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Original Article

Long-term molecular changes in WHO grade II astrocytomas following radiotherapy

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Abstract

Monitoring the long-term radiotherapy-associated molecular changes in low-grade gliomas (LGGs) facilitates the understanding of LGG response to radiotherapy. In this study, we used immunohistochemistry to analyze the expression of Ki-67, tumor protein P53 (TP53), P21, and P27 in 8 paired WHO grade II astrocytoma samples. The interval between radiotherapy (RT) and the second surgery was more than 3 months in all cases. The average Ki-67 labeling index (LI) was 5.3% in pre-RT samples and 11.54% in post-RT samples. Ki-67 LI was higher in the primary tumors that underwent malignant transformation observed at the second surgery after radiation. Post-RT Ki-67 LI decreased in 2 cases with an interval of less than 12 months between RT and the second surgery. TP53 expression was found in 3 out of 4 pre-RT samples with malignant transformation and in 1 out of 4 pre-RT samples without malignant transformation. Post-RT TP53 increased in 2 cases in which increased expression of P21 or P27 was also observed. Our study suggests that radiotherapy can inhibit WHO grade II astrocytoma proliferation as reflected by Ki-67 LI, but the effect attenuates with time. In addition, there is a tendency of malignant transformation for WHO grade II astrocytomas with a high Ki-67 level or TP53 expression in initial samples.

Key words Radiotherapy, WHO grade II astrocytoma, malignant transformation, Ki-67, TP53

Low-grade glioma (LGG) is a frequent primary brain tumor in young adults[1]. The prognosis of patients with LGG is generally good, but the infiltrative nature and the tendency of malignant progression make the management of LGGs challenging[2]. Although no statistical difference in survival was observed between LGG patients treated with early postoperative radiotherapy (RT) and those treated with delayed RT, the early employment of RT has been demonstrated to prolong progression-free survival (PFS) and control seizures better in LGG patients[3]. Therefore, RT is still an important treatment strategy and is usually recommended for patients with high-risk LGGs, such as those older than 40 years and those with radiographic residual disease[4].

Monitoring cellular and genetic changes following RT is therefore valuable for understanding the effects of RT and for tailoring treatment for LGG patients. Tracing molecular changes in LGGs after RT can be difficult because of the rarity of paired surgical samples before and after RT. Henson *et al.*[5] have reported molecular alterations in astrocytomas following RT, but the study included astrocytomas of different histological grades and only demonstrated short-term changes following RT. In the current study, we collected 8 paired surgical samples of WHO grade II astrocytomas with an interval of more than 3 months between RT and the second surgery. We compared Ki-67, tumor protein P53 (TP53), P21 and P27 in the paired samples to determine molecular changes of LGGs following RT.

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Patients and Methods

Patients

We reviewed the medical records of glioma patients treated at Sun Yat-sen University Cancer Center and Zhongnan Hospital of Wuhan University between 1994 and 2009. Patients were included according to the following criteria. First, the patient was pathologically diagnosed with WHO grade II astrocytoma initially. Second, the patient underwent RT following the first surgery. Third, a second surgery was performed, and the interval between RT and the second surgery was longer than 3 months. Fourth, tissue samples from 2 surgical procedures were available. The present study was approved by the ethics committee of Sun Yat-sen University Cancer Center.

Immunohistochemical analysis

From paraffin-embedded specimens, 4-μm sections were stained with hematoxylin and eosin (HE) to confirm diagnosis. The WHO classification (2007) was strictly applied. Histological changes associated with RT, such as endothelial proliferation, vascular hyalinization, reactive astrocytosis, and necrosis with calcification, were also examined. All sections were independently reviewed by two neuropathologists.

