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# Abstract

Ki-67 is a commercially available mouse monoclonal antibody (MoAb), which reacts with a nucleolar antigen (the Ki-67 antigen) expressed in proliferating eukaryotic cells. The author examined the precise localization of the Ki-67 antigen in C-6 cells using immunohistochemical and immunoelectron microscopic methods and estimated the proliferative activity of human brain tumors in situ. Positive nucleoplasmic reactions (early G1 phase) and nucleolar staining (late G1 phase) were observed. The cells showed very weak positive reactions in only one or two nucleoli (S phase) and multiple spicule reactions in the nucleoplasm (G2 phase). During the mitotic phase, the Ki-67 antigen was stained on the surfaces of all chromosomes and finely dispersed in the cytoplasm. By immunoelectron microscopic study, positive reactions were observed on the granular and dense fibrillar components. Therefore, the Ki-67 antigen seems to participate in the processing and assembly of preribosomal particles. In human brain tumors, the Ki-67 score (positive cells/total neoplastic cells), ranging 0 to 36.7%, correlated well with the histopathological grade of malignancy of the tumor. These findings suggest that immunohistochemical staining with the MoAb Ki-67 can be used as a convenient procedure for the simple evaluation of the proliferative activity of brain tumors.

**KEYWORDS:** monoclonal antibody Ki-67, immunohistochemistry, cell proliferation, brain tumors, nucleolar organizer regions

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# Cell Kinetic Analysis of Brain Tumors Using the Monoclonal Antibody Ki-67: In Vitro and In Situ Study

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Ki-67 is a commercially available mouse monoclonal antibody (MoAb), which reacts with a nucleolar antigen (the Ki-67 antigen) expressed in proliferating eukaryotic cells. The author examined the precise localization of the Ki-67 antigen in C-6 cells using immunohistochemical and immunoelectron microscopic methods and estimated the proliferative activity of human brain tumors *in situ*. Positive nucleoplasmic reactions (early  $G_1$  phase) and nucleolar staining (late  $G_1$  phase) were observed. The cells showed very weak positive reactions in only one or two nucleoli (S phase) and multiple spicule reactions in the nucleoplasm ( $G_2$  phase). During the mitotic phase, the Ki-67 antigen was stained on the surfaces of all chromosomes and finely dispersed in the cytoplasm. By immunoelectron microscopic study, positive reactions were observed on the granular and dense fibrillar components. Therefore, the Ki-67 antigen seems to participate in the processing and assembly of preribosomal particles. In human brain tumors, the Ki-67 score (positive cells/total neoplastic cells), ranging 0 to 36.7%, correlated well with the histopathological grade of malignancy of the tumor. These findings suggest that immunohistochemical staining with the MoAb Ki-67 can be used as a convenient procedure for the simple evaluation of the proliferative activity of brain tumors.

# Key words : monoclonal antibody Ki-67, immunohistochemistry, cell proliferation, brain tumors, nucleolar organizer regions

An increasing number of studies have stressed the usefulness of cell kinetic estimations as an aid in the prognostic and diagnostic evaluation of human brain tumors. Recently, immunohistochemical assessment of proliferating cells has been attempted using a mouse monoclonal antibody (MoAb) to bromodeoxyuridine (BrdU) (1) and anti-proliferating cell nuclear antigen (PCNA/cyclin) antibody (2).

In 1983, Gerdes *et al.* (3) reported that they had succeeded in producing MoAb Ki-67 which detects an unknown nuclear antigen expressed during the proliferative phase S ( $G_1$ , S,  $G_2$  and M) of the eukaryotic cell cycle, but not in the nuclei of cells in the resting phase ( $G_0$ ). Burger *et al.* (4) described a simple and rapid method of determining growth fractions *in situ* by immunostaining frozen sections with the MoAb Ki-67. Strong correlations between the degree of positive reaction to this antibody and the histopathological grade of malignancy have been reported in breast tumors (5), lung tumors (6) and lymphomas (7).

However, there have been no studies employing cultured brain tumor cells or comparing the Ki-67 antigen with other proliferation related antigens. The detailed subcellular localization and

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cellular biological function of this antigen are not yet fully known.

The present study was performed to determine the detailed distribution of the Ki-67 antigen during the cell cycle and to evaluate the proliferative activity of cultured glioma cells and biopsied materials of various brain tumors. The author also compared the results with those obtained from two other methods of using anti-PCNA/ cyclin or anti-BrdU antibody for detecting the proliferating cells.

