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Proliferation Potentials of Human Intracranial Neoplasms Assessed with Ki-67 (MIB-1) Labeling Index and Argyrophilic Nucleolar Organizer Regions

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To evaluate the accuracy of the Ki-67 labeling index (LI) as an index of brain tumor proliferation, specimens of 303 intracranial neoplasms were examined for tumor proliferation potentials as assessed by MIB-1 (Ki-67 antigen) immunostaining and/or counting the argyrophilic nucleolar organizer regions (Ag-NORs). The Ki-67 LIs were higher in the histologically high grade tumors, i.e., the metastatic carcinomas, malignant lymphomas, glioblastomas, medulloblastomas, anaplastic astrocytomas, anaplastic oligodendrogliomas, anaplastic ependymomas, and anaplastic meningiomas compared to the histologically low grade tumors, i.e., the pilocytic astrocytomas, grade II astrocytomas, oligodendrogliomas, ependymomas, meningiomas other than those of the anaplastic variety, and pituitary adenomas. The number of Ag-NORs was not correlated with the tumor grade, and was inconsistent with the growth potential in some tumors. Moreover, in the consecutive sections of 87 tumors stained for both Ki-67 and Ag-NORs, the number of Ag-NORs did not correlate with the tumor Ki-67 LI in a regression analysis ($r = 0.139$). The patients with a high grade astrocytoma (anaplastic astrocytoma and glioblastoma) and a tumor Ki-67 LI > 10 had significantly shorter survival periods than those of the patients with a Ki-67 LI < 10 ($p = 0.029$). Our results thus suggest that Ki-67 is a potential marker of tumor growth activity and that the Ki-67 LI may be of use as an indicator of the prognosis of patients with brain tumor. The number of Ag-NORs may not always reflect the growth potential of some tumors.

Key Words : Brain Neoplasm, Ki-67, Labeling Index, Ag-NOR, Histologic Grade, Correlation, Survival

Introduction

Neoplastic diseases are characterized by un-coordinated cell growth. Cellular proliferation follows an orderly progression through the cell cycle, which is governed by many protein complexes. The growth of brain tumors results from the relative proportion of cells contained in three populations : a) cycling/proliferative ; b) quiescent (G0)/static, and c) terminally differentiated/dying. Intracranial neoplasms are critical due to their growth rate, origin and location, which directly reflect the surgical outcome, and a proportion of these tumors recurs even after complete surgical resection. The growth rate directly affects the patient's prognosis. Thus, quantification of the proliferative potential would help to predict the biological behavior of these tumors and may influence their treatment. Among the cell proliferation markers, BUdR is an experimental agent which estimates the S-phase fraction of cells. Its identification requires the pretreatment of living cells[1-3]. Proliferating cell nuclear antigen (PCNA) is a 36-kDa DNA polymerase-delta auxiliary protein which accumulates in the nucleus during the S phase of the cell cycle[4]. Labeling with the monoclonal antibody Ki-67 recognizes the native Ki-67 antigen and recombinant fragments of the Ki-67 molecules expressed in the G1, S, G2, and M phases of the cell cycle but absent in the quiescent cells[5]. The antibody to Ki-67 has been used extensively in recent years both as a laboratory index and as a clinical index of tumor proliferation[6-12]. In addition, the number of argyrophilic nucleolar organizer regions (Ag-NORs) has been reported to correlate closely with cellular proliferations[13-16]. The present study was undertaken to evaluate the accuracy of the Ki-67 LI by using MIB-1 immunostaining and the number of Ag-NORs as indicators of brain tumor proliferation, with a special emphasis on astrocytic tumors which constitute 50% of all brain tumors.

Materials and Methods

Specimens of 303 brain tumors obtained from patients who had undergone surgical resection at our Department of Neurosurgery, Nagasaki University School of Medicine, Nagasaki, Japan, were retrospectively analyzed for the proliferative nuclear Ki-67 antigen and Ag-NOR. All of the tumors samples had been formalin-fixed and paraffin-embedded. The 5-μm tumor sections were cut and stained with hematoxylin and eosin for histologic classification and grading according to the World Health Organization

Table 1. Ki-67 labeling index (LI) in intracranial neoplasms.