All archival tissues were analyzed for expression of Ki-67, TP53, P27, and P21 using immunohistochemical staining. Sections with paraffin-embedded specimens were de-paraffinized using xylene and absolute ethanol, rinsed in distilled water, exposed to 3% H_2O_2 for 10 min at 37℃, and placed in EDTA antigen-unmasking solution. The antigen-unmasking solution and sections were heated in an oven at 90℃ for 15 min. The sections were cooled to room temperature in unmasking solution, rinsed with PBS, and subsequently blocked with 10% normal horse serum in PBS. For staining, sections were incubated overnight at 4℃ with the following primary antibodies (ready to use): TP53 (Zymed, ZM-0480, Clone: Do-7, which reacts with wild-type and mutant TP53 protein), P21 (Zymed, ZM-0206, Clone: DSC-60.2), P27 (Zymed, ZM-0340, Clone: 1B4), or Ki-67 (Zymed, ZM-0165, Clone: 7B11). Subsequently, sections were incubated at 36.5℃ with biotinylated anti-mouse Ig in PBS for 30 min, followed by incubation with streptavidin conjugated to horseradish peroxidase in PBS for 30 min and rinsing in PBS 3 times for 3 min each. The sections were exposed to diaminobenzidine tetrahydrochloride chromogen for up to 5 min and rinsed in distilled water. Sections were counterstained with Mayer hematoxylin for 1 min and covered with a permanent mounting medium. Formalin-fixed, paraffin-embedded breast carcinoma sections with strong immunoreactivity for TP53, P21, P27, and Ki-67 were used as positive controls. Sections without primary antibody treatment served as negative controls. Only the nuclear staining was considered positive, and only sections without background staining were used for analysis. Ten fields containing approximately 500 tumor cells were counted in each case. The Ki-67 labeling index (LI) was calculated as a percentage of positive cells to the total number of tumor cells counted. Immunohistochemical positivity of TP53, P21, and P27 was scored on a scale of 0 to 3+, with 0 indicating no staining; 1+ indicating ⩽10% of tumor cells were stained; 2+ indicating 11% to 49% of tumor cells were stained; and 3+ indicating ≥ 50% of tumor cells were stained.

Results

Patient characteristics

Eight patients with a median age of 32.5 years (range, 26 to 45 years) at the initial diagnosis of WHO grade II astrocytomas, including 2 men and 6 women, were identified (Table 1). Co-60 beams and linear accelerator were used for RT in 6 cases and 2 cases, respectively. The average dosage of RT was 57.3 Gray (Gy). The interval between RT and the second surgery ranged from 6 to 90 months (average, 36.4 months).

Histopathologic findings

Among 8 patients, malignant transformation was observed in 4 cases, and the histological tumor grade remained unchanged (no malignant transformation) in 4 cases (Table 1). In post-RT tumor samples, apparently viable tumor cells were observed in all cases. Typical changes associated with radiation was identified in post-RT tumor samples (Table 2, Figure 1). Vascular hyalinization was found in 5 cases, endothelial activation in 5 cases, large cells with copious cytoplasm in 5 cases, necrosis with calcification in 4 cases, and reactive astrocytosis in only 1 case.

Immunohistological findings

Ki-67 is an important marker for cell proliferation. We examined Ki-67 LI in the paired tumor samples. The average Ki-67 LI increased from 5.3% in all pre-RT samples to 11.5% in all post-RT samples (Table 3, Figure 2), from 8.91% to 19.48% after RT in the samples with malignant transformation, and from 1.7% to 3.6% after RT in the samples without malignant transformation (Table 3, Figure 3). Post-RT Ki-67 LI

Table 1. Clinical characteristics of 8 grade Ⅱ astrocytoma patients with a long interval between radiotherapy (RT) and the second surgery

Case No	Sex	Age at diagnosis (years)	WHO grade		Interval between first and second surgery (months)	Interval between RT and second surgery (months)	Radiation (Gy)
			First surgery	Second surgery			
1	F	38	II	III	61.5	59	60
2	M	26	II	III	49	46	60
3	F	36	II	III	10	8	61.6
4	F	41	II	IV	93	90	51
5	F	45	II	II	22	19	60
6	F	28	II	II	45	43	50
7	F	28	II	II	21	20	56
8	M	29	II	II	8	6	60

F, female; M, male.