# Materials and Methods

Cell cultures and growth conditions. Three cultured human glioma cell lines (OK, KC and KH) originally derived from patients with malignant glioma, and one rat glioma cell line (C-6) were used. The author also used a cultured human larynx epidermoid carcinoma cell line (HEp-2) for comparison with cultured cell lines derived from other tissues. The cultured cells were maintained in glass tissue culture flasks (TD-40) containing 10 ml Eagle's minimum essential medium (MEM; Gibco, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (FBS; Gibco) and 2001U/ml of penicillin G (Gibco) at 37 °C in a humidified 5 % CO<sub>2</sub> atmosphere. The culture medium was replaced with fresh culture medium every 3 days.

Immunohistochemical staining procedures. The cells were detached from the surface of culture flasks with phosphate buffered saline (PBS) containing 0.25% trypsin and 0.2% EDTA and plated on Lab Tek<sup>®</sup> chamber slides (Nunc, Inc., Naperville, IL, USA) at a concentration of  $2 \times 10^5$  cells/well. After preincubation for 48h (in exponential growth), the medium was aspirated, and the slides were washed in PBS for 30 min. Then, they were fixed in cold acetone for 10 min and air-dried for 90 min.

Immunohistochemical staining was performed by the peroxidase-antiperoxidase (PAP) method (8) as follows. To block nonspecific binding, after washing with PBS three times for 5 min, the slides were covered with 1 % normal goat serum (NGS; Sigma Chemical Co., St. Louis, MO, USA) in PBS for 10 min at room temperature in a humidified chamber. Excess NGS solution was removed, and the fixed cells were incubated under a 1:20 dilution of MoAb Ki-67 (DAKO-PC; code no. M722;

Dakopatts, Denmark) or nonspecific mouse immunoglobulin (Dakopatts) as a control in PBS containing 1 % bovine serum albumin (BSA; Sigma) and 0.5 % Tween 20 (Sigma) for 2h at room temperature. After washing 3 times for 5 min with PBS, the cells were incubated with rabbit anti-mouse IgG antibody (Dakopatts) diluted 1:40 for 40 min. Cells were washed in PBS, and peroxidase anti-peroxidase complex (Dakopatts) diluted 1:40 was applied for 40 min. After another rinse with PBS, peroxidase activity was detected with a substrate mixture of 3,3'-diaminobenzidine 4 HCl (DAB; Sigma) (20 mg/ 100ml) in Tris HCl buffer (pH 7.6) containing 0.05 % hydrogen peroxide. Slides were finally counterstained with methylgreen or hematoxylin and mounted. The percent of Ki-67 positive nuclei among total neoplasmic nuclei (Ki-67 score) was determined by 500-cell counts.

In order to compare the Ki-67 method with other proliferation analysis methods, indirect immunohistological methods using either anti-PCNA/cyclin antibody (2) or anti-BrdU antibody (9) were conducted.

Cell cycle study. Cells in the  $G_1$  phase were estimated by their transition into the  $G_1$  phase after mitotic selection. The mitotic cells were collected by shaking the flasks. The medium containing mitotic cells was centrifuged, and after the cell pellets were incubated for 2 h (early  $G_1$  phase) or 8h (late  $G_1$  phase) in chamber slides, the cells were fixed and processed for immunohistochemistry.

In order to evaluate the patterns of Ki-67 staining during the S phase, the author performed immunocytochemical double-staining for the Ki-67 antigen and DNAincorporated BrdU. Exponentially growing cells were incubated in the medium containing  $1.25 \,\mu$ M BrdU (Sigma) for 30 min. After washing with PBS, the cells were fixed in cold acetone for 10 min and air dried for 90 min. The immunostaining procedure for Ki-67 was performed as described above. Then the DNA was denatured by immersing the slides for 30 min in 2N HCl, followed by the neutralization with  $0.1 \text{ M Na}_2\text{B}_4\text{O}_7$ . The slides were treated with a 1:20 dilution of FITCconjugated anti-BrdU MoAb (Boehringer Mannheim, Indianapolis, IN, USA) for 60 min. After rinsing several times with PBS, the slides were mounted in phosphatebuffered glycerin, and examined under a Nikon fluorescence microscope.

