The Combination of Low Cytoplasmic and High Nuclear Expression of p27 Predicts a Better Prognosis in High-grade Astrocytoma

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Abstract. *Background: The function of the cyclin-dependent kinase (cdk) inhibitor p27 is regulated by translocation between the nucleus (activate) and the cytosol (inactivate). No previous reports have examined the subcellular localization of p27 in glioma which was evaluated here regarding the prognosis in high-grade astrocytomas. Patients and Methods: The pattern of subcellular localization of p27 expression was examined immunohistochemically in 49 patients with high-grade astrocytoma who were over 20 years of age. The relationship between p27 localization and the prognosis was statistically examined. Results: Kaplan-Meier survival analysis showed that cytoplasmic p27 expression was statistically associated with a worse prognosis (p=0.0203), while nuclear p27 expression showed some tendency towords a better prognosis (p=0.1180). Cox multiple regression analysis showed the combination of high nuclear and low cytoplasmic p27 expression associated with a significantly better prognosis in high-grade astrocytoma. Conclusion: A combination of low cytoplasmic and high nuclear expression of p27 predicts a better prognosis in high-grade astrocytomas and thus the subcellular localization of p27 expression is useful for predicting the prognosis for these patients.*

High-grade astrocytoma is the most frequently occurring malignant brain tumor. The prognosis of patients harboring high-grade astrocytoma remains poor, despite treatment with combinations of surgery, radiotherapy and chemotherapy. Due to the fact that it is difficult to remove the entire tumor surgically, patients are often treated with radio- and chemotherapy. However, the overall one-year survival rate of patients with high-grade astrocytomas is reported to only be 40% and it is only slightly higher at 46% following combined radio- and chemotherapy (1).

Cellular progression through the cell cycle is governed by cyclin-dependent kinase (cdk) which is regulated by phosphorylation, activated by the binding of cyclins and inhibited by cdk inhibitors (2-4). Based on their protein sequence homologies and putative cdk targets, cdk inhibitors belong to one of two families: namely, the CIP/KIP family ($p21^{Waf1/Cip1}$, $p27^{kip1}$ and $p57^{kip2}$), which inhibits a broad range of cyclin/cdk complexes, and the INK4 family ($p15^{Ink4b}$, $p16^{Ink4a}$, $p18^{Ink4c}$ and $p19^{Ink4d}$), which mainly inhibits cdk4 and cdk6. The coordinated expression of cyclins, cdks and cdk inhibitors is often deregulated in cancer (5). The cdk inhibitor $p27^{kip1}$ regulates the cellular progression from G_1 to S-phase, the restriction point of the cell cycle, p27 acts primarily by complexing with cyclins D1, E and A, thereby inhibiting the function of these cdks. Several studies have shown that the loss of p27 expression is associated with a poor prognosis in malignant tumors, while it still remains controversial as to whether the expression or loss of expression of $p27^{kip1}$ has any prognostic significance in human cancer (6-14). Most previous reports have examined the relationship between the nuclear expression of p27 and the prognosis. However, the intracellular transition between the nuleus and the cytosol has an effect on the function of p27 and the cytoplasmic displacement of p27 may thus indicate a mechanism of p27 inactivation, which is different from that of proteasomal degradation (15). The cytoplasmic translocation of p27 has been increasingly recognized in primary human tumors associated with a poor survival (16-19), while the prognostic significance of subcellular localization has not been previously investigated in glioma patients.

Abbreviations: LI: labeling index; ACNU: 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosoureal hydrochloride.

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Therefore, in this paper, firstly the subcellular localization (nuclear or cytoplasmic) of $p27^{kip1}$ was evaluated immunohistochemically and then the relationship between p27 localization and the prognosis in high-grade astrocytomas was analyzed.

