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Histopathological Parameters Predicting Recurrence and Malignant Transformation of Adult Low-Grade Astrocytomas with Special Reference to MIB-1 and p53 Immunohistochemistry

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Low grade astrocytomas generally have a good prognosis and the patients can survive a long time without recurrence. It is, however, well known that some low grade astrocytomas can show malignant transformation and they recur in a rather short period. These tumors demonstrate the characteristics of anaplastic astrocytoma or glioblastoma at the time of recurrence. As it is important to predict whether malignant transformation or recurrence will occur after the first operation, we have studied the histopathological characteristics of nonrecurrent and recurrent adult low grade astrocytomas. The population density of hematoxylin-eosin stained tumor cells and MIB-1 and the p53 immuno-stained index (SI) of parafin embedded surgical samples taken from 20 recurrent and 10 non-recurrent cases of low grade astrocytoma (grade II) were histopathologically studied. The numbers of stained tumor cells per 1 mm^2 (SI) were counted from the average population density per 1 mm^2 in 5 quadratic grids of the microscopic field. Formalin-fixed paraffin-embedded tissue sections were immunohistochemically stained for MIB-1 and p53. No correlation was noted between the age of the patients, the recurrence and the recurrence time. Male patients seemed to be more susceptible to recurrence than females. In the majority of recurrent cases, the SI of MIB-1 was higher and the SI of p 53 was lower than that observed in the non-recurrent cases at the time of the initial operation. As a statistically significant difference in the SI of MIB-1 and SI of p53 was found between the non-recurrent and recurrent astrocytomas at the time of the first operation, such analysis might be valuable for prediction of the recurrence of low grade astrocytoma.

Introduction

Low grade astrocytomas (grade II) generally shows good outcome after surgical removal. It is, however, well known that some low grade astrocytomas show malignant transformation to higher grade astrocytomas (grade III or grade IV) and demonstrate, devastating prognosis[1)2)]. It is, therefore, necessary to foresee such malignant transformation at the time of initial surgery to establish a proper therapeutic plan for the patient.

Although several analytical methods are available to determine the grading of astrocytoma, there is, currently, no definite nor standard method to measure the grading quantitatively. The estimation of tumor grading has so far been based on the morphological characteristics of

hematoxylin-eosin (HE) stained tumor tissue specimens. The significance of the staining index (SI) of MIB-1 or tumor suppressor gene (for example, p53 suppressor gene) has been anticipated for predicting malignant transformation[3)4)]. To our knowledge, at present MIB-1 antibody has an advantage over all the other markers of cell proliferation in that it can be used after formalin-fixing in paraffin-embedded tissues. The p53 gene is a well-recognized tumor suppressor gene observed on chromosome 17p. Studies of fibrillary astrocytic neoplasmas have suggested that the p53 gene may play a role in the malignant transformation of tumor cells and in the progession of certain low grade tumors to higher grade neoplasms.

In our study, we have measured the tumor cell density by HE staining and obtaining the SI of MIB-1 and p53 at the initial operation and time of recurrence. By comparing these cytological characteristics, it was found that the SI of MIB-1 and p53 were important factors to predict the malignant transformation of low grade astrocytoma.

Materials and Methods

The neuropathological laboratory files at the Tokyo Women's Medical University were searched for cases of astrocytoma that fullfllled the World Health Organization International Histological Classification of Tumors of the Central Nervous System definition of low grade astrocytomas (LGA, 1993). Tumors were obtained over a 20 years period (between 1969 and 1989). The tumor tissues were obtained from 30 patients of LGA (grade II, WHO grading) diagnosed at the time of initial surgery. Ten of the 30 tumors had not shown recurrence for more than 5 years (5 to 15.5 years) and were defined as a non-recurrent group. The other tumors had shown recurrence. Of the recurrent tumors that were removed in the second surgery, 4 tumors showed re-recurrence later. All the removed tumor samples from the second and third operations were analyzed using the same methods as those for the primary tumor. All the observed patients the recieved pure adjuvant chemotherapy after first operation because the same kind of cases were diagnosed to be LGA grade II.