Table 2. Histological features of tumors after radiation

Case No	Viable tumor cells	Vascular hyalinization	Endothelial activation	Necrosis with calcification	Reactive astrocytosis	Large cells with copious cytoplasm
1	+	+	–	+	–	+
2	+	–	+	–	+	–
3	+	+	+	+	–	+
4	+	–	–	+	–	+
5	+	+	+	+	–	+
6	+	+	+	–	–	+
7	+	+	+	–	–	–
8	+	–	–	–	–	–

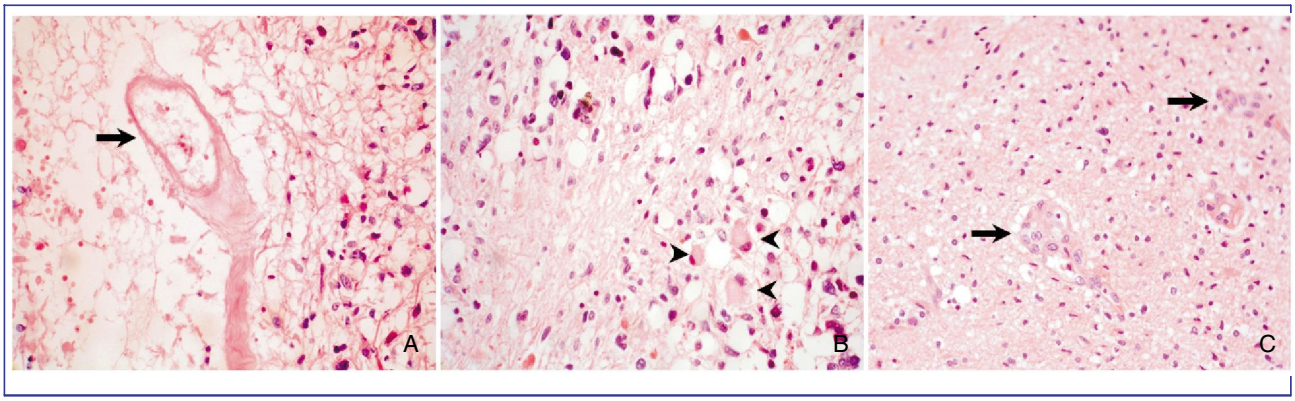

Figure 1. **Typical pathologic presentations of low-grade gliomas associated with radiation.** Vascular necrosis (arrow in A, HE ×400), large cells with copious cytoplasm (arrow heads in B, HE ×400), and endothelial activation (arrows in C, HE ×200) were found in post-radiotherapy (RT) samples.

decreased in 2 patients with the interval between RT and the second surgery shorter than 12 months, and increased in the other 6 patients with the interval longer than 12 months (Table 3, Figure 4).

Table 3. Expression of cell cycle regulators in pre-RT and post-RT astrocytomas

Case No	Ki-67 LI (%)		TP53		P21		P27	
	Pre-RT	Post-RT	Pre-RT	Post-RT	Pre-RT	Post-RT	Pre-RT	Post-RT
1	1.4	8.2	+	++	++	+++	++	++
2	7.4	40.4	++	++	+++	+++	+++	+++
3	26.0	19.4	+++	+++	+++	+++	++	++
4	0.8	9.9	–	–	++	++	+++	+++
5	1.0	8.4	–	++	++	++	++	+++
6	0.2	5.4	–	–	+++	++	++	+++
7	0.2	0.4	–	–	+++	+++	++	+
8	5.4	0.2	+	+	+++	+++	++	++

LI, labeling index.