Patients and Methods

Clinical data and patient selection. Prior written informed consent was obtained from the patients and/or their guardians. The records of 49 patients who were over 20 years old with high-grade astrocytoma (17 astrocytoma; 32 glioblastoma) who had undergone initial surgery at Hiroshima University Hospital between January 1991 and December 2003 were studied retrospectively. The patients ranged in age from 20 to 76 years (mean age [± standard deviation], 52.6±16.4 years; median age, 55 years). The follow-up data of the patients were obtained from hospital records. High-grade astrocytomas of the brain stem, basal ganglion or cerebellum were excluded. Pilocytic astrocytomas and diffuse astrocytomas that were classified as low-grade astrocytomas were excluded. Other types of glioma, such as oligodendroglial tumors, ependymal tumors and gangliogliomas were also excluded because they are pathologically distinct from astrocytic tumors. Any patients who did not receive radiation therapy were excluded because a craniotomy and adjuvant radiotherapy are standard treatments for high-grade astrocytoma. The remaining 49 patients were enrolled in this study; 44 of them received 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosoureal hydrochloride (ACNU)-based chemotherapy. Chemotherapy was not taken into consideration in the present study, because it was not administered uniformly in all patients and, unlike radiotherapy, it has not yet been proven that chemotherapy prolongs the survival in patients with high-grade astrocytomas. The relationship between p27 subcellular localization and prognosis was statistically examined, using a multivariate analysis including other clinicopathological factors (age, sex, WHO grade and MIB-1 labeling index, LI). The details of the 49 patients are shown in Table I. The mean survival was 27.09 months (range, 2.33-120.03 months).

Tissue specimens and immunohistochemical staining. All the tumor specimens were obtained by surgical resection and then were fixed in 10% formalin before paraffin processing. Representative slides were stained with hematoxylin and eosin for standard histological diagnosis. The tumors were classified into the histological subtypes by one of the authors (K.S.), according to the WHO criteria. Four-micrometer tissue sections were deparaffinized with xylene and antigen retrieval was carried out by using the heat-induced epitope retrieval method with citrate buffer solution, pH 6.0. Endogenous peroxidase blocking was carried out by dipping the slides into a solution made by mixing 19 ml of 30% H_2O_2 and 90 ml of 99% methanol for 30 min. After each step, the slides were rinsed and washed with phosphate-buffered saline (PBS) solution, pH 7.5, 3 times for 5 min each. The labeled streptavidin biotin (SAB) method, which is an indirect method of immunostaining, was employed for antibody incubation, using a histofine SAB (m) kit (Nichirei Company, Tokyo, Japan). The antibodies consisted of a mouse polyclonal antibody at a 1:50 dilution for p27, and a mouse monoclonal antibody (Dako Japan, Kyoto, Japan) at a 1:50 dilution for MIB-1. The pathological specimens (thickness, 4 μm) were mounted on gelatin-coated slides and deparaffinized by 15 min xylene treatment. To block endogenous peroxidase, the slides were immersed for 30 min in 3% hydrogen peroxidase in methanol. Each specimen was rinsed 3 times for a total of 15 min in PBS, PH 7.5, with gentle stirring. They were then incubated overnight with the primary antibody at 4˚C. Next, the SAB procedure was performed. After washing in PBS, the sections were exposed for 5 min to tetrahydrochloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in 0.05 M Tris buffer, pH 7.6, containing 0.003% hydrogen peroxide. To facilitate the visualization of cytoplasmic immunostaining, the slides were counterstained with Mayer's hematoxylin. Control sections in which the primary antibody was omitted revealed a total lack of immunoreactivity. All the experiments including the controls were each performed twice on different days. One author (S.T.) who had no knowledge of either the pathological diagnosis or any of the clinical and radiological data determined the MIB-1 LI with % positive staining cells by counting at least 500 tumor cell nuclei.

Evaluation of $p27^{kip1}$ expression. The specimens were scored for nuclear and cytoplasmic staining separately, by two authors (H.S. and P.S.) who had no knowledge of either the pathological diagnosis or any clinical and radiological data. They counted at least 1,000 tumor cells per sample from randomly selected fields at ×400 magnification. p27 LI was determined with % positive staining cells separately by subcellular localization as cytoplasmic or nuclear.