Tumor specimens were stained with HE as the general staining method. Sections from paraffin embedded specimens were further prepared with 3% H_2O_2 and they were soaked in 10 mM citrate buffer pH solution. For MIB-1 immunostaining, isoantigen activation was carried out using microwaves (4 μm parallel settlement, 90 ℃, 15 min). MIB-1 was stained by using a LSAB Kit (DAKO A/S, Denmark, France) and the MIB-1 antibody (1: 10 dilution; Immunotech) for 60 min at room temperature. The p53 protein was stained using the p53 antibody (1 : 100 dilution; DO-7, DAKO). The WHO grading of the tumor was defined by the HE staining. The durations from the initial to the second surgery and from the second to the third surgery were recorded.

The tumor cell densities per mm^2 by HE staining, MIB-1 positive cell ratio (MIB-1 SI) and p53 gene positive cell ratio (p53 SI) were counted. Five suitable places were chosen in the microscopic field and 100 square grids (100×100 μm) for each plate were analyzed to count the cells. In particular, the counting of all cells stained positively with MIB-1 or p53 antibodies ware included in the square grids, which could be seen by special coloring under an microscope (OLYMPUS-BH-2, ×400).

A statistical analysis was made by the Duncan's multiple comparison method, a one-way analysis of variance. A p-value <0.05 was considered to be statistically significant.

Results

Clinical features

The age and sex of the 30 patients are shown in Table 1. The average age of non-recurrent pa-

Table 1 Clinical and histological features in 10 non-recurrent and 20 recurrent low grade astrocytomas (grade II)

Type	Cases No.	age	sex	Histological type T1	Histological grading T1	T2	T3	Duration time (Month)
Non-recurrent	1	36	M	g	II			186.0
	2	43	F	f	II			51.0
	3	32	F	g	II			114.0
	4	41	F	f	II			86.0
	5	47	F	f	II			79.0
	6	23	M	g	II			60.0
	7	40	M	f	II			63.6
	8	51	M	f	II			63.6
	9	35	M	g	II			60.0
	10	35	M	g	II			63.6
Average		38.30±7.96	M/F=6/4	g/f=5/5				82.68±40.56*1
Recurrent	1	36	M	f	II	II		8.0
	2	46	M	f	II	II		2.0
	3	58	M	f	II	III		21.0
	4	60	M	f	II	III		7.0
	5	25	M	f	II	III	IV	52.0
	6	36	M	g	II	III	III	29.0
	7	28	M	g	II	III	III	31.0
	8	50	M	f	II	III		8.0
	9	34	M	g	II	III		72.0
	10	34	M	g	II	IV		79.0
	11	44	M	g	II	IV	IV	21.0
	12	39	F	f	II	IV		53.0
	13	41	M	f	II	IV		13.0
	14	46	M	g	II	IV		51.0
	15	33	F	g	II	II		41.0
	16	37	M	g	II	II		9.0
	17	24	M	f	II	III		46.0
	18	47	M	g	II	IV		3.0
	19	31	M	g	II	IV		46.0
	20	32	M	f	II	IV		153.0
Average		39.20±9.91	M/F=18/2	g/f=10/10				37.25±35.71*2

f: fibrillary astrocytoma, g: gemistocytic astrocytoma, T1: first operation, T2: second operation, T3: third operation.
*1: duration time from T1 to the last day of follow up, *2: duration time from T1 to T2.

tients was 38.3 years old (23～51 years old), and the age of recurrent patients was 39.2 years old (24～60 years old). Regarding sex, males were 24 and females were 6 cases. Regarding the histological types, 15 cases were fibrillary and 15 cases were gemistocytic astrocytoma. In 20 recurrent astrocytoma cases, the duration time from the onset to the first operation was 18.20±20.43 (1.0±58.0) months. The mean duration time from the first operation to the second operation in the same cases was 37.25±35.71 (2.0～153.0) months. The mean duration time from the second to the third operation (4 cases) was 9.00±2.94 (6.0～13.0) months. Regarding the 10 non-recurrent cases, the mean duration time from the onset of symptoms to the operation was 82.68±40.56 (51.0～186.0) months (Table 1). The recurrence was more predominant in males (n=18) than females (n=2). No other correlation was recognized between age or sex and recurrent time.