Cells in the G<sub>2</sub> phase were estimated by their transition into the G<sub>2</sub> phase after synchronous culture with aphidicholin (10). Exponentially growing cells were treated in the medium with  $0.5 \,\mu$ g/ml aphidicholin (Wako Pure Chemical Ind., Ltd., Osaka, Japan) for 24 h resulting in a significant accumulation of cells in the S phase. After washing with PBS, the cells were reincubated in MEM for 3h. Then, these cells were fixed and immunohistochemically stained.

Mitotic cells were distinguished from the cells in interphase on the basis of the difference in chromatin structure observed under a microscope.

Flow cytometric analysis. Cultured brain tumor cells were detached from the flask and fixed in 70 % ethanol for 10 min at room temperature. Then, the cells were resuspended in PBS at a cell concentration of 107/ml and MoAb Ki-67 or nonspecific mouse immunoglobulin as a control was added at a final dilution of 1:20 and incubated at room temperature for 60 min. After the incubation, the cell suspensions were washed twice with PBS, resuspended in 1:40 FITC-conjugated rabbit anti-mouse IgG antibody (Dakopatts) and incubated for another 30 min at 37°C with 0.2 ml RNase (Worthington Diagnostics, San Francisco, CA, USA). Finally 0.2 ml propidium iodide (PI; Calbichem-Behring Corp.) was added to stain nuclear DNA. The stained cells were measured on Spectrum III (Ortho Diagnostic Systems, Inc., Raritan, NJ, USA). Forward scatter (cell size) and right angle scatter (fine structure of cells) were first plotted as a two-dimensional scattergram by DS-1 computer (Ortho), then the cell doublets and larger aggregates were excluded. The selected single cells were replotted in a second scattergram according to red fluorescence of PI (relative DNA content) and green fluorescence of FITC (FITCconjugated antibody).

Immunoelectron microscopic study. C-6 cells in the exponential growth phase were washed with PBS containing with 10 % sucrose (S-PBS) and fixed in cold methanol for 10 min. The fixed cells were washed three times with S-PBS and incubated in 10 % NGS for 10 min. After excess NGS was removed, they were incubated with a 1:5 dilution of MoAb Ki-67 for 3h at room temperature. After washing the slides with S-PBS, a peroxidase-conjugated rabbit anti-mouse IgG antibody was applied for 2h. After another S-PBS washing, the cells were fixed with 1 % glutaraldehyde for 5 min at 4°C. After another washing with PBS, peroxidase activity was detected with DAB. After washing with PBS, the cells were fixed with 2 % OsO4 for 45 min at room temperature. After dehydration, a gelatin capsule was placed upon the slides, and cells were embedded in Epon mixture (11). Ultra-thin sections were cut and examined under a Hitachi H-500 electron microscope.

Nucleolar organizer regions staining (Ag-NORs staining). Since the Ki-67 staining reaction was mainly

localized in nucleoli, the relationship between the Ki-67 antigen and nucleolar organizer regions (NORs) associated proteins, which belong to the proteins composing nucleoli, was examined. NORs-associated proteins were silver stained (Ag-NORs) by the one-step method of Howell and Black (12). Briefly, 1 volume of gelatin dissolved in 1 % aqueous formic acid at a concentration of 2 % and 2 volumes of 50 % aqueous silver nitrate solution were reacted on the surface of the slides for 30 min at room temperature. The slides were rinsed three times with distilled water, and mounted. The Ag-NORs staining pattern was compared with the Ki-67 immunostaining pattern. In addition, the changes in the Ag-NORs pattern of the cells after pretreatment with MoAb Ki-67 for 60 min were also examined.

In situ study. Two normal brain and 55 brain tumor tissue samples were examined. These tissues were obtained at surgery. They were first minced into small blocks and fixed in periodate-lysine-paraformaldehyde (PLP) solution (11) for 4 h at 4°C. After fixation, they were washed with S-PBS overnight at 4°C. The tissues were quickly frozen in iso-pentane solution, chilled in a drvice-acetone mixture, and cut into  $10\,\mu$ m-thick sections on a cryostat. Then, the sections were stained immunohistochemically with MoAb Ki-67 diluted 1:10 according to the PAP method as described in immunohistochemical staining procedures. In order to block the endogenous peroxidase activity, the sections were immersed in 70 % methanol containing 0.3 % H<sub>2</sub>O<sub>2</sub> for 30 min at room temperature and then rinsed in distilled water prior to treating with PAP complex. Slides were lightly counterstained with hematoxylin or methylgreen and mounted.

The percentage of Ki-67-positive cells in the total cell population (Ki-67 score) was assessed by counting at least 500 cells in representative areas at  $\times 400$  magnification. Only clearly labeled nuclei were counted.