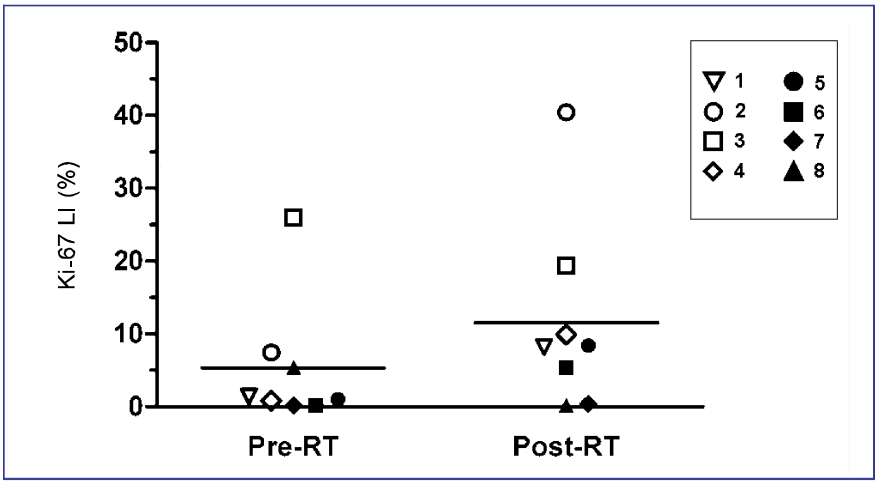

Figure 2. Distribution of Ki-67 labeling index (LI) in 8 paired pre-radiotherapy (pre-RT) and post-RT samples of WHO grade II astrocytomas. The average of Ki-67 LI in 8 post-RT samples was 11.54% , which was higher than that in pre-RT samples (5.3%).

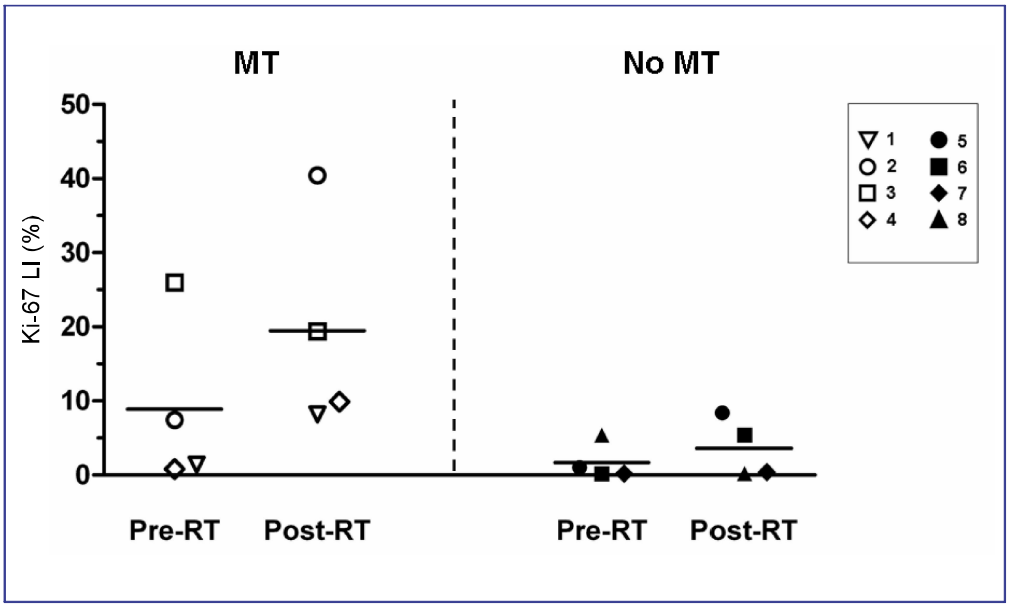

Figure 3. Scattergram demonstrating the percentage of Ki-67 LI in paired pre-RT and post-RT samples from patients with or without malignant transformation. The average of Ki-67 LI was higher in pre-RT samples from patients with malignant transformation (8.91%) than in those without malignant transformation (1.7%). In addition, there was increased expression of Ki-67 in post-RT samples from patients with or without malignant transformation, compared to their pre-RT counterparts.