Histology	Ki-67 LI*	Ag-NOR/cell*
Pilocytic astrocytomas	1.20±1.56 (9)	ND
Astrocytomas (gradeII)	1.52±3.18 (32)	2.04±0.54 (8)
Anaplastic astrocytomas	13.47±11.40 (34)	2.40±0.77 (7)
Glioblastomas	19.54±18.34 (59)	2.71±1.13 (24)
Oligodendrogliomas	2.95±3.89 (6)	1.91 (2)
Anaplastic oligodendrogliomas	13.08±3.47 (3)	1.92±0.99 (6)
Oligoastrocytomas	4.85 (2)	ND
Ependymomas	1.67±2.89 (3)	2.30 (2)
Anaplastic ependymomas	28.64 (2)	2.97 (2)
Medulloblastomas	12.31±10.78 (11)	2.99±1.49(19)
Meningiomas		
Meningotheliomatous	2.68±4.74 (13)	2.03±0.48 (29)
Fibrous	1.19±1.97 (8)	1.98±0.54 (26)
Transitional	1.27±1.79 (8)	1.94±0.66 (20)
Anaplastic	16.76 (2)	3.53±1.78 (4)
Angiomatous	1.33±2.31 (3)	1.63±0.41 (3)
Pituitary adenomas	1.52±4.03 (17)	2.09±0.49 (29)
Metastatic brain tumors	37.69±21.37 (14)	2.99±1.49 (19)
Malignant lymphomas	36.78±38.64 (4)	2.27 (2)

*, mean±standard deviations. The numbers of tumors examined by each method are in parentheses. ND : notdone

Histological Classification of Central Nervous System Tumors[17]. Consecutive tumor sections were stained for the Ki-67 antigen and Ag-NOR. The brain tumors are detailed shown in Table 1.

Immunohistochemical staining of Ki-67 antigen

The staining method used was that which we described previously[7]. Deparaffinized tissue sections were rinsed in distilled water for rehydration, placed in a glass container filled with 10 mM citrate buffer (pH 6.0), processed in a domestic micro-oven (Hitachi MR-A330, Tokyo, Japan) for 15 minutes at 500 W, and then cooled to room temperature. After pretreatment of the sections with 0.2% trypsin followed by a rinse in phosphate-buffered saline (PBS), the tissue endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 30 minutes. The sections were then incubated with the monoclonal mouse anti MIB-1 "paraffin Ki-67" antibody (Immunotech S. A., Marseille, Cedex, France) diluted 1 : 50 in PBS, and incubated overnight at 4℃. The bridge antibody applied was biotinylated goat anti-mouse immunoglobulin (1 : 100 in PBS), at room temperature for 1 hr, and then the avidin biotin complex (Vector Laboratories, Burlingame, CA) was applied for 45 minutes. After each antibody incubation, the sections were washed three times for 5 minutes each time in PBS. The substrate chromogen was DAB. The sections were counterstained in Mayer's hematoxylin, dehydrated, and mounted. The Ki-67-positive cells were counted from the areas of a given tumor section where the maximum numbers of tumor cells were immunostained. In each section, all of the tumor cells (immunopositive and negative) of five to ten (varied for available tumor cells) such light microscopic high-power fields set with a video monitoring screen were counted. The Ki-67 labeling index (LI) of the immunopositive cells was obtained as the percentage of the total number of tumor cells in the visualized fields of that section.

Staining of Ag-NORs

After tissue sections were deparaffinized, they were stained according to the method described by Shibuya et a l[10]. Briefly, tissue sections were covered with a solution consisting of 2% gelatin in 1% aqueous formic acid mixed with 50% aqueous silver nitrate solution (1 : 2 v/v) for 30 minutes in a dark place. The sections were then rinsed briefly in distilled water and dehydrated in graded alcohols, cleared in zyline and finally mounted. All tumor cells in the maximum cellular areas of the visualized microscopic high-power fields were randomly counted irrespective of the presence or absence of Ag-NORs as black dots. A total of 200 cells were counted, and the average number of Ag-NORs per cell was obtained.