Statistical analyses were performed by means of the appropriate Student's t test.

#### Results

Immunocytochemistry of cultured cells. Cells positive for MoAb Ki-67 are clearly distinguishable from the background of unlabeled cells and showed heterogenoeus nuclear staining patterns in individual cells (Fig. 1A). In cultured glioma cells at exponential growth, the percentage of Ki-67

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positive cells ranged from 43.4 to 59.4 %, whereas the percentage of cells positive for BrdU and for PCNA/cyclin ranged from 28.6 to 39.3 % and from 30.9 to 41.4 %, respectively. The percentage of Ki-67 positive cells correlated well with the percentage of BrdU and PCNA/cyclin positive cells (about 1.4–1.6 higher). In HEp-2 cells, the mean percentage of Ki-67-positive cells was 93.0 %, which was much higher than that in glioma cell lines examined (Table 1).

The staining pattern was characteristic for each antibody, that is, BrdU demonstrated a fine granular form in the entire nucleus (Fig. 1B) and PCNA/cyclin showed a diffuse positive reaction in the entire nucleus and/or spotted pattern in the nucleoli (Fig. 1C).

Distribution of the Ki-67 antigen during the cell cycle. The distribution of the Ki-67 antigen varied in accordance with the phases of the cell cycle (Fig. 2).

Postmitotic daughter nuclei were easily recognized, and these nuclei displayed diffusely stained spots or homogeneously distributed speckles (Fig. 3A).

Positive nucleoplasmic staining with variable nucleolar staining was observed 2 h after starting synchronization of the M phase (early  $G_1$  phase; Fig. 3B). One or more larger and irregularly shaped nucleoli became obviously stained 8 h after synchronization (late  $G_1$  phase; Fig. 3C).

In the study of the double staining procedure

 Table 1
 Percentage of Ki-67, BrdU and PCNA-positive nuclei in cultured glioma cell lines

	Positive nuclei/total nuclei (%) <sup>a</sup>				
Cell line	Ki-67	BrdU <sup>b</sup>	PCNA <sup>c</sup>		
C-6	$53.9 \pm 4.7$	$36.9 \pm 2.2$	$39.6 \pm 0.8$		
OK	$43.4 \pm 2.2$	$28.6 \pm 1.7$	$30.9 \pm 3.4$		
KC	$46.2 \pm 2.2$	$33.1 \pm 2.3$	$33.3 \pm 1.7$		
кн	$59.4 \pm 2.7$	$39.3 \pm 1.0$	$41.1 \pm 1.5$		
HEp-2	$93.0 \pm 2.1$	n. d.	$43.9 \pm 3.9$		

a: Mean ± SEM b: Bromdeoxyuridine c: Proliferating cell nuclear antigen. PCNA : anti - proliferating cell nuclear antigen. Brdu : Bromodeoxyuridine with either MoAb Ki-67 or anti-BrdU MoAb (S phase), the same cells which showed very weak



Fig. 1 The staining pattern of C-6 cells with each antibody. A: Ki-67 monoclonal antibody presents different intranuclear staining patterns in individual cells ( $\times$  400). B: BrdU demonstrates a fine granular form in the entire nucleus ( $\times$  400). C: PCNA/cyclin showed a fine granular pattern in the entire nucleus and a spotty pattern in the nucleolus ( $\times$  400).

positive reaction in only one or two nucleoli by MoAb Ki-67 were stained clearly by anti-BrdU MoAb (Fig. 4A, B).

Almost all cells showed multiple spotty reactions or irregular speckles 3h after S phase synchronous culture (G<sub>2</sub> phase; Fig. 3D).

The Ki-67 antigen was present in all dividing cells (M phase), and its distribution was restricted to the chromosomes. Occasionally staining was found in the nuclear matrix as well. During prophase a meshwork throughout the nucleoplasm tended to be stained (Fig. 4A). In metaphase (Fig. 5B) and anaphase (Fig. 5C) the Ki-67 antigen was restricted to the surface of condensed chromosomes. In telophase a diffuse or punctated staining of the entire nucleoplasm was observed (Fig. 5D).

*Flow cytometry.* Fig. 6 shows the twodimensional display of two-color staining for PI (abscissa) and FITC-labeled MoAb Ki-67 (ordinate) on C-6 glioma cells at exponential growth. It was clearly shown that the intensity of fluorescence of MoAb Ki-67 decreased in the S phase as observed in the immunohistochemical study.

