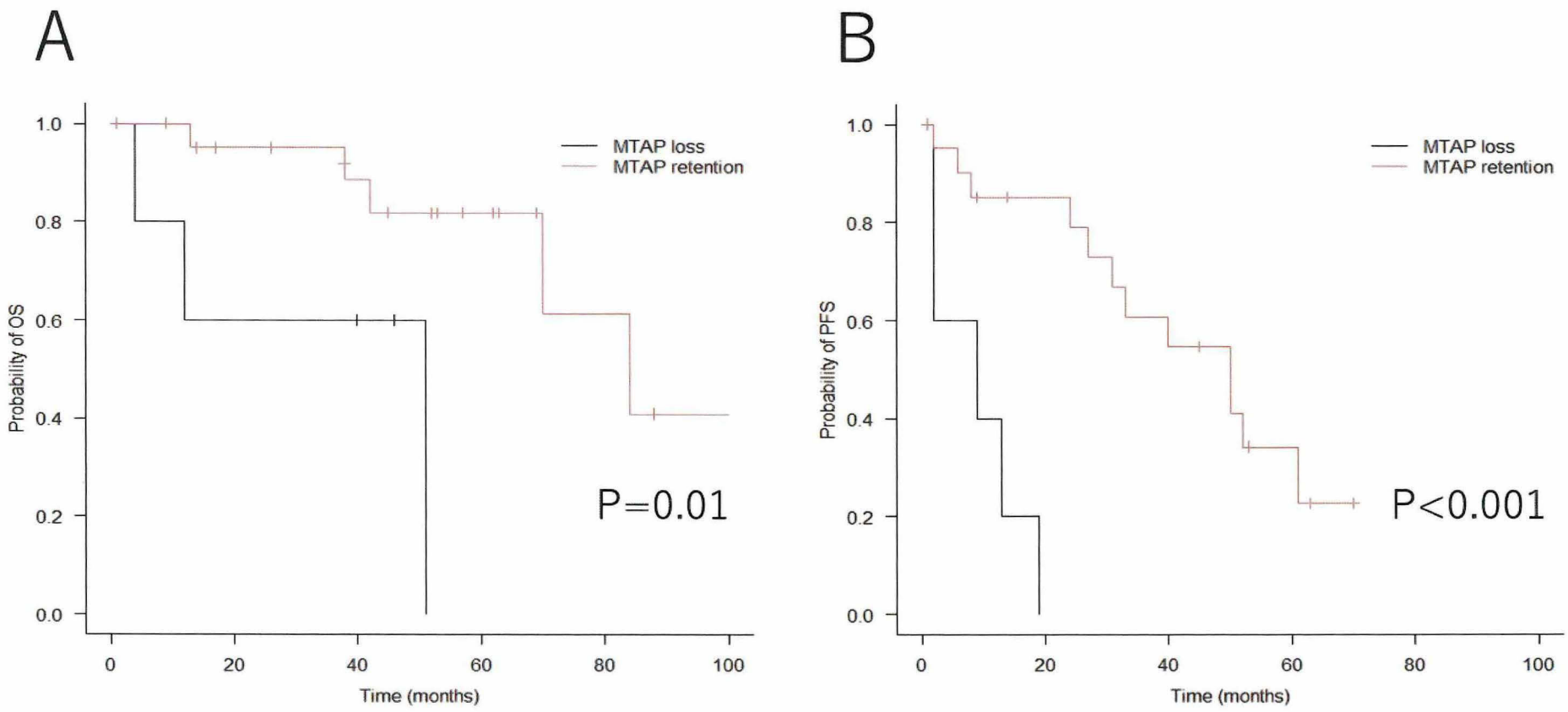
A
1.0
0.8
0.6
0.4
0.2
0.0
Probability of OS
0
20
40
60
80
100
Time (months)
MTAP loss
MTAP retention
P=0.01
B
1.0
0.8
0.6
0.4
0.2
0.0
Probability of PFS
0
20
40
60
80
100
Time (months)
MTAP loss
MTAP retention
P<0.001

Table 1. Clinicopathological characteristics of the MTAP loss and MTAP retention groups

	Total (n=30)	MTAP loss (n=6)	MTAP retention (n=24)	*p*
Age, years	68±15	71±18	67±15	0.42
Female	18 (60)	5 (83)	13 (54)	0.36
Tumor location				0.05
Convexity	11 (37)	3 (50)	8 (33)	
Parasagittal/falx	10 (33)	1 (17)	9 (37)	
Skull base	6 (25)	0 (0)	6 (25)	
Tentorial	2 (7)	2 (33)	0 (0)	
Infratentorial	1 (3)	0 (0)	1 (4)	
Simpson grade				0.25
Grade I, II or III	13 (43)	2 (33)*	11 (46)**	
Grade IV	16 (53)	3 (50)*	13 (54)**	
Grade V	1 (3)	1 (17)*	0 (0)	
Highest CNS WHO grade				0.09
Grade 2	27 (90)	4 (67)	23 (96)	
Grade 3	3 (10)	2 (33)	1 (4)	
Mitotic counts, per 10 HPF	3.9±5.5	9.8±6.0*	2.4±4.3**	0.001
Ki-67, %	13±10.1	20.8±8.9*	11±9.7**	0.03
CDKN2A homozygous deletion	5 (17)	5 (100)	0 (0)	<0.001
Length of available follow-up, months	43.1±31.4	25.7±22.5	42.8±29.7	0.15
Recurrence	19 (63)	5 (83)	14 (58)	0.37
Dead	8 (27)	3 (50)	5 (21)	0.3
Adjuvant radiotherapy	16 (53)	5 (83)	11 (46)	0.18

Data are expressed as means±SD or number (%). Missing data were excluded in the given percentages. HPF, high-power field.
* at surgery for the first meningioma with MTAP loss. ** at first surgery.

Table 2. Overview of patients with meningiomas with MTAP loss

Patient #	Sex	Age at first surgery (years)	Tumor location	CNS WHO grade	MTAP loss status	Mitoses* per 10 HPF	Ki-67* (%)	*CDKN2A* status	Simpson* grade	Number of surgeries	Recurrence**	PFS† (months)	Dead	Length of follow-up† (months)	RT
1	F	73	Falx	2	heterogeneous (spatial and temporal)	4, MP(-) 4, MP(+)	19, MP(-) 14, MP(+)	homozygous deletion: subclone with heterozygous deletion	I	4	Yes	19	No	40	Yes
2	F	88	Convexity	2	uniform	5	14	N/A	V	1	Yes	2	Yes	4	Yes
3	F	36	Tentorial	3	uniform	12	25	homozygous deletion	IV	5	Yes	9	Yes	51	Yes
4	F	77	Tentorial	2	uniform	18	18	homozygous deletion	IV	1	Yes	13	No	46	Yes
5	M	79	Convexity	3	heterogeneous (spatial)	15, MP(-) 1, MP(+)	35, MP(-) 12, MP(+)	homozygous deletion: subclone with heterozygous deletion	IV	1	Yes	2	Yes	12	Yes
6	F	72	Convexity	2	heterogeneous (spatial)	5, MP(-) 5, MP(+)	12, MP(-) 14, MP(+)	homozygous deletion	II	1	No	1	No	1	No

F, female; M, male; HPF, high-power field; PFS, progression-free survival; RT, radiotherapy; MP(+), MTAP retention area; MP(-), MTAP loss area.
*at surgery for the first meningioma with MTAP loss.
** recurrence after surgery for the first meningioma with MTAP loss.
†from the time when surgery for the first meningioma with MTAP loss was performed.