Patient Survival and statistical analysis

Our analysis of patient survival was restricted to the patients with astrocytic tumors who did not have a tumor recurrence. All of the patients with astrocytic tumors had been followed-up for at least for six months at the time of the preparation of this present manuscript. The maximum follow-up period for the patients with astrocytomas was 13 years. The Ki-67 LI of the astrocytic tumors was analyzed in relation to the survival of patients. The mean values and standard deviations (SD) for the Ki-67 LI,

Ag-NOR count and survival time were calculated, and the p-values were obtained using Student's t-test (Ki-67), regression analysis, or the log rank test (survival).

Results

Ki-67 labeling index in the intracranial neoplasms

The staining pattern of the Ki-67 antigen is shown in Fig. 1. The distributions of Ki-67 antigen-immunopositive cells were either diffusely or focally distributed. The Ki-67 LIs (mean±SD) of the various brain tumors are shown in Table 1. In general, the histologically high grade tumors showed higher Ki-67 LIs. Among the astrocytic brain tumors, the mean Ki-67 LI was 1.2±1.56 in the pilocytic astrocytomas, 1.52±3.18 in the grade II astrocytomas, 13.47±11.40 in the anaplastic astrocytomas, and 19.54± 18.34 in the glioblastomas. The anaplastic astrocyomas and glioblastomas had significantly higher Ki-67 LIs compared to the histologically low grade (pilocytic, grade I and fibrilary/protoplasmic, grade II) astrocytomas ($p< 0.0001$). Among the oligodendroglial tumors, the mean Ki-67 LI was 2.95±3.89 in the pure oligodendrogliomas, 13.08±3.47 in the anaplastic oligodendrogliomas, and 4.85 in the two mixed oligoastrocytomas. The Ki-67 LI in the benign ependymomas was 1.67 ± 2.89, whereas in the anaplastic ependymomas it was 28.64. In the meningeal tumors, the mean Ki-67 LI in the meningotheliomatous meningiomas was 2.68 ± 4.74 ; in the fibroblastic meningiomas, 1.29 ± 1.97; in the transitional meningiomas, 1.27±1.79 ; in the angiomatous meningiomas, 1.33 ± 2.31;