*Immunoelectron microscopic study.* As shown in Fig. 7, granular components of nucleoli were strongly stained for Ki-67 antigen, and fibrillar components were weakly stained. The



Fig. 2 Schematic illustration of the immunohistochemical localization of the Ki-67 antigen during various cell cycle phases observed in cultured C-6 cells.

fibrillar center seemed to be negative. In the dividing cells, most positive staining was observed on the chromosome surface, and some positive reactions were detected in the cytoplasm. However, positive staining was not detected in the centrioles or spindles (Fig. 8).

Ag-NORs staining. Ten to twenty silver granules of Ag-NORs staining were observed in the nuclei of C-6 cells. Since the nucleolar staining patterns of MoAb Ki-67 were different from those of Ag-NORs staining (Fig. 9A), the Ki-67 antigen and NORs-associated proteins were not thought to be identical. This hypothesis was further confirmed by the observation that NORs staining patterns after pretreatment with MoAb Ki-67 were not different from the original Ag-NORs staining (Fig. 9B).

*In situ study.* In normal human brain tissue, no positive immunostaining with MoAb Ki-67



**Fig. 3** C-6 cells with various synchronous populations stained by MoAb Ki-67. A: Postmitotic daughter nuclei are diffusely stained ( $\times$  1,000). B: Early G<sub>1</sub> phase. The nucleoplasm and relatively large nucleoli are stained diffusely ( $\times$  1,000). C: Late G<sub>1</sub> phase. A small number of nucleoli are positively stained ( $\times$ 1,000). D: G<sub>2</sub> phase. Multiple spotty stainings are visible in almost all cells ( $\times$  400).

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Fig. 4 Immunocytochemical double staining for the Ki-67 antigen and DNA-incorporated BrdU.
A: A variety of Ki-67 staining patterns in exponentially growing C-6 cells are visible (× 400).
B: Immunofluorescence staining of the same cells in Fig. 3A with anti-BrdU monoclonal antibody. Cells with only one or two nucleoli stained for the Ki-67 antigen are clearly fluorescent (× 400).

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Fig. 5 Distribution of the Ki-67 antigen in C-6 cells in different stages of mitosis (×1,000).
A: Prophase; B: Metaphase; C: Anaphase; D: Telophase.

was found in neuronal, glial, or any other cells. The range of the Ki-67 scores in various brain tumors is summarized in Table 2.

In low and high grade astrocytomas, the averages of Ki-67 scores were 1.8 and 8.5%, respectively. The latter was about 4.7 times higher than the former. Positive reactions were mostly diffuse in nucleoplasm, and positive cells were scattered (Fig. 10A).

In glioblastomas, the average Ki-67 score was 19.5 %, and was higher than that of astrocytomas. The distribution of positive cells was much more variable. The size and shape of the nuclei showing positive reactions were markedly variable due to the pleomophism of the tumor cells (Fig. 10B).

The averages of Ki-67 scores in oligodendrogliomas and in choroid plexus papillomas were only 1.1 and 3.2%, respectively. However, in anaplastic ependymoma, the average was 15.1%, and the positive rate was as high as in glioblastoma (Fig. 10C).

Medulloblastoma and primitive neuroectodermal tumor (PNET) demonstrated high average Ki-67 scores of 14.2 and 30.1 %, respectively, and showed a patchy distribution of positive nuclei throughout the section. In the former, diffuse positive reactions were observed widely (Fig. 10D), but in the latter, the staining patterns were varied, and mitotic cells as well as cells with positive staining of nucleoli were noted frequently

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Fig. 6 Two-parameter flow cytometric analysis of C-6 cells after labeling with FITC for Ki-67 and staining with propidium iodide (PI) for the DNA content. The intensity of fluorescence of MoAb Ki-67 decreased in the S phase.
A: Control B: Ki-67 staining



Fig. 7 Electron microscopic immunolocalization of the Ki-67 antigen in nuclei of C-6 glioma cells.

A: Conventionally fixed C-6 glioma cells ( $\times$  5,000). FC: fibrillar center, DFC: dense fibrillar component, GC: granular component B: Peroxidase-antibody complexes are selectively enriched over the granular and dense fibrillar components and are absent from the fibrillar centers ( $\times$  4,000).

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Fig. 8 Electron microscopic immunolocalization of the Ki-67 antigen in metaphase chromosomes. Peroxidase-antibody complexes are visualized over the surface of chromosomes (arrows) and in the cytoplasm (arrow head) ( $\times$  6,000).