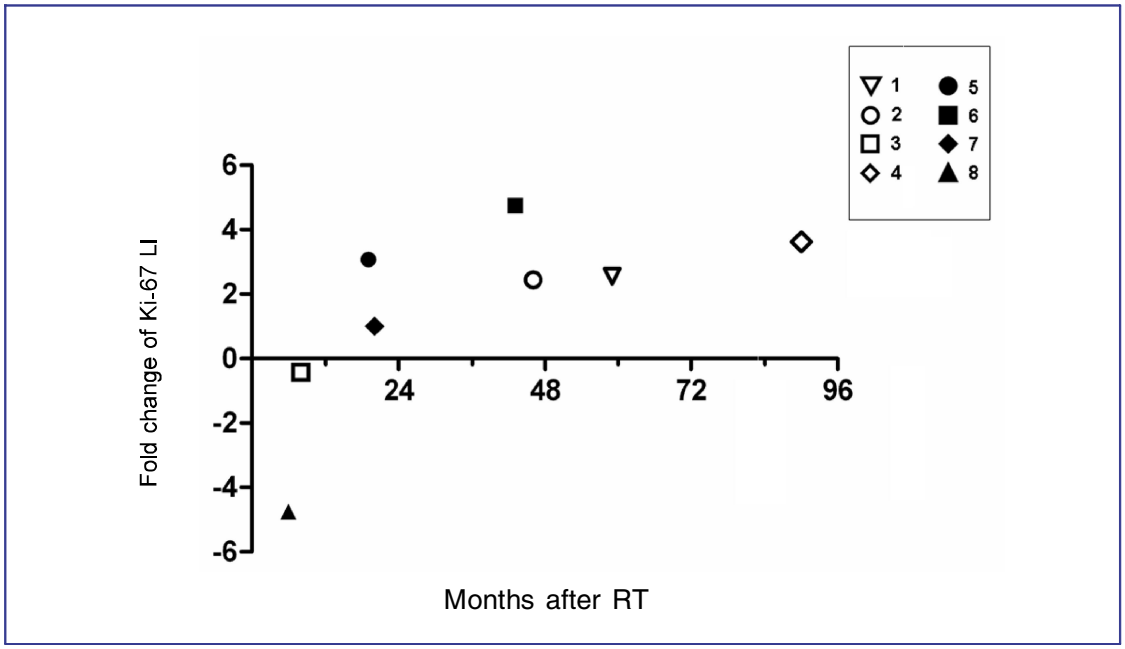

Figure 4. **Fold change of Ki-67 LI after radiotherapy.** The following formula was used for the calculation: fold change = $\log_2\left(\frac{\text{post-RT Ki-67 LI}}{\text{pre-RT Ki-67 LI}}\right)$. A fold change of zero indicated an equal of Ki-67 LI in post-RT and pre-RT samples. Among the 8 cases, post-RT Ki-67 LI decreased in 2 patients (case #3 and case #8), which resulted in a negative fold change. The interval between RT and the second surgery was shorter than 12 months in both cases.

TP53, P21, and P27 are cell cycle regulators and are suggested to play an important role in the repair of radiation-induced DNA damage. Therefore, we also investigated the expression of these molecules in the paired samples. TP53 was detected in pre-RT samples from 3 patients with malignant transformation and 1 without malignant transformation. P21 and P27 were detected in all samples. After RT, TP53 expression was increased in 2 cases, P21 expression was increased in 1 case and decreased in 1 case, P27 expression was also increased in 1 case and decreased in 1 case (Table 3).