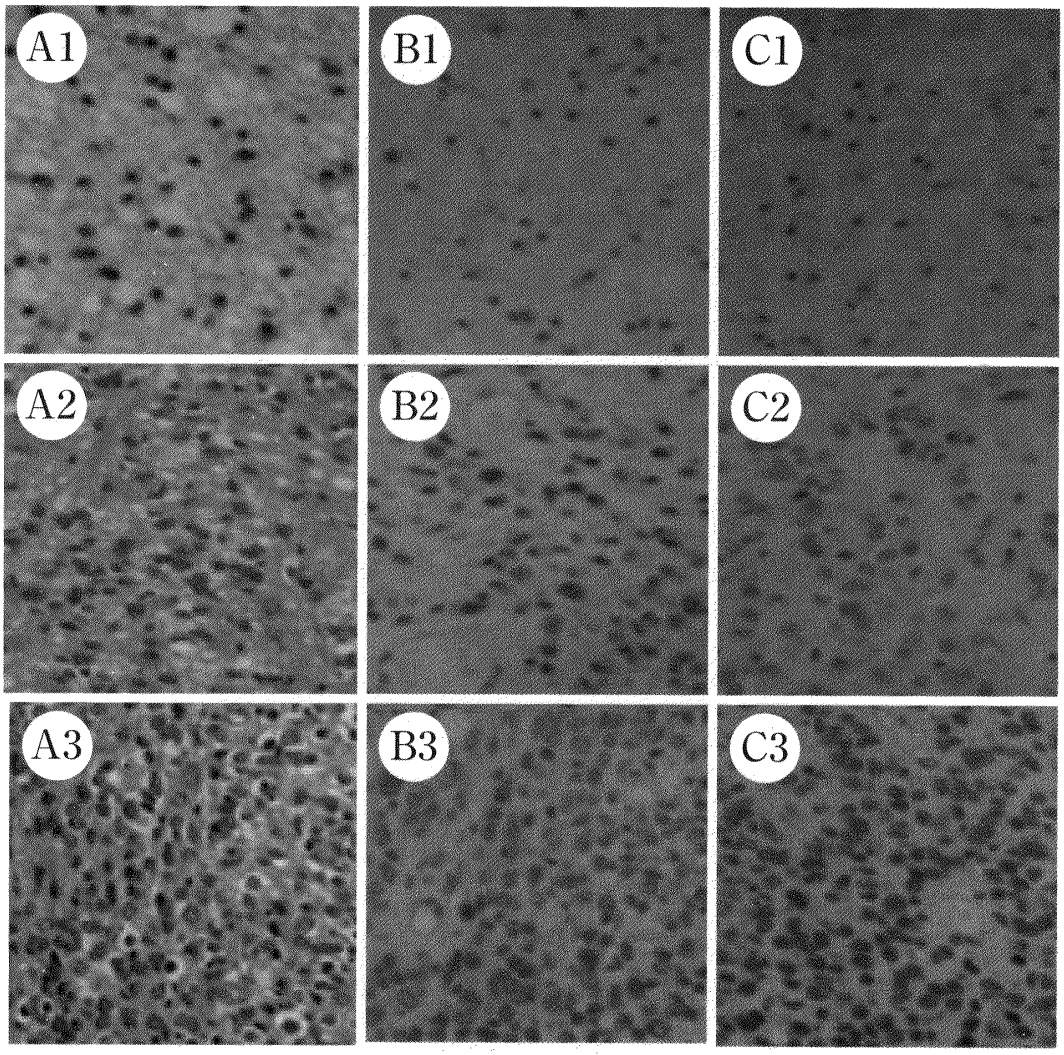

Fig. 1 Histological changing pattern of recurrent astrocytoma in 3 surgical operations for patient No. 5
A : HE staining, B : MIB-1 staining, C : p53 staining.
1 : first operation (grade II), 2 : second operation (grade III), 3 : third operation (grade IV).

In this regard, a significant difference was seen for the mean the duration times from the onset of symptoms to operation between the non-recurrent and recurrent ($p<0.05$).