#### Fig. 9 Ag-NORs staining.

A: Ag-NORs staining of exponentially growing C-6 glioma cells. Many black dots are present in each nucleus ( $\times$  1,000). B: Pretreatment with monoclonal antibody Ki-67. All cells show almost the same Ag-NORs dots as in Fig. 9A ( $\times$  1,000). Shiraishi



Fig. 10 Ki-67 Staining of various human brain tumors.

- A: Astrocytoma. Positive reactions are diffuse in nucleoplasm ( $\times$  400).
- ${\sf B} :$  Glioblastoma. The Ki-67 score is higher than those of astrocytomas ( $\times\,400).$
- C: Anaplastic ependymoma. The Ki-67 score is 15.1  $\%~(\times\,400).$
- $\mathsf{D} \colon$  Medulloblastoma. Diffuse positive reactions are observed widely ( $\times$  200).
- E: PNET. Positive reactions of chromosomes and nucleoli are noted frequently ( $\times$  400).
- F: Recurrent meningioma. Positive reaction of chromosome is observed ( $\times$  400).

## (Fig. 10E).

In a recurrent meningioma (Fig. 10F) and a clival meningioma which showed faster growth clinically than common meningiomas, relatively high average Ki-67 scores of 3.0 and 3.7 % were noted. There was no correlation between the Ki-67 scores and the histological types of meningioma. Neurinomas and pituitary adenomas showed low average Ki-67 scores of 0.9 and 1.2 %, respectively.

No positive reactions were observed in an

epidermoid, a ganglioglioma, a hemangioblastoma, or a craniopharyngioma.

Metastatic tumors demonstrated a high average Ki-67 score of 17.8 %, ranging from 2.0 to 31.2 %. The positive cells were homogeneously dispersed throughout the neoplasms.

Although the positive reaction for the MoAb Ki-67 was mostly diffuse in nucleoplasm, there was a tendency for it to be intense in the nucleoli and in the chromosomes of tumor cells of high grade tumors.

Pathology	WHO	Namber of	Ki-67 score $(\%)$	
	grade	cases	Range	Mean±SEM
Meningioma	1	12	0 - 3.7	$1.3 \pm 1.7$
Neurinoma	1	9	0 - 3.0	$0.9 \pm 1.1$
Ganglioglioma	1	1	/	0
Hemangioblastoma	1	1	1	0
Craniopharyngioma	1	1	1	0
Epidermoid	1	1	1	0
Pituitary adenoma	1	2	0.4 - 2.0	$1.2\pm 0.8$
Low grade astrocytoma	2	3	0 - 3.0	$1.8 \pm 1.3$
Oligodendroglioma	2	4	0 - 2.3	$1.1 \pm 0.8$
Choroid plexus papilloma	2	1	/	3.2
High grade astrocytoma	3	5	4.0-14.0	8.5± 3.3
Anaplastic ependymoma	3	2	17.0 - 25.0	$15.1 \pm 1.9$
Glioblastoma multiforme	4	2	16.5 - 22.5	19.5± 3.0
Medulloblastoma	4	2	13.1 - 15.2	$14.2 \pm 1.1$
$PNET^{a}$	4	1	/	30.1
Malignant lymphoma	4	1	/	36.7
Metastatic tumor		6	2.0 - 31.2	$17.8 \pm 11.5$

Table 2 Ki-67 scores of various brain tumors

a: Primitive neuroectodermal tumor

Table 3	List	of	nucleolar	proteins
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Protein	Molecular weight (kD)	Location	Cell cycle expression	Function
RNA polymerase I	14-210	Fibrillar center	All	Pre-rRNA transcription
Fibrillarin	34	Fibrillar center	All	Associated with U3 RNP
Nucleolin	110	Fibrillar center	All	Pre-rRNA transcription ribosome assembly
U3 RNP	_	Nucleoli	All	Ribosome assembly, transport
U8 RNP	_	Nucleoli	All	Ribosome assembly, transport
P40	40	Nucleoli	Late $G_1$	Associated with cell proliferation
P105	105	Nucleoli	Early G <sub>1</sub> -S	Associated with cell proliferation
p120	120	Nucleolar matrix	Early G <sub>1</sub>	Binding protein to attach rDNA and preribosomes to the nucleolar matrix
p145	145	Granular component	Diving cell	Processing or assembly of preribosomal particles

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A comparison of average Ki-67 scores with the histological grades of brain tumors (WHO) (13) revealed a correlation between these variables: grade 1 showed 0.9 %; grade 2, 1.6 %; grade 3, 10.4 %; and grade 4, 20.2 %. There was no difference in the Ki-67 score between grades 1 and 2. Significant differences (p < 0.01) in the Ki-67 score between grades 1 and 3 and between grades 1 and 4 were noticed. There was a slight difference (p < 0.05) in the Ki-67 score between grades 3 and 4. The borderline between the benign group (WHO grades 1 and 2) and malignant group (grades 3 and 4) as determined by the Ki-67 scores was found at approximately 5 % (Fig. 11).