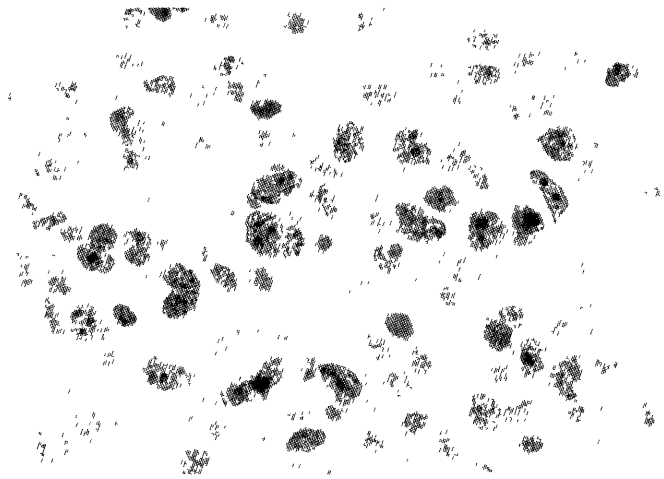

Fig. 1a

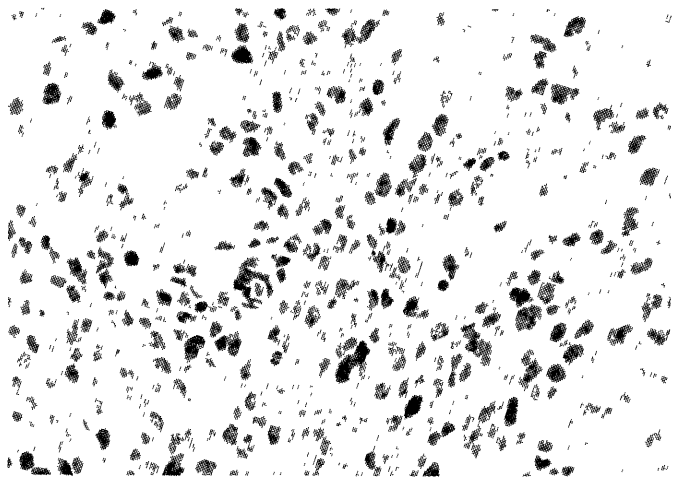

Fig. 1b

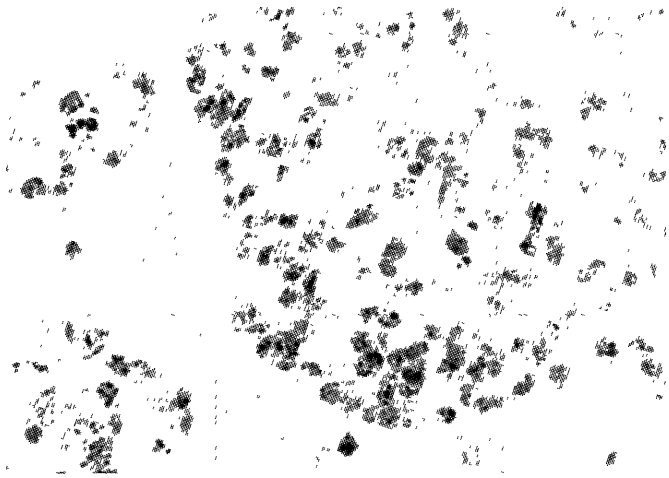

Fig. 1c

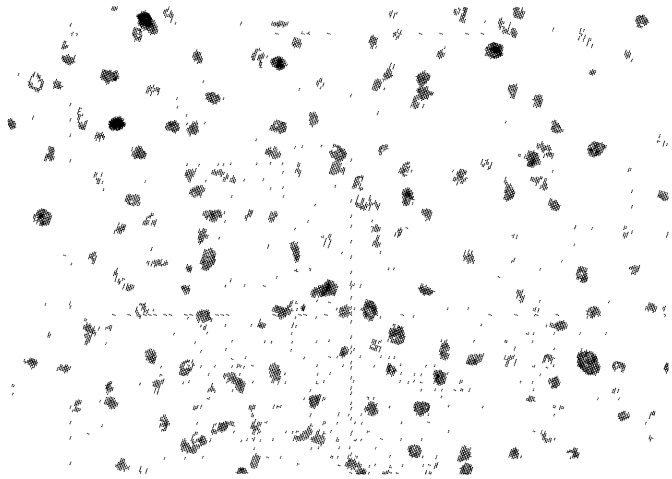

Fig. 1d

Fig. 1. The Ki-67 immunoreactivity in intracranial neoplasms stained with MIB-1 antibody (avidin biotin complex method) showed many Ki-67-immunopositive cells in glioblastomas (a), anaplastic meningiomas (b), and metastatic brain tumors (c), and few Ki-67-positive cells in meningotheliomatous meningiomas (d).