Histological features

1) HE staining

All 30 cases of astrocytomas were diagnosed as grade II (WHO grading) at the time of the first operation, in which 15 cases were fibrillary astrocytoma and 15 cases were gemistocytic astrocytoma (Table 1). The primary astrocytoma showed a scattered cell density with a few mitosis as shown in Fig. 1. No abnormal vascular formation nor necrosis were observed.

In the 20 recurrent tumor tissues obtained in the second operation, 8 cases showed malignant transformation to grade III anaplastic astrocytoma, and 8 cases to grade IV astrocytoma (glioblastoma) (Table 2). As shown in Fig. 1, in the tumor cells taken during the second operation for recurrence, cell density had increased and hyperchromatic mitotic nuclei and vascular hyperplasia were found. The nuclei of the tumor cells were hyperechromatic with mitotic figures.

Table 2 Cellular density (number of tumor cells) of HE staining, and the immunohistochemical staining indices (SI) of MIB-1, and p53, and the observed duration in 10 non-recurrent and 20 recurrent low grade astrocytomas

Cases mean ± SD	HE NTC (/mm²)	MIB-1 SI (%)	p53 SI (%)	Duration time (Month)
1. non-recurrent (n = 10, grade II)	106.92 ± 33.68	0.98 ± 0.90	28.47 ± 7.14	82.68 ± 40.56*1
2. recurrent (n = 20)				
T1 (n = 20, grade II)	117.13 ± 58.07	6.27 ± 3.87	15.66 ± 9.18	18.20 ± 20.43*2
grade II (n = 4)*5	82.74 ± 37.53	6.00 ± 4.96	23.90 ± 8.84	
grade III (n = 8)	100.14 ± 46.76	6.92 ± 5.04	11.38 ± 6.20	
grade IV (n = 8)	147.11 ± 64.84	5.75 ± 1.95	15.81 ± 9.86	
T2 (n = 20)	177.68 ± 73.18	16.31 ± 10.37	28.49 ± 16.51	37.25 ± 35.71*3
grade II (n = 4)	112.25 ± 24.33	10.61 ± 6.44	26.80 ± 11.93	
grade III (n = 8)	159.78 ± 46.33	19.14 ± 11.72	18.75 ± 8.43	
grade IV (n = 8)	228.31 ± 79.96	16.34 ± 10.43	39.06 ± 19.23	
T3 (n = 4)	259.47 ± 72.33	16.81 ± 5.91	29.66 ± 18.01	9.00 ± 2.94*4
grade III (n = 2)	207.44 ± 67.26	12.05 ± 0.28	14.79 ± 7.69	
grade IV (n = 2)	311.50 ± 18.53	21.56 ± 3.77	44.53 ± 5.47	

NTC: number of tumor cells/mm², SI: staining index, T1: first operation, T2: second operation, T3: third operation.
*1: duration time from T1 to the last day of follow up, *2: duration time from the onset to the first operation, *3: duration time from T1 to T2, *4: duration time from T2 to T3, *5: These grades indicate those from the second operation.