Statistical analysis. Statistical analysis was performed with the Student's *t*-test using the Stat View™ software program for Windows, version 5.0 (Abacus Consepts Inc., Berkeley, CA, USA). Differences of $p<0.05$ were considered to be statistically significant. The survival time was recorded in days from the date of histological diagnosis at the time of surgery to the date of death or the last follow-up. Survival was plotted and the median survival time was estimated by the Kaplan-Meier method. Associations between the relative risk of death and each prognostic variable (cytoplasmic and nuclear p27 expression) were evaluated according to the Cox proportional hazards regression model for censored data after dummy code-transformation of the predictor. The survival curves were computed using the Kaplan-Meier product-limit method and then were compared using the log-rank test.

Results

Patient treatment and tumor pathology. Tumor specimens from 49 patients were analyzed. According to the WHO classification, 17 of the tumors (34.7%) were anaplastic astrocytomas and 32 (65.3%) were glioblastomas. All the patients had undergone a craniotomy and all received adjuvant radiation therapy (40-63 Gy in 2-Gy fractions) and many also received combined adjuvant chemotherapy. Out of the 49 patients enrolled in this study, 45 patients had received ACNU-based chemotherapy.

Subcellular localization of p27 and clinical characteristics of high-grade astrocytomas. Out of the 49 high-grade astrocytomas examined, positive nuclear expression of p27 was observed in 45 cases; the mean LI was 38.36% (range 0-95.11%). In addition, positive cytoplasmic expression was seen in 32 cases, and the mean LI was 4.62% (range 0-52.17%). Figure 1 shows representative samples of nuclear and

Table I. *Demographic, clinical and pathological data.*

		p27 Nuclear expression group			p27 Cytosolic expression group		
	Total	Low	High	*p*	Low	High	*p*
Number of patients	49	27	22		40	9	
Age (years)	52.6±16.4	52.3±16.0	53.0±17.2	0.9362	50.3±16.6	63.2±10.4	0.9761
Gender							
Female	19 (38.8%)	9 (33.3%)	10 (45.5%)	0.7733	16 (25.0%)	3 (33.3%)	0.3868
WHO grade							
AA	17 (34.7%)	11 (40.7%)	6 (27.3%)	0.5512	14 (35.0%)	3 (33.3%)	0.3772
MIB-I index (%)	17.1±11.6	17.6±11.6	16.5±11.8	0.6383	17.7±12.0	14.7±9.93	0.5092

Continuous values are the mean ± SD, and categorial values are the number of patients (percentage). To test the correlation between the two expression levels (low and high) in each subcellular localization (nuclear or cytoplasmic), Fisher's exact test was used to compared categorical variables and the Mann-Whitney test was used to compare continuous variables. AA: Anaplastic astrocytoma.

cytoplasmic expression of p27. The cytoplasmic p27 LI tended to be lower than the nuclear p27, as the scatter chart shows (Figure 2). The nuclear and cytoplasmic expressions of p27 were categorized into two groups, namely, high and low expression, using as the cut-off point the mean LI value of each. Thus, the high nuclear expression group was ≥40% (n=27) and the low nuclear expression group was <40% (n=22), while the high cytoplasmic expression group was ≥5% (n=9) and the low cytoplasmic expression group was <5% (n=40).

The 49 cases were also categorized into four expression groups according to both the nuclear and cytoplasmic expression level: a high nuclear/high cytoplasmic expression group (n=3); a high nuclear/low cytoplasmic expression group (n=19); a low nuclear/high cytoplasmic expression group (n=6) and a low nuclear/low cytoplasmic expression group (n=21).

Table I shows the distribution of the p27 localization and the characteristics for all 49 patients. No significant correlation was observed between low and high expression in each p27 group (nuclear or cytoplasmic) for any of the clinical factors (age, sex, WHO grade and MIB-1).

Kaplan-Meier survival analysis of the subcellular p27 expression in high-grade astrocytoma patients. Figure 3 shows the Kaplan-Meier survival curves according to the subcellular p27 expression (nuclear or cytoplasmic) indicating that the cytoplasmic p27 expression had a highly significant effect on the overall survival (p=0.0203). While a high nuclear p27 expression tended to indicate a better prognosis, no statistical significance (p=0.1180) was demonstrated for nucleus p27 expression.