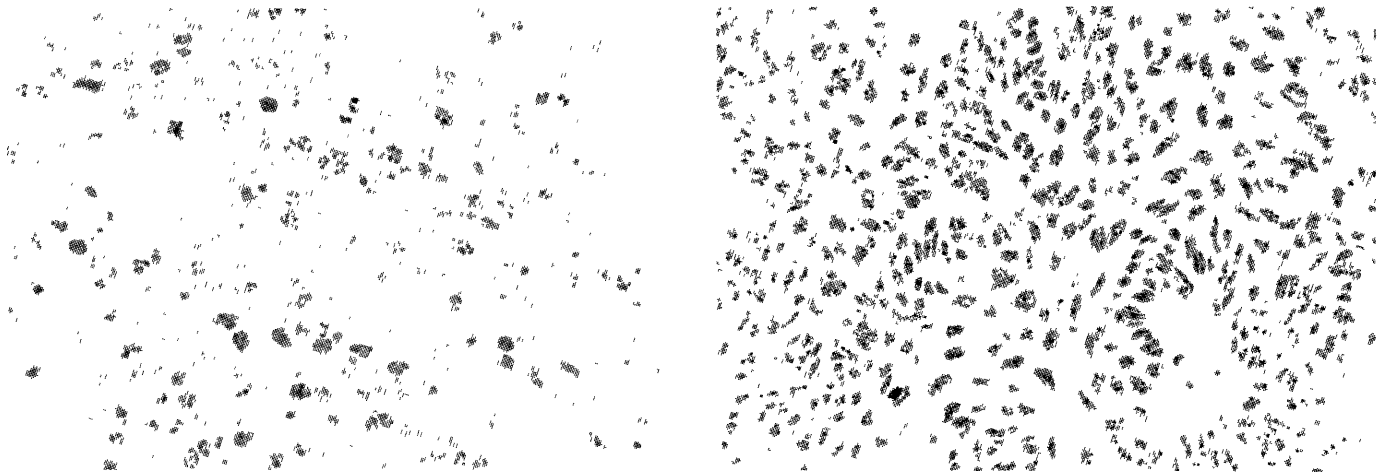

Fig. 2a Fig. 2b

Fig. 2. Ag-NOR staining in a pituitary adenoma (a) and in a metastatic brain tumor (b). Ag-NORs are seen as black dots in the nucleus.

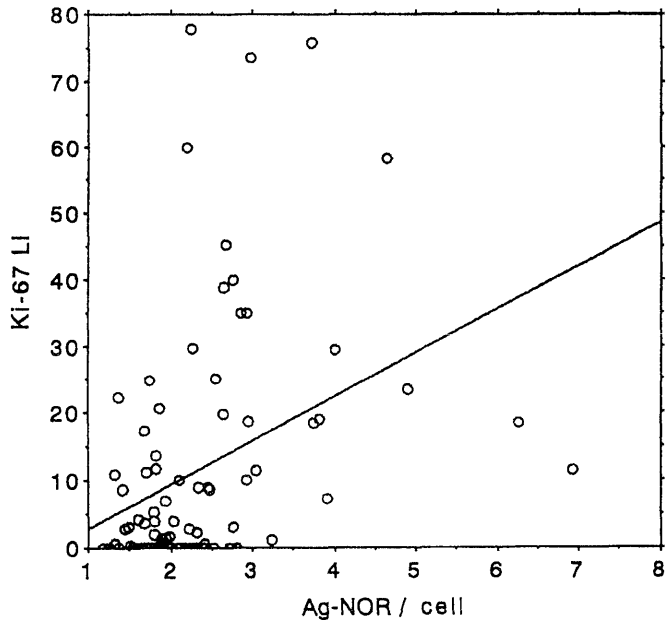

Fig. 3. The correlation between Ki-67 LI and Ag-NOR in the consecutive sections of 87 brain tumors stained for both Ki-67 (MIB-1 antibody) and Ag-NOR. A linear regression analysis showed no significant correlation ($r = 0.139$).

and in the anaplastic meningiomas, 16.76. The mean Ki-67 LI in the medulloblastomas was 12.31 ; in the pituitary adenomas, 1.52 ± 4.03 ; and in the metastatic brain tumors, 37.69 ± 21.37.