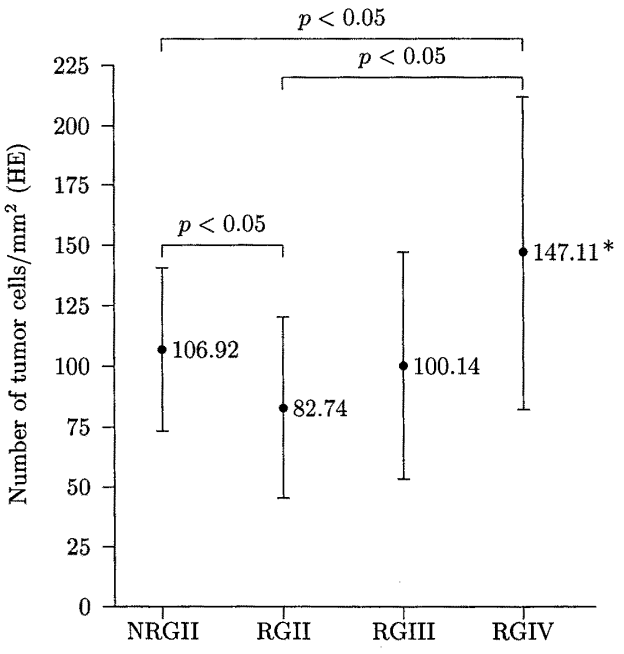

Fig. 2 Number of tumor cells/mm² of HE staining from the first operation in recurrent and non-recurrent cases
NRGII : non-recurrent (n = 10), RGII : recurrent grade II from the second operation (n=4), RGIII : recurrent grade III from the second operation (n = 8), RGIV : recurrent grade IV from the second operation (n=8), *p < 0.05 RGIV vs NR group.

Necrosis was not seen in any of the tumors in primary operation. Grade IV astrocytoma showed the typical pattern of glioblastoma multiforme.

In the 10 non-recurrent tumors, the cell density in HE stained sections at the operation was 106.92 ± 33.68/mm².

Regarding the recurrent tumors, the cell density in the HE stained sections from the initial operation was 117.13 ± 58.07/mm² in all the cases, and 82.74 ± 37.53/mm² for the patients whose tumors showed grade II (n=4) from the second operation, 100.14 ± 46.76/mm² for the patients whose tumor changed to grade III (n=8) from the second operation, and 147.11 ± 64.84/mm² for the patients whose tumor changed to grade IV (n=8) from the second operation (Table 2, Figs. 1, 2). No relationship was seen between the recurrent time and the grading. The number of tumor cells for non-recurrent astrocytoma is not associated with the grade II of recurrent astrocytoma ($p > 0.01$).

2) MIB-1 staining

The average MIB-1 SI at the initial surgery of non-recurrent cases was 0.98 ± 0.90% and in the

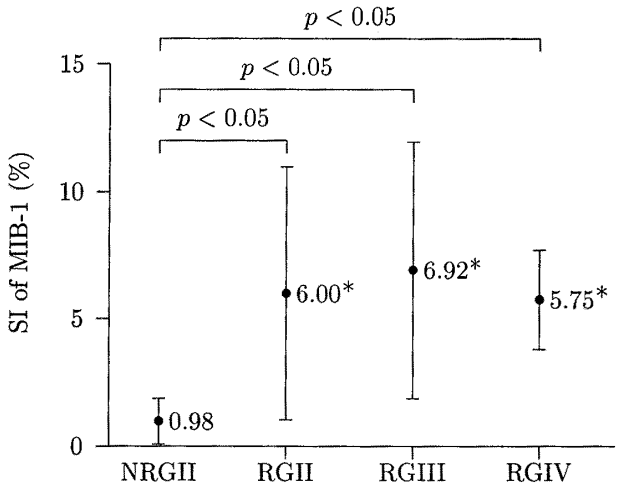

Fig. 3 Staining index of MIB-1 from the first operation in recurrent and non-recurrent tumors
NRGII : non-recurrent (n=10), RGII : recurrent grade II from the second operation (n=4), RGIII : recurrent grade III from the second operation (n=8), RGIV : recurrent grade IV from the second operation (n=8), *p< 0.05 NR vs each group.

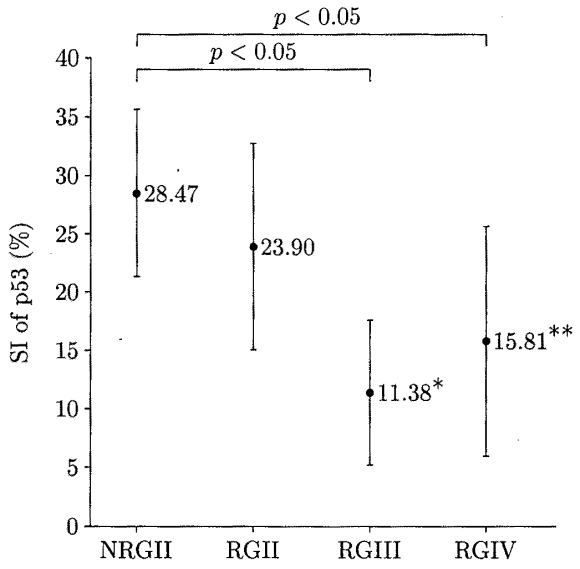

Fig. 4 Staining index of p53 from the first operation in recurrent and non-recurrent patients
NRGII : non-recurrent (n=10), RGII : recurrent grad II from the second operation (n=4), RGIII : recurrent grade III from the second operation (n=8), RGIV : recurrent grade IV from the second operation (n=8), *p< 0.05 NR vs RGIII, **p< 0.05 NR vs RGIV.