Discussion

LGGs are usually considered indolent tumors, with a 5-year overall survival (OS) rate higher than 50% [6]. However, a subset of LGGs tends to present with more aggressive clinical behavior and therefore require more aggressive interventions. Radiotherapy is an important treatment option for LGGs. In the current study, we examined several molecular parameters in 8 paired surgical samples of WHO grade II astrocytomas with an interval longer than 3 months between RT and the second surgery. We found that RT decreased Ki-67 LI in WHO grade II astrocytomas, but the effect attenuated with time. In addition, we found that malignant progression may be associated with a high Ki-67 LI or P53 expression in the initial surgical samples of WHO grade II astrocytomas.

Radiotherapy is the mainstay for managing high-grade gliomas and has been demonstrated to be beneficial for selected patients with LGGs. Kortmann *et al.*[7] summarized the outcome of surgery alone and surgery plus RT in LGG patients treated between 1956 and 2002 and found that symptoms were controlled in up to 80% of LGG patients treated with postoperative RT. A prospective, randomized clinical trial conducted by the European Organization for Research and Treatment of Cancer (EORTC, trial 22845) reported similar results[3]. In that study, immediate postoperative RT was demonstrated to increase the median PFS by approximately 2 years compared to the observation group. The 5-year PFS rate was 44% for patients treated with early RT and 37% for those in the observation group (P = 0.02). In addition, seizures were better controlled by early RT. Notably, in EORTC 22845, early employment of RT failed to improve OS, with 5-year OS rates of 63% in the early RT group and 66% in the delayed RT group (P = 0.49). The results of EORTC 22845 indicated that RT is effective but not durable enough in managing LGGs. Henson *et al.*[5] investigated MIB-1, a monoclonal antibody for detecting Ki-67, in 8

paired pre-RT and post-RT astrocytoma samples, of which RT was performed within 8 weeks after the resection. Reduction of cellular proliferation reflected by MIB-1 LI was observed shortly following RT. Pierce *et al.*[8] assessed the proliferative activity using proliferating cell nuclear antigen (PCNA) and Ki-67 LI in primary and recurrent astrocytomas of various histopathologic grades following RT. The levels of both PCNA and Ki-67 were found to be significantly higher in 20 of 28 post-RT specimens. The authors postulated that the increased proliferation was a result of repopulation in the tumor pool or of the selection of astrocytoma cells with more aggressive phenotype by radiation. In the current study, we found that Ki-67 LI increased in most cases (6 of 8) with a long-term interval after RT. Interestingly, decreased Ki-67 LI was observed in 1 patient with malignant transformation and 1 without malignant transformation, both having finished RT less than 12 months after the first surgery (8 months in Case #3 and 6 months in Case #8). On the basis of our findings and the studies mentioned above, we assume that RT can inhibit the proliferation of WHO grade II astrocytoma, but the effect was not durable and probably lasted less than 12 months. The growth of tumor cells gradually accelerated 1 year after the completion of RT, which can partly explain why early RT improves the seizure control and PFS but fails to prolong OS. If our assumption is correct, more aggressive treatment, such as chemotherapy following RT, could be administered for WHO grade II astrocytoma patients with high risk.

TP53 is a critical tumor suppressor involved in the regulation of cell growth, apoptosis, transcription, and malignant progression[9], and it plays a critical role in the repair of DNA damage induced by radiation. Activation of TP53 results in cell cycle arrest partly via regulating P21 and P27. Increased expression of TP53 and P21 and decreased proliferation have been observed in astrocytomas within 8 weeks after RT. We measured the expression of TP53, P21, and P27 in our series of WHO grade II astrocytomas with a long interval after RT. Increased level of TP53 was only found in 2 out of 8 cases, both in which the expression of P21 or P27 paralleled that of TP53 whereas the proliferation reflected by Ki-67 expression did not decrease. One explanation for this finding is that tumor cells may overcome the cell cycle arrest induced by TP53 through other mechanisms.