To clarify the correlation between the nuclear and cytoplasmic p27 expression, Kaplan-Meier survival analysis of the four expression groups (high nuclear/high cytoplasmic, high nuclear/low cytoplasmic, low nuclear/high cytoplasmic and low nuclear/low cytoplasmic expression group) was assessed and a statistically significant difference was observed (p=0.0178), thus suggesting that a high nuclear/low cytoplasmic expression group was a better prognostic factor. This result was confirmed by Kaplan-Meier analysis of the high nuclear/low cytoplasmic group and the others, where a statistically significant difference was observed (p=0.0246) (Figure 4).

The effect of subcellular p27 expression on the prognosis in the high-grade astrocytoma patients. To determine which of the clinical variables (age, sex, MIB-1 index and a high nuclear/low cytoplasmic p27 expression) significantly helped to predict survival in high grade astrocytoma patients, Cox multiple regression analysis was applied (Table II). A highly significant difference was obtained for age (p=0.0346), sex (p=0.0261) and a high nuclear/low cytoplasmic p27 expression (p=0.0237).

Therefore, the combination of a high-nuclear expression and a low cytoplasmic expression of p27 was found to be a significant good predictor of the clinical survival in high-grade astrocytomas.

Discussion

In general, the cytoplasmic p27 expression tended to be lower than that in the nucleus, which was consistent with previous findings (15, 16). Although the mechanism responsible for restricting p27 to the cytosolic compartment is not known, it is interesting to note that the high nuclear and low cytoplasmic p27 expression in the high-grade astrocytomas did correlate closely with increased survival.