recurrent cases, it was 6.27 ± 3.87% for all the cases from the first surgery, 16.31 ± 10.37% from the second surgery and 16.81 ± 5.91% from the third surgery (Table 2). There was a significant difference of MIB-1 SI between the non-recurrent and recurrent cases at the initial operations. No significant difference between the MIB-1 SI of the recurrent cases from the first operation and the transformed grading (Figs. 1, 3) was observed. The SI of MIB-1 for non-recurrent cases is correlated with the each group of changed grade of the second operation from first operation in recurrences ($p < 0.05$).

3) p53 staining

In the non-recurrent tumors, p53 SI was 28.47 ± 7.14%. In the recurrent cases, p53 SI at the time of initial surgery, was 15.66 ± 9.18%, 28.49 ± 16.51% from the second surgery and 29.66 ± 18.01 % from the third surgery (Table 2). There was a correlation between p53 SI of tumor cells transformed to grade III and IV astrocytoma from the initial operation and the second operation (Figs. 1, 4). The SI of p53 for the non-recurrent cases was correlated with the changed grade III and grade IV of the second operation from the first operation in the recurrences ($p<0.05$). But in the recurrent cases, the SI of p53 for grade II was not correlated with the non-recurrence ($p>0.01$).

Discussion

The recurrence of brain tumors and the survival time thereof can be predicted generally from the age of the patient, clinical grading, size, site, histological type and so on[5)~7)]. It has gradually become possible to study the mechanism of malignant transformation at the gene level. It is necessary to analyze the proliferative potential and suppressor genetic function of a brain tumor in order to predict the malignant transformation. The measurement of MIB-1 SI as a factor of proliferation and p53 SI as a suppressive function has become possible by using immunohistochemical staining. Therefore, by measuring the MIB-1 or p

53 staining index, it should be possible to predict recurrence, and the patients' survival expectancy at the time of the initial operation.

Cellular density and grading

In general, a higher cellular density is an indication of malignancy. It has been, however, noted that the distribution of proliferating cells in neoplasm tissue is not homogeneous. At present, the grading of malignancy is generally based on cellular pleomorphism, the ratio of mitotic cells, vascular proliferation, and the presence of necrosis. The grade of malignancy of astrocytoma must be defined by the cellular density and the proliferation along with the suppressive characteristics of the cells in the tumor. As a significant difference of cellular density was observed among the grades II, III, and IV (WHO classification), automatic computerized analysis will be developed in the future to assess the grading of malignancy.

MIB-1 staining

Since 1982, the proliferative potential of tumor cells has been analyzed using MIB-1 (Ki-67). Although the MIB-1 antibody was identified with the Ki-67 antigen, it is known that cell growth inside tumor tissue is not generally homogeneous and the distribution of growing cells also is not uniform. The cell cycle time for each MIB-1 positive cell is different, and the SI of MIB-1 is measured by ignoring such heterogeneity.

Shibata et al (1989) studied the MIB-1 SI of 141 cases of astrocytoma and reported that the MIB-1 SI is 9.4～11.1%. There was a correlation between the MIB-1 SI and histological malignancy grading[1]. Kordek et al (1996), classifying 56 cases of astrocytoma into grades II, III, and IV, reported that there was a correlation between the MIB-1 SI (1.9, 6.8, 9.8%) and each grading[8]. Prayson and Estes (1996) reported that, in tumor tissues of 32 astrocytoma patients, the MIB-1 SI was 0.0～4.3% in 1,000 tumor cells[9].

Ide et al (1996), counting the number of MIB-1 positive cells in 57 cases of meningioma, reported that the positive rate of MIB-1 SI was 0.06～6.8% and there was a correlation between MIB-1 SI and histological grading of malignancy[10]. However, in these reports, the concept of cell density was not considered.

