Expression of Cell Cycle Regulator p27Kip1 Is Correlated with Survival of Patients with Astrocytoma

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ABSTRACT

p27Kip1 is a cyclin-dependent kinase inhibitor that negatively regulates cell proliferation by mediating cell cycle arrest in G1. This study was undertaken to assess the prognostic value of p27Kip1 for astrocytomas. Tissue samples from 130 astrocytomas (WHO grade 1, 5 cases; grade 2, 23 cases; grade 3, 64 cases; grade 4, 38 cases), including 92 primary and 38 recurrent tumors, were examined immunohistochemically for Ki-67 and p27Kip1 expression. Patient charts were reviewed for clinical presentation, and survival was followed. The p27Kip1 labeling index (LI) ranged from 2.3 to 98.4%, with a mean value of 47.5% (± 23.4%). The p27Kip1 LI decreased with increasing tumor grade but did not correlate with other parameters. There was no correlation between Ki-67 LI and p27Kip1 LI. For patients with primary astrocytomas, the 50% survival times of those with low p27Kip1 LI (<50%) and those with high p27Kip1 LI (≥50%) were 17.1 and 69.6 months, respectively. For patients with high-grade tumors, the 50% survival times were 13.1 months for those with low p27Kip1 LI and 33.7 months for those with high LI. On multivariate analysis, p27Kip1 was one of the most significant prognostic factors, indicating that low p27Kip1 LI was associated with poor prognosis (primary, risk ratio = 2.5, P = 0.0023; high-grade, risk ratio = 2.2; P = 0.0139). The expression of p27Kip1 was inversely related to tumor grade and positively related to favorable outcome of patients with astrocytoma, suggesting that p27Kip1 may be a candidate for prognostic factor for this tumor.

INTRODUCTION

Recently, cell cycle regulators have been shown to be disrupted in human cancers (1). Cyclin and CDK3 complexes play an important role in controlling the cell cycle. The G1-S transition is regulated mainly by cyclin Ds, CDK2, and CDK4 (2). The CDK inhibitors inhibit the kinase activities of cyclin and CDK complexes and block transitions of the cell cycle. Two families of CDK inhibitors have been identified. One is the INK4 family, including p15/INK4B, p16/CDKN2/INK4A, p18, and p19, which binds cyclin D-dependent kinases, especially CDK4 and CDK6. The other is the kinase inhibitor protein family, including p21/Cip1, p27/Kip1, and p57/Kip2, which inhibit a variety of CDKs.

p27Kip1 is a CDK inhibitor that regulates progression from G1 into S phase by binding to and inhibiting the cyclin E-CDK complex (3). p27Kip1 is present in large amounts in quiescent cells, and its level declines when cells proliferate in response to mitogenic signals, such as growth factors and cytokines (4, 5). Although deletions and mutations of CDK inhibitors, such as those in p16, p15, and p53, occur in a variety of human malignancies (6, 7), those of the p27 gene are rarely observed in human tumors (8–11). Regulation of p27Kip1 occurs primarily at the posttranslational level by ubiquitin-mediated degradation (12). Therefore, changes in p27Kip1 protein level in tumor are common and may indicate tumor behavior (13). Decreased p27Kip1 protein may be a useful prognostic factor for breast (13–15), colon (16), gastric (17), and prostate cancers (18).

Astrocytomas are the most common primary intracranial neoplasms and are classified according to their histological characteristics of malignancy (19). Factors predicting the prognosis of patients are important for therapeutic planning. Some of these factors have already been reported and used for clinical patients, such as WHO grading, Ki-67 proliferation index, and p53 mutation, but they do not always reflect the prognosis or survival of patients. We are attempting to identify markers that are useful not only for representation of histological or biological malignancy but also for prediction of the prognosis of patients with these tumors. Therefore, we examined the expression of p27Kip1 in astrocytomas, the relationships between p27Kip1 expression and cellular proliferation, and survival of patients with astrocytoma.