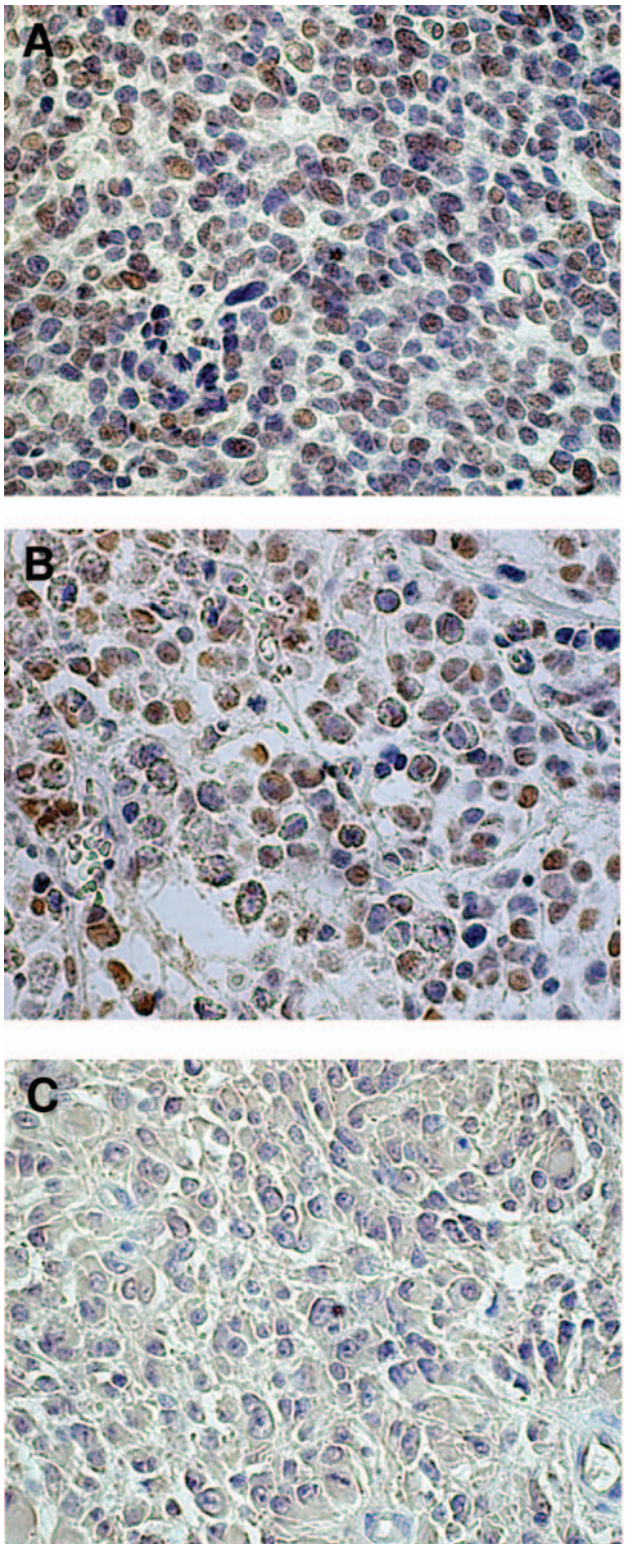

Figure 1. *Immunohistochemical staining for p27 expression in high-grade astrocytomas. A, p27 expressed only in the nucleus (original magnification ×400). B, p27 expressed in both the nucleus and cytoplasm (original magnification ×400). C, p27 not expressed (original magnification ×400).*

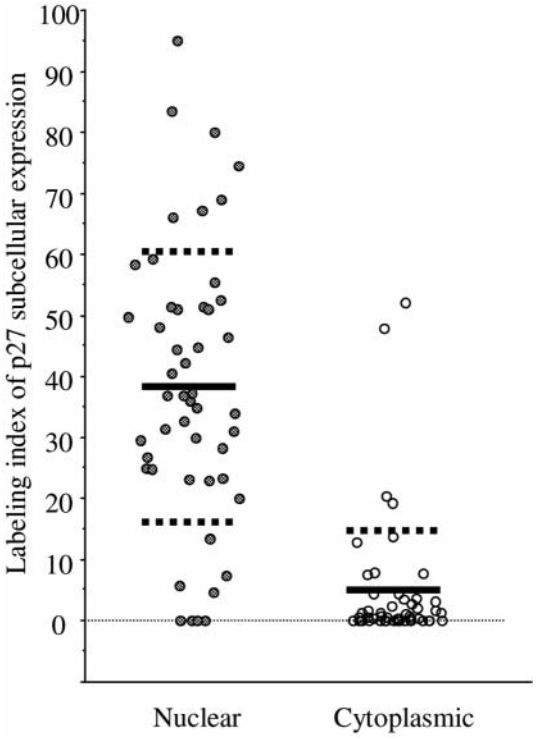

Figure 2. *Scatter diagrams showing differences in the labeling index of p27 between nuclear and cytoplasmic subcellular expression groups. Each circle represents a patient, and the line indicates the median.*

Some studies have indicated a loss of p27 nuclear expression to be associated with a worse prognosis in several carcinomas including lung cancer, colon cancer, gastric cancer, adenocarcinoma of the esophagus, hepatocellular carcinoma, breast cancer, prostate cancer, and thyroid cancer (17-19, 22-26). p27 is maximally expressed during the quiescent (G_0) and prereplicative (G_1) phases of the mammalian cell cycle, while it thereafter decreases as the cells enter the replicative phases (27, 28). p27 is highly expressed in cells belonging to the non-proliferative compartment of tissues and tumors (29) and is likely to play a role in the differentiation process (30). Concerning high-grade astrocytoma, several reports have demonstrated that the expression of p27 in the nucleus showed a reduction with malignancy, while a decrease in its expression was considered to reflect a poor prognosis (20, 31, 32).

The function of p27 is thought to be regulated by translocation between the nucleus and cytoplasm, and the cytoplasmic localization of p27 was associated with a poor prognosis in some carcinomas, including breast cancer, ovarian cancer and Barrett's-associated adenocarcinoma. The present report is the first to examine the effect of cytosol p27 on the survival of patients with high-grade astrocytoma, and to demonstrate that cytoplasmic p27 expression reflects a poor prognosis.