The MIB-1 SI was found to be 6.27 ± 3.87% at the time of primary surgery in recurrent cases, and 0.98 ± 0.90% in non-recurrent cases in our study. As a significant difference in the MIB-1 SI was noted between the recurrent and non-recurrent low grade astrocytomas, it might be possible to predict malignant transformation, as well as recurrence, by using MIB-1 SI after the primary surgery.

p53 gene

From the results of experiments regarding cell growth, it was reported that, the presence of the p53 gene in cancer cells combines with DNA and this acts to arrest cell growth in the G1 phase, hence prompting cell death by apoptosis[11]. This p53 protein suppression is a common genetic event in astrocytoma progression and may signal the transition from benign to malignant tumor grading[12]. It is thought in general that the p53 gene shows a suppressive action over the G1 phase in all growing cells.

The tumor suppressor gene has been seen in many tumors and excess manifestation of the p53 with staining heterogeneity has been reported[13][14]. In our study, we found the cellular density and MIB-1 SI to have a marked increased effect on the course of tumor recurrent. p53 SI to have a increased effect on the course of tumor recurrent, and malignant transformation a significant is seen from the first operation at the second operation was diagnosed grade II cases of low grade astrocytoma. This again illustrates a sharp feature in all types of astrocytomas.

A high MIB-1 SI and low p53 SI would indicate a higher degree of potential malignancy and shor-

ter life expectancy. The balance between MIB-1 SI and p53 SI in low grade astrocytoma (WHO grade II) found at the time of the initial operation are useful indicators for the prediction of recurrence as well as malignant transformation to a higher grade of astrocytoma.

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成人低悪性度星細胞腫の再発・悪性転化の予測に関する組織学的研究
—再発時に MIB-1 と p53 遺伝子産物発現について—

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悪性度の低い星細胞腫が，悪性に転化して，きわめて不良な予後を示すことがあることは臨床的にしばしば経験することである．初回手術時に低悪性度の星細胞腫と判断された腫瘍が悪性化するか否かを予測することは臨床上きわめて重要である．本研究では，この予測を可能にすることができるか否かを明らかにするために，再発なしに良好な経過をたどっている症例と悪性転化して再発した症例について，初回手術，ならびに再発時に得られた腫瘍標本について病理組織学的検討を行った．

WHO 分類で診断が確定した astrocytoma grade II（low grade astrocytoma）30 例のうち，再発 20 例，非再発 10 例を検討した．再発例と非再発例の低悪性度星細胞腫（low grade astrocytoma）の手術時に得られた組織を用い，HE 染色，MIB-1 と p53 免疫染色を行った．初発時（手術），再発時（再発手術の行われた例のみ），再再発時の手術で摘出した腫瘍組織切片について，それぞれの組織切片で細胞密度の高い部位 5 カ所を選んで検鏡し，1mm^2 あたりの HE 染色による腫瘍細胞数 NTC（numbers of tumor cells），および MIB-1 と p53 の免疫染色陽性細胞指数 SI（staining index）を測定した．HE 染色の NTC，MIB-1 と p53 の SI および悪性度 G(grade)，再発までに要した時間 t(recurrence time) の相互の関連性について検討した．

初発時および再発時の HE 染色による細胞数と腫瘍の悪性度，再発までの時間には有意差は認められなかった．再発例（$\pi=20$）では MIB-1 の SI と再発について分析すると MIB-l SI の高いほど悪性化して再発しやすく（SI 5.75～21.56%），p53 SI の低いほど再発しやすい（SI 6.20～44.53%）．非再発例（$\pi=10$）では MIB-1 の SI が似値で（SI 0.98 ± 0.90%，$p<0.05$），p53 の SI が高値であった（SI 28.47 ± 7.14%，$p<0.05$）．腫瘍の悪性化，再発を予知する指標としては，細胞密度，MIB-1 および p53 の SI 値が重要な指数と考えられた．腫瘍再発を予知するためには，増殖と抑制双方の因子のバランスを検討することが必要である．