MATERIALS AND METHODS

Patient Selection. Astrocytomas (n = 130) were obtained from patients who underwent surgery in Okayama University Hospital (Okayama, Japan) between January 1979 and December 1997. All tumors were graded according to WHO...
Table 1  Clinical characteristics of 130 patients with astrocytomaa

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Tumor grade, no. of cases (%)</th>
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<tr>
<td></td>
<td>Grade 1</td>
</tr>
<tr>
<td>No. of cases (%)</td>
<td>5 (3.8)</td>
</tr>
<tr>
<td>Primary</td>
<td>4 (4.3)</td>
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<tr>
<td>Recurrence</td>
<td>1 (2.6)</td>
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<tr>
<td>No. of males/no. of females</td>
<td>2/3</td>
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<tr>
<td>Age (yr)</td>
<td>Mean ± SD</td>
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<td></td>
<td>Range</td>
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<tr>
<td>Ki-67 LI (%)</td>
<td>Mean ± SD</td>
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<tr>
<td></td>
<td>Median</td>
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<td></td>
<td>Range</td>
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<tr>
<td>p27 LI (%)</td>
<td>Mean ± SD</td>
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<td></td>
<td>Median</td>
</tr>
<tr>
<td></td>
<td>Range</td>
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<tr>
<td>Survival of primary patients</td>
<td>No. alive/no. dead</td>
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<td></td>
<td>50% survival time (months)</td>
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*a Mean values are expressed as mean ± SD.

criteria (20–22). Ninety-two specimens were from primary tumors, and 38 were from recurrent tumors. All patients with primary tumor had received no prior therapy, such as chemotherapy or radiation therapy, before surgery. Most of the patients with recurrent tumor had been treated after the first surgery with radiation therapy and adjuvant chemotherapy, mainly composed of a nitrosourea compound and IFN, when the primary tumor was diagnosed to be high grades [grade 3 (anaplastic astrocytoma) and grade 4 (glioblastoma)]. The clinical course of patients was followed until death or the end of the year 1997. Immunohistochemistry. A total of 130 formalin-fixed and paraffin-embedded tumor tissue samples were available for this study. Expression of Ki-67 and p27Kip1 protein was immunohistochemically investigated. Antibodies used for this study were affinity-purified MM-1 (Novocastra Laboratories, Newcastle, United Kingdom), which is a Ki-67-specific monoclonal antibody, and anti-p27Kip1 monoclonal antibody (Novocastra Laboratories). MM-1 was used at a 1:100 dilution, and anti-p27Kip1 monoclonal antibody was used at a 1:40 dilution. Paraffin sections were cut at a 5-μm thickness, deparaffinized with xylene, rehydrated, and treated with 0.1 mol/liter citrate (pH 6.0) in a 500-W microwave oven for 15 min for antigen retrieval. Immunostaining was performed with an Elite avidin biotin peroxidase kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s specifications (6, 7). The sections were counterstained with hematoxylin. Normal human tonsil tissue was used for positive control of both Ki-67 and p27Kip1. Negative controls were produced by substituting normal mouse serum for the primary antibodies.

Scoring of Tumors Cells. The LIs of Ki-67 and p27Kip1 were calculated microscopically field at a magnification of ×400 by counting at least 500 tumor cells from selected fields in accordance with WHO grading of photomicrographs. Strong and diffuse reactions in the nuclei were considered positive for both Ki-67 and p27Kip1. Positive reactions only in the cytoplasm or doubtful staining in the nuclei were omitted. Results were expressed as mean ± SD. Prior studies have shown good correlation between p27Kip1 LIs and p27Kip1 levels, as determined by Western blot (23).

Statistical Methods. The Mann-Whitney U test and the Kruskal-Wallis test were used to compare the distributions of clinicopathological characteristics among the subsets of the patients. Nonparametric Spearman rank correlation coefficients were used to assess the degree of linear association between Ki-67 LI and p27Kip1 LI. Survival rates were estimated with Kaplan-Meier (24) curves, and those of the patient subsets were compared with the log-rank test (25). To assess the correlation of survival time with multiple clinicopathological characteristics, multivariate analyses were performed using Cox proportional hazards models (26).