Table II. *Multivariate analysis of prognostic factors.*

	Total		
	β Coeff.	*p*-Value	RR (95% CI)
Age	0.026	0.0346	1.026 (1.002-1.051)
Gender: Female (%)	–0.861	0.0261	0.423 (0.198-0.903)
MIB-1 index (%)	0.001	0.9398	1.001 (0.968-1.036)
N>40 and C<5	0.865	0.0237	2.375 (1.122-5.026)
WHO grade: AA	–0.263	0.5726	0.769 (0.309-1.916)

N: Nuclear p27; C: cytoplasmic p27.

p27 is considered to be a tumor suppressor gene and the loss of its function has been associated with the development of many types of human cancer. The tumor suppressor function of p27 was first implicated in the context of cell cycle regulation (5). The cytoplasmic translocation of p27 has been increasingly recognized in primary human tumors to be associated with a poor survival, whereas nuclear expression confers a more favorable outcome (33, 34). The regulation of p27 degradation appears to be linked to phosphorylation of p27 by cyclin E/cdk2 (35-37) and its export from the nucleus (38). The cytoplasmic displacement of p27 may indicate a mechanism of p27 inactivation, which may thus be an alternative mechanism to proteasomal degradation (39). Recent studies have shown oncogenically activated kinase Akt/PKB to also be able to phosphorylate p27kip1 at T157, thus inducing its relocalization to the cytoplasm (40, 41). The Akt-mediated cytosolic accumulation of p27 is critical for Akt mitogenic signaling. The presence of cytoplasmic p27 (induced by phosphorylation at T157) has been shown to predict a poor prognosis in breast cancer (40, 41). The elucidation of pathways leading to p27 phosphorylation and degradation following mitogenic stimulation may provide mechanistic links between oncogene activation and cell cycle deregulation in carcinomas since sufficient evidence has accumulated to suggest that cytoplasmic relocalization of p27 might facilitate tumor development. p27 may act as a tumor suppressor or as an oncogene depending on its subcellular localization.

These observations indicate that the nuclear expression of p27 influences cell cycle regulation and a high expression level might lead to the growth inhibition of astrocytic tumor cells. On the other hand, the cytoplasmic expression of p27 might indicate an inactivated status of p27 and cooperation with the oncogenic process, *i.e.* Akt/PKB. Therefore, as demonstrated, a high nuclear and low cytoplasmic p27 expression would indicate a better prognosis among patients with high-grade astrocytoma.

This study was limited by the small number of patients, especially regarding those with a high cytoplasmic expression (n=6). Therefore, further studies should be performed with a larger number of participants.