WHO grade II astrocytomas are heterogeneous and the prognosis can be significantly different, which makes the identification of prognostic factors necessary[10]. For patients with unfavorable factors, interventions should be applied early and more aggressively. The EORTC conducted a detailed analysis of prognostic factors for OS of patients with LGGs based on trials 22844 and 22845 [11]. The analysis revealed that age older than 40 years, astrocytoma histology, maximum tumor diameter no less than 6 cm, tumor crossing the corpus callosum, and the presence of a neurologic deficit before surgery negatively affected survival. The evaluation of tumor proliferation may offer great insight into the malignant potential of LGGs. Immunohistochemistry for Ki-67 antigen was shown to provide prognostic information in LGGs independent of histopathologic grade and tumor location[12]. McKeever *et al.*[13] found an MIB-1 LI higher than 2% predicted a shorter survival for patients with grade II astrocytomas. By performing a survival analysis of 50 patients with grade II astrocytomas, Schiffer *et al.*[14] demonstrated that MIB-1 LI was an independent prognostic factor along with the extent of resection, postoperative Karnofsky status, and age. The survival of astrocytoma patients grouped by MIB-1 LI was significantly different, with a median survival of 2.9 years in the group with high MIB-1 LI (greater than or equal to 8%) versus 4.6 years in the group with low MIB-1 LI (less than 8%). Nevertheless, there are still conflicting opinions on the prognostic value of Ki-67 for LGGs. Hilton *et al.*[15] demonstrated that the level of Ki-67 failed to predict the survival of patients with supratentorial fibrillary astrocytomas. In the current study, we found that the average level of Ki-67 LI was 8.9% in the initial samples of WHO grade II astrocytoma with malignant transformation, which was much higher than that in those without malignant transformation (1.7%). We speculate that increasing Ki-67 expression in some well-differentiated astrocytomas is an early molecular event before the phenotypic transformation.

TP53 dysfunction is frequently observed in LGGs, and several studies have shown the prognostic relevance of TP53 status in LGGs. Ishii *et al.*[16] reported association of malignant progression and poor prognosis of WHO grade II astrocytomas with mutated TP53. In that study, 92% of WHO grade II astrocytomas with TP53 mutation recurred and 57% progressed to higher grades, whereas 64% without TP53 mutation experienced recurrence and 41% had malignant progression. Watanabe *et al.* [17] found that TP53 mutation was associated with tumor recurrence and a shorter time-to-progression in low-grade astrocytomas. Sarkar *et al.*[18] analyzed TP53 expression in paired astrocytomas upon recurrence. They found that overexpression of TP53 protein was related to malignant progression. In our previous study, we reported TP53 overexpression and mutation as molecular markers to predict malignant progression in astrocytomas[19]. However, the role of TP53 in predicting prognosis for LGGs is controversial. In an immunohistochemical study of TP53 expression in 100 cases with grade II astrocytomas, Vital *et al.* [20] demonstrated that TP53 expression did not reach statistical significance as an independent predictive factor. In our current study, we demonstrated that the expression of TP53 was more frequently observed in the

initial surgical samples of WHO grade II astrocytomas with malignant transformation than in those without malignant transformation. We postulate that TP53 is partly involved in the malignant transformation of WHO grade II astrocytomas.

The major limitation of the present investigation is the small sample size, which weakens the power of the study. However, our study provides a valuable insight into the molecular nature and long-term changes associated with radiation in WHO grade II astrocytomas. Our findings suggest that WHO grade II astrocytomas with a high Ki-67 LI or TP53 expression are more likely to progress to higher grade astrocytomas. Furthermore, RT is capable of inhibiting WHO grade II astrocytoma proliferation, as reflected by Ki-67 LI, but the effect occurs for only a limited time. Therefore, we may consider more aggressive treatment, such as chemotherapy following radiation, for WHO grade II astrocytomas with a high risk of malignant transformation. Further studies and appropriate prospective trials are warranted to test our hypothesis.

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