Ag-NORs in ihe ntracranial neoplasms

The staining pattern of Ag-NORs is shown in Fig. 2. The mean ($\pm$SD) numbers of Ag-NORs per cell in the various tumors are shown in Table 1. Among the astrocytic brain tumors, the mean number of Ag-NORs per cell was 2.04 ± 0.54 in the grade II astrocytomas, 2.40 ± 0.77 in the anaplastic astrocytomas, and 2.71 ± 1.13 in the glioblastomas. This values were not significantly different. The mean number of Ag-NORs per cell in the benign oligodendrogliomas (2 cases) was 1.91, whereas in the anaplastic oligodendrogliomas (6 cases), the value was 1.92 ± 0.99. The mean Ag-NOR numbers in the benign ependymomas and anaplastic ependymomas were 2.30 and 2.97, respectively. Among the meningiomas, although a higher Ag-NOR number was noted in the anaplastic meningiomas (4 cases, mean 3.53 per cell), this value was not significantly different compared to the value obtained for the benign meningiomas. In the pituitary adenomas, the mean Ag-NOR number was 2.09 ± 0.49, in the metastatic brain tumors it was 2.99 ± 1.49, and in the malignant lymphomas (2 cases), it was 2.27. Thus, the number of Ag-NORs was not consistent with the growth potential of some tumors. Consecutive sections of 87 brain tumors were stained for tboth he Ki-67 antigen and Ag-NOR, and the correlation between the two proliferation index values was obtained by regression analysis. No correlation was obtained between the Ki-67 LI and Ag-NOR values in these tumor sections ($r = 0.139$), as shown in Fig. 3.

Ki-67 LI and survival of patients with astrocytic tumors

Ninety-five percent of the patients with pilocytic or grade II astrocytomas were still alive, when this study was performed. The survival periods of only the patients with anaplastic astrocytomas or glioblastomas were analyzed in relation to the tumor Ki-67 LIs. Among the anaplastic astrocytoma-patients with a mean Ki-67 LI<10, the survival time was 499.14 ± 334.89 days ; among such patients with a Ki-67 LI > 10, the survival time was