RESULTS

Patient Characteristics. The characteristics of the 130 patients included in this study are shown in Table 1. There were 64 males (49.2%) and 66 females (50.8%), with an overall mean age of 39.9 years (range, 0–84 years). Five tumors (3.8%) were classified as WHO grade 1, 23 (17.7%) were classified as grade 2, 64 (49.2%) were classified as grade 3, and 38 (29.2%) were classified as grade 4. Ninety-two (70.8%) were primary tumors, and 38 (29.2%) were recurrent. Fifty-eight (63.0%) patients with primary tumor underwent total or subtotal tumor resection. By the end of the year 1997, 56 patients with primary tumor (60.9%) had died, and the median duration of follow-up review was 5.1 years for the 36 surviving patients.
Ki-67 and p27 Expression and LIs. Table 1 provides a summary of the LIs of Ki-67 and p27 Kip1 for the group of all patients and the subsets of the patients differentiated by WHO grading. Ki-67 and p27 Kip1 immunoreactivity was characterized by dark brown reaction product confined to the nuclei of tumor cells (Fig. 1). Ki-67 and p27Kip1 were statistically analyzed for categories of sex (male/female), age (<40 years old/≥40 years old), and tumor grade (grades 1 and 2/grade 3/grade 4). All of the tumor specimens were positive for Ki-67, and the LI ranged from 0.5 to 40.0%, with the mean value of 12.6 ± 9.4% and a median value of 10.5%. There was no statistically significant relationship between Ki-67 LI and sex (P = 0.2738), as shown in Fig. 2A. The Ki-67 LI increased significantly with tumor grade (P < 0.0001; Kruskal-Wallis test), as reported previously, and age (P = 0.0076), as shown in Fig. 2, B and C. All of the tumors were also positive for p27 Kip1, although expression of p27 Kip1 was quite variable. p27 Kip1 LI ranged from 2.3 to 98.4%, with a mean value of 47.5 ± 23.4% and a median value of 50.7%. Fig. 2, A and B, show that the p27 Kip1 LI was not significantly correlated with either patient sex (P = 0.7799) or age (P = 0.1609). Fig. 2C shows that p27 Kip1 LI tended to decrease with tumor grade (P = 0.0309). We also analyzed the relationship between Ki-67 and p27 LIs. There was not a significant correlation between these parameters, not only for the group of all patients (P = 0.1246) but for each grade of tumor as well.

Correlation of Variables with Survival. The correlation between survival and Ki-67 or p27 Kip1 was analyzed for all 92 patients with primary tumor, including 4 (4.3%) with grade 1, 18 (19.6%) with grade 2, 39 (42.4%) with grade 3, and 31 (33.7%) with grade 4 tumors. For the 70 patients with high-grade tumors, the correlations between survival and Ki-67 and p27 Kip1 LIs were also analyzed. The patients were divided into low (<50%, 43 patients) and high (≥50%, 49 patients) p27 Kip1 groups. Low (<10%, 44 patients) and high (≥10%, 48 patients) Ki-67 groups were also defined. Survival data shown in Table 2 and Fig. 3 display the Kaplan-Meier survival curves for groups with high and low Ki-67 and p27 Kip1 LIs.

Group of All Patients with Primary Astrocytoma. Fifty-six patients (60.9%) with primary astrocytoma were dead and 36 (39.1%) patients were alive at the end of 1997. The 50% survival time was 31.3 months. The 1-, 2-, and 5-year survival rates were 73.8, 54.9, and 35.0%, respectively. Fig. 3A displays Kaplan-Meier survival curves for the group of all patients with primary astrocytoma, comparing patients with low Ki-67 LI (n = 44) with those with high LI (n = 48). The 50% survival times for the low and high Ki-67 groups were 102.4 and 21.6 months, respectively. The 1-, 2-, and 5-year survival rates for the low Ki-67 group were 77.3, 65.9, and 54.7%, respectively, and for the high Ki-67 group, the rates were 70.4, 43.1 and 14.4%, respectively. These findings indicate that the mortality risk of patients was significantly correlated with increase in Ki-67 expression (P = 0.0004; log-rank). Fig. 3B shows the Kaplan-Meier survival curves, comparing the patients with low p27 Kip1 LI (n = 43) with those with high LI (n = 49). The 50% survival times for the...
patients with low p27Kip1 LI and those with high LI were 17.1 and 69.6 months. The 1-, 2-, and 5-year survival rates for the low LI group were 58.2, 33.8, and 14.7%, and those for the high LI group were 87.2, 72.3, and 51.9%, respectively. These findings also indicate that p27Kip1 expression was strongly correlated with survival of patients with primary astrocytoma ($P < 0.0001$). Multivariate analysis identified low expression of p27Kip1 as a novel independent prognostic factor for survival time (relative risk = 2.5, $P = 0.0023$; Table 2), as well as Ki-67 ($P = 0.0240$).