Fig. 11 Scateergram showing Ki-67 scores according to the histopathological grades of tumor malignancy.

# Discussion

Recently, Gratzner (14) developed a monoantibody that specifically binds to clonal bromodeoxyuridine (BrdU), a thymidine analogue which is incorporated into the cell nucleus during DNA synthesis. Hosino et al. (1) reported that the labeling index with BrdU of tumors correlated closely with the prognosis or survival of patients with gliomas. However, in this method it is necessary to inject the BrdU into patients before biopsy of tissue. Therefore, this method raises ethical problems due to the potentially mutagenic hazard of the thymidine analogue to normal tissues.

PCNA, also known as cyclin (15), a nonhistone, acidic nuclear protein with an apparent molecular weight of 37,000 daltons, appears at the  $G_1/S$  boundary in the cell cycle (16). Recently Tabuchi et al(2) accomplished a simple immunocytochemical demonstration of PCNA/ cyclin both in vitro and in situ in human glioma cells. They reported that cell kinetic studies with PCNA/cyclin offered an advantage over the BrdU method and gave results within a few hours after biopsy that benefited the individual patient by indicating the rate of tumor growth. However, the frequency of the appearance of anti-PCNA/ cyclin antibody in SLE is low, and the antibody titer is not constant (17). Besides, the anti-human IgG antibody, which is the secondary antibody used in this method, also reacts with the patient' s IgG contained in the tumor tissue, and that sometimes makes it difficult to judge the positive reactions.

Immunocytochemical staining of tumor cells with MoAb Ki-67 has been shown to correlate well with conventional measures of cell proliferation by autoradiography and flow cytometry (18). Schrape *et al.* (7) reported that quantification of the relative proportions was parallel between cells in the S phase (BrdU-labeling cells) and cells in the cell cycle (Ki-67 positive cells).

The results obtained in this study showed that the Ki-67 scores correlated well (about 1.4–1.6 times higher) with the percentage of BrdU or PCNA/cyclin positive cells, and there was a significant correlation between the histological grade of malignancy of brain tumors and the Ki-67 scores. These findings suggest that immunohisotchemical staining with MoAb Ki-67 can be a potent tool for evaluation of proliferative activity of brain tumors.

In addition, Ki-67 immunohistochemical staining for in situ characterization of the proliferative activity of brain tumors has a number of advantages: 1) Since this method requires no preoperative injection of chemicals, there are no side effects and no restrictions in applying it clinically. 2) Since the MoAb Ki-67 reacts with cells not only in the S phase, but also in the  $G_1$ ,  $G_2$  and M phases, the mean value of the positive rate with MoAb Ki-67 is higher than those with BrdU and with PCNA/cyclin. Therefore, we can more accurately assess the proliferative activity of a tumor. 3) Since the distribution of the staining for the MoAb Ki-67 varies in accordance with the phases of the cell cycle, the cell cycle can be studied in situ. 4) MoAb Ki-67 is now commercially available, and this method can be used easily for routine procedures in surgical pathology laboratories.

Yet, because the titer of MoAb Ki-67 is relatively low, the positive rate may vary depending on various factors such as the fixing procedure, specimen storage state, staining method, or difference in antibody titer by lots. It is sometimes difficult to determine if the reaction is positive or not, and judgement may vary by individual investigators. Therefore, it may be difficult to make a strict comparison of the results obtained by different laboratories, and it seems necessary to standardize the staining procedure for determining the Ki-67-positive rate. The Ki-67 antigen easily loses its antigenic activity in conventional fixation, and thus it is not suitable for retrospective studies of stored paraffinembedded tissue specimens.

The number of cases examined in this study was not large enough, and the follow-up period was not long enough to estimate the prognostic significance of the Ki-67 score. Follow-up study is necessary to establish the correlation between the Ki-67 score and either the postoperative survival period or response to various treatment modalities in individual brain tumor cases.