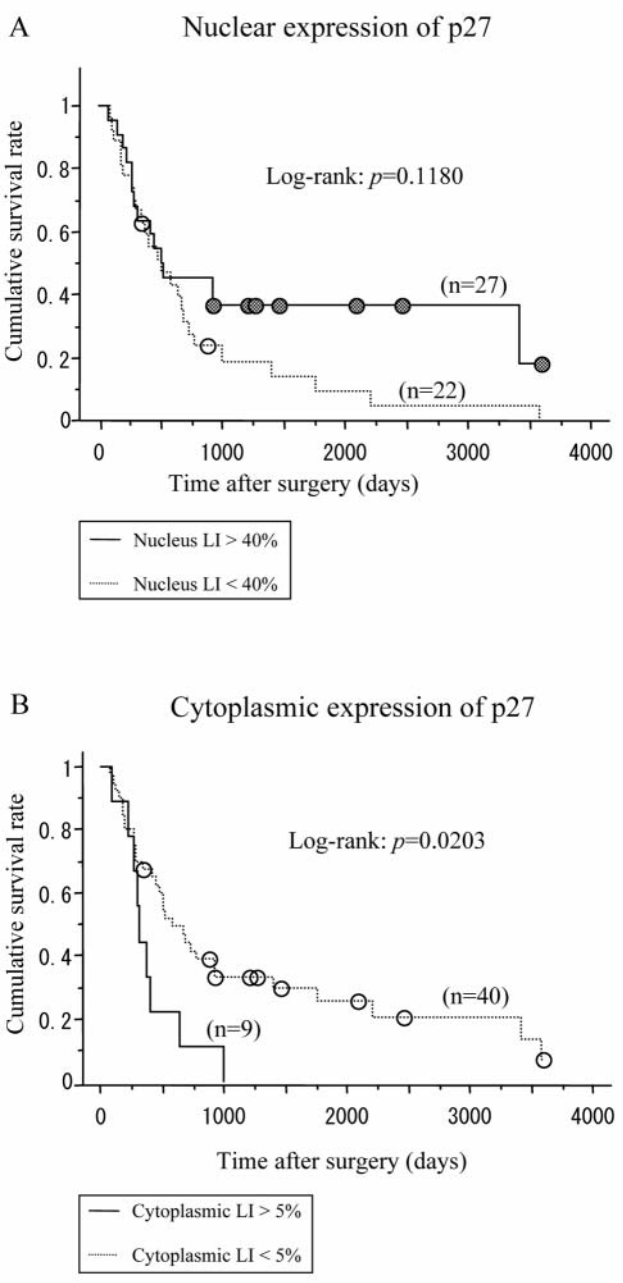

Figure 3. *The Kaplan-Meier curves of overall survival rates of high-grade astrocytoma patients with nuclear (A) or cytoplasmic (B) expression of p27. Log-rank test: Cytoplasmic LI <5% vs. >5% p=0.0203 and nuclear LI <40% vs. >40% p=0.1180.*

Conclusion

High nuclear and low cytoplasmic expression of p27 is associated with a better prognosis in high-grade astrocytoma patients, and the expression of cytosolic p27 could thus be useful for predicting the prognosis of high-grade astrocytoma patients. Moreover, such patients might be treated more

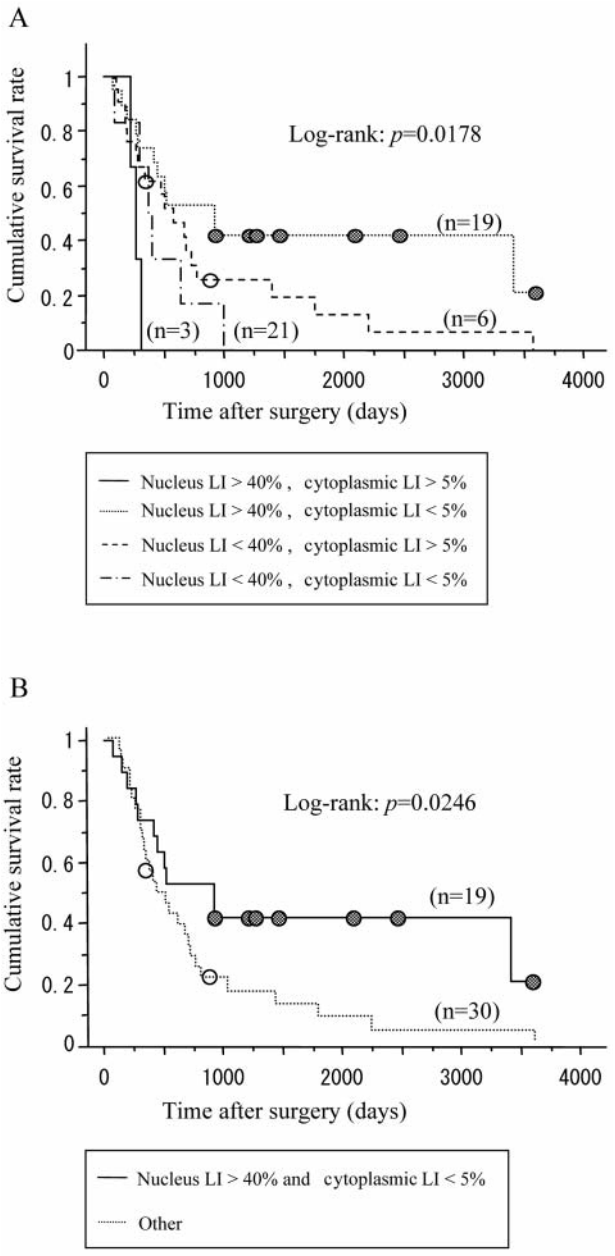

Figure 4. *Kaplan-Meier curves of overall survival rates between the four p27 expression localization groups (A) and between the high nuclear/low cytoplasmic expression group and the others (B).*

effectively by targeting p27 mislocalization; however, the mechanism responsible for p27 localization still needs to be more fully elucidated.

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