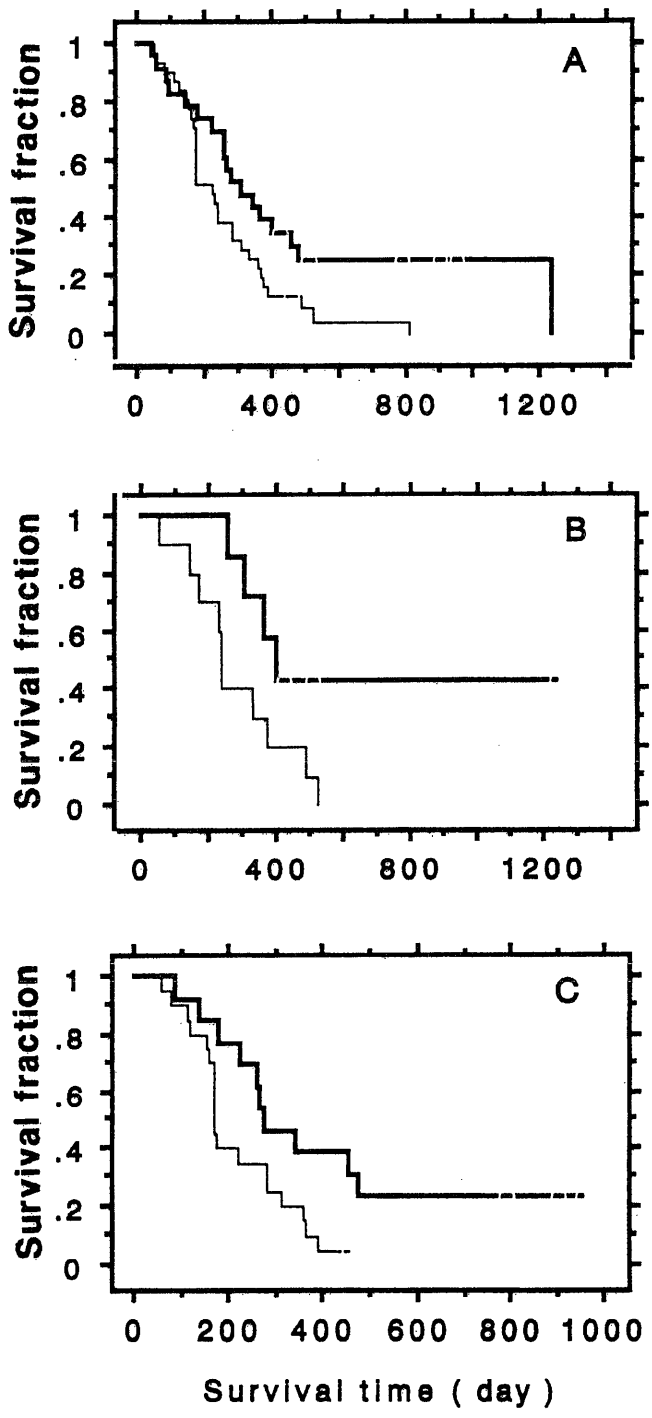

Fig. 4. The Kaplan-Meier curves for the patient groups defined by the tumor MIB-1 Ki-67 LI < 10 (bold line) and > 10 (plain line). (A) patients with high grade astrocytomas (p = 0.039, log rank), (B) patients with anaplastic astrocytomas (p = 0. 051, log rank), and (C) patients with glioblastomas (p = 0.031, log rank).

278.1±149.55 days (p = 0.051, log rank test). The same Ki-67 LI cut-off values were applied to the patients with glioblastomas, and the survival times were 416.15±300.32 and 216.65±110.88 days, respectively (p = 0.038, log rank test). Again, with the above Ki-67 LI cut-off values, the patients with combined high grade astrocytomas (anaplastic astrocytomas and glioblastomas) with a Ki-67 LI < 10 had significantly shorter survivals (p = 0.029). The Kaplan-Meier curves for patient survival are shown in Fig.4.

Discussion

In the present study we used two markers to assess the proliferative potential of various brain tumors i.e., the Ki-67 LI (MIB-1) and the number of Ag-NORs. The previously available antibody to Ki-67 could be applied only to frozen tissue sections ; the present antibody used here to detect Ki-67, MIB-1, can effectively be applied to paraffin-embedded tissue sections, and MIB-1 immunostaining has been reported to be superior to that of Ki-67 in individual tumors[18]. In the present series, the malignant lymphomas, glioblastomas, medulloblastomas, and tumors metastasized to the brain,t which represent the most malignant form of brain tumors in terms of histology and prognosis, had higher Ki-67 LIs compared to the schwannomas, pituitary adenomas, pilocytic astrocytomas, astrocytomas, pure oligodendrogliomas, pure ependymomas, and benign meningiomas. The histologically anaplastic groups of tumors, i.e., the anaplastic astrocytomas, anaplastic oligodendrogliomas, anaplastic ependymomas, and anaplastic meningiomas also had higher Ki-67 LIs than their benign counterparts. The relationship between the Ki-67 LI and the histological grade of brain tumors has been investigated in many laboratories[6-12,18,19]; however, the range of LI within histological grades varied widely. In almost all of those studies, the authors used a microwave oven for the antigen unmasking of paraffin-embedded tissues before MIB-1 immunostaining. We have always used a domestic micro-oven for tissue processing[7] and the Ki-67 LIs of the tumors obtained in the present study were in no way inferior to those robtained with the use of a microwave oven[19]. The Ki-67 LIs we observed in different tumors are in agreement with those of the previous reports, in which the authors also used the same MIB-1 antibody to detect Ki-67 antigen in paraffin-embedded tissue sections.

Astrocytomas are the most common primary gliomas, with the highly anaplastic glioblastomas being the most frequently occurring astrocytic tumors. Distinctive histological features permit astrocytic tumors to be graded into levels of anaplasia, and these histological grades correlate with the biological behavior of the tumor and patient prognosis. However, there is also a strong correlation