It is widely accepted that determination of the growth fraction of a malignant tumor is of prognostic value. Since all phases of the cell cycle are thought to be reactive with MoAb Ki-67, it seems that MoAb Ki-67 could be a useful tool for determining the growth fraction of various tumors (5).

However, it is questionable as to whether the Ki-67 scores represent the actual growth fraction. When the growth fraction was determined by exposing cultured glioma cells in exponential growth to BrdU four times, every 8h for 30 min each time (19), a value of approximately 97 % was obtained in C-6 cells (data not shown). It is suggested that most cultured C-6 cells in exponential growth are in the proliferating cell cycle. However, the Ki-67 scores in glioma cell lines were about 30 to 40 %, which were lower than the expected rates, and it seems that the actual growth fraction is not represented. On the other hand, the mean Ki-67 score of HEp-2 cells was 93 %. In this cultured cell line, the Ki-67 score may be regarded as expressing approximately the actual growth fraction.

This discrepancy between the Ki-67 score and growth fraction can be explained as follows: 1) Expression of the Ki-67 antigen may be lower in cultured glioma cells than in neoplastic cells derived from other organs. 2) As indicated by the BrdU labeling index and flow cytometric DNA analysis, the level of expression of the Ki-67 antigen may decrease in the S phase, and these cells may be regarded as negative. Considering the present results', it seems that the Ki-67 score does not necessarily express the actual growth fraction of a brain tumor.

The nucleolus is a subcellular organelle producing ribosomes and composed of the three components discernible under an electron micro-

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scope (20). Firstly, the fibrillar centers of a nucleolus are the so-called nucleolar organizer regions (NORs) which are composed of loops of DNA (rDNA) transcripting ribosomal RNA. Secondly, the dense fibrillar components are composed of an assembly of 5 nm ribonucleo-protein fibers whose function is transcription of RNA from rDNA. Thirdly, the granular components, which are composed of an assembly of 15 nm ribosomal precursor particles, are considered to conduct the processing of preribosomes.

The present immunoelectron microscopic study showed that the Ki-67 antigen was localized in the granular component of nucleoli in interphase cells as described by Verheijen et al. (21). Therefore, the Ki-67 antigen is considered to be involved in processing of preribosomes. Although the nucleolus is known to disappear during cell division (nucleolar dissociation), certain nucleolar associated proteins as well as ribonucleoproteins, are known to remain on the surface of the chromosomes. After being distributed to daughter cells, those proteins are considered to participate in formation and association of nucleoli (nucleolar association) (20). The present study also revealed that the Ki-67 antigen was localized on the surface of chromosomes in metaphase cells, and in the cytoplasm as well. Verheijen et al. (22) also reported the same results. It is suggested that the Ki-67 antigen is involved in the formation of nucleoli in daughter cells entering into the G1 phase. In the cells entering into the G<sub>0</sub> phase after cell division, the Ki-67 antigen seems to disappear rapidly (23).

NORs are considerd to play a central role in the ribosome-forming function of nucleoli. NORs-associated proteins, RNA polymerase I (24) and nucleolin (25) have been shown to be argyrophilic, and they are easily identified under a microscope. Ochs *et al.* (26) reported that the antibody specific for protein C23 (nucleolin) markedly decreased the intensity of silver-staining in interphase nuclei. Therefore, they thought that C23 must be the major silver-staining protein of the nucloeli. However, the silver staining patterns of NORs associated proteins are not changed by pretreatment with MoAb Ki-67. Most NORs associated proteins are found to be localized in the fibrillar center as well as in the dense fibrillar component. Therefore, it is difficult to presume the Ki-67 antigen as a member of the NORs associated protein family.

Recently, the group of Busch *et al.* (27–30) identified four different nucleolar proliferationassociated antigens. However, their subcellular locations and cell cycle expression were different from those of the Ki-67 antigen. In contrast, Hügle *et al.* (31) reported that the ribosomal S1 protein was associated with the interphase nucleolus and distributed over the chromosomal surface during mitosis. The subcellular localization of ribosomal S1 protein resembles that of the Ki-67 antigen.

Those proliferation - associated nucleolar antigens, including the Ki-67 antigen, are proteins which are probably involved in regulation, as well as modification, of the function of the nucleolus, rather than functioning themselves. The author is convinced that the clarification of the function of the Ki-67 antigen will yield a clue which will help in the understanding of the regulatory mechanism of cell growth as well as tumorigenesis.

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