among the patient age, tumor grade, and prognosis. More objective indicators of tumor proliferative potential, such as BUdR or Ki-67, are currently being investigated with the hope that these will be a more accurate means of predicting patient survival. In the present study, we found that the anaplastic astrocytomas and glioblastomas had significantly higher Ki-67 LIs compared to the pilocytic astrocytomas and grade II astrocytomas. The distribution of Ki-67 LI values for the anaplastic astrocytomas and glioblastomas overlapped to the extent that these two grades were mostly indistinguishable by the Ki-67 LI in individual tumors, although the mean Ki-67 LI of the glioblastomas was high, as previously reported by other authors[6-12,19]. Survival analyses of high grade glioma patients did not identify significantly different subgroups with respect to the Ki-67 LI (9 and present study). In this respect, proliferation indices appear to be a less significant prognostic variable within the group of high grade lesions than are other features used in the morphological assessment of these lesions, such as the presence of necrosis or vascular proliferation. We found that in the group of glioblastoma patients, the survival time decreased as the tumor Ki-67 LI increased. Other authors have also analyzed the survival time of patients with high grade astrocytomas (glioblastoma or anaplastic astrocytomas) in respect to tumor Ki-67 LIs with different cut-off points[9-19]. The mean Ki-67 LIs for anaplastic astrocytomas (14.3) and glioblastomas (18.9) reported by Montine et al[9]. were in the same range as those we obtained for these two tumor subclasses. Using the Ki-67 LI cut-off values of <7.5 and >7.5, Montine et al. suggested that the LI<7.5 result indicated a long-term survivor within the high grade glioma group ; however, it did so with low specificity. One recent study[19] included a survival analysis of glioma patients using the Ki-67 LI and PCNA as the prediction values, and the authors suggested that the survival time was significantly reduced when either the Ki-67 LI or PCNA LI was increased. In that study, the Ki-67 LI cut off values were LI<5 and $\geqq 5$. In th epresent study, we divided anaplastic astrocytomas and glioblastomas into two groups according to their Ki-67 LI (<10 and >10), and we demonstrated that patients with a tumor Ki-67 LI >10 survived for a significantly shorter time than the patients with a tumor Ki-67 LI <10. Specifically, we showed a significant correlation of the Ki-67 LI and the survival of the glioblastoma patients using the above Ki-67 LI cut-off values. Although the survival periods of the patients with anaplastic astrocytomas with a Ki-67 LI <10 were longer, the difference was not significant. This may be due to differences in the number of tumors in each group, wide standard deviations, the Ki-67 LI cut-off values used, and other factors related to tumor prognosis. Another study[8] suggested that the Ki-67 LI has in general the best sensitivity and specificity in placing patients correctly in groups of survivors and non-survivors. Our ersults support this hypothesis.

Other proliferative indices also correlate to the tumor proliferation activity and tumor histological grades[10,11,19,20]. In brain tumors, a good correlation between the Ki-67 LI and the LI of S-phase-labelled PCNA and BUdR has been reported[10,11,20]. The labeling index of DNA polymerase alpha sometimes showed higher values than the Ki-67 LI in brain tumors[21]. Another study compared the thalium TI-201 single photon emission computed tomography (SPECT)-imaging findings of brain tumors and their Ki-67 LI ; the radionucleotide uptake of TI-201 in the tumor specimens was high in the tumors with a high Ki-67 LI[22]. NORs are loops of DNA that contain the RNA gene and are transcribed by RNA polymerase 1[23]. These regions are important in the regulation of protein synthesis[24] and can be identified easily by the argyrophilic technique. Some studies have shown a close correlation between the Ag-NOR number with cell proliferation and the clinical behavior of the tumors[13-16], while other studies did not[25]. In the present study, although some of the high grade tumors had somewhat higher Ag-NOR numbers than the low grade tumors, the overall difference was not significant. We found that the pituitary adenoma, a benign tumor that rarely recurs or shows malignant transformation, had a mean Ag-NOR number greater than those of the astrocytomas, oligodendrogliomas, and meningiomas. The Ki-67 LI was found to be increased in oligoastrocytomas and anaplastic astrocytomas than the well differentiated oligodendrogliomas (26 and present study). However, the Ag-NOR value we obtained in the oligodendroglial tumors did not correlate with the histologic grades. Morever, we did not find any correlation between the more reliable tumor marker Ki-67 with the Ag-NOR number in the same tumor samples. Although there is some relationship between NORs and the cell cycle[27], the number of Ag-NORs may not measure the cellular proliferation activity directly.

In conclusion, the present study suggests that the assessment of the tumor Ki-67 Labeling index is useful criteria to distinguish between benign and malignant brain tumors, and may influence the treatment of choice and outcome of patients.

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