### Patients with High-Grade Astrocytoma

Because the patients with low-grade astrocytoma had low Ki-67 LI, high p27Kip1 LI, and good prognosis in this study, we focused on high-grade astrocytomas (grades 3 and 4). Of 70 patients with primary high-grade astrocytoma, 50 (71.4%) were dead and 20 (28.6%) were alive. The 50% survival time was 20.8 months, and the 1-, 2-, and 5-year survival rates were 65.0, 42.5, and 17.9%, respectively. Fig. 3C shows Kaplan-Meier survival curves, comparing the patients with low Ki-67 LI ($n = 27$) with those with high LI ($n = 43$). The 50% survival times for the

### Table 2 Univariate and multivariate analyses of prognostic factors for all patients with primary astrocytoma and those with high-grade astrocytoma

<table>
<thead>
<tr>
<th>Prognostic factors</th>
<th>No. of cases (%)</th>
<th>Log-rank ($P$)</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$P$</td>
<td>$RR^2$ (CI)</td>
</tr>
<tr>
<td>Overall</td>
<td>92 (100)</td>
<td></td>
<td>0.3420$^c$</td>
<td>0.3433</td>
</tr>
<tr>
<td>Sex (female)</td>
<td>49 (53.3)</td>
<td>0.0001$^c$</td>
<td>&lt;0.0001$^c$</td>
<td>$&lt;0.0001^c$</td>
</tr>
<tr>
<td>Age ($\geq 40$ yr)</td>
<td>51 (55.4)</td>
<td>0.0001$^c$</td>
<td>0.0001$^c$</td>
<td>10.2 (3.1–33.8)</td>
</tr>
<tr>
<td>WHO (grades 3 and 4)</td>
<td>70 (76.1)</td>
<td>&lt;0.0001$^c$</td>
<td>0.0001$^c$</td>
<td>3.1 (1.7–5.8)</td>
</tr>
<tr>
<td>Radiotherapy (performed)</td>
<td>58 (63.0)</td>
<td>0.0001$^c$</td>
<td>0.0001$^c$</td>
<td>2.1 (1.7–7.5)</td>
</tr>
<tr>
<td>Chemotherapy (performed)</td>
<td>59 (64.1)</td>
<td>0.0001$^c$</td>
<td>0.0001$^c$</td>
<td>2.0 (1.1–3.5)</td>
</tr>
</tbody>
</table>

$^a$ Cox regression model used to evaluate multivariate predictive value of prognostic factors; results are shown as $P$ (regression coefficient $\pm$ SE).

$^b$ RR, relative risk, determined by Cox regression model; CI, 95% confidence interval (in parentheses).

$^c$ P < 0.01.

$^d$ P < 0.05.
patients with low Ki-67 LI and high LI were 33.7 months and 20.3 months. The 1-, 2-, and 5-year survival rates for the patients with low Ki-67 LI were 63.0, 51.9, and 34.7%, respectively, and those for the high LI group were 66.6, 35.2, and 7.8%. These findings indicate that the increased mortality risk was associated with increase in Ki-67 expression ($P = 0.0459$; log-rank) but the increase was not highly significant, as was the case for the group of all patients. Fig. 3 shows Kaplan-Meier survival curves, comparing the low p27 LI ($\leq 10\%$) group ($n = 39$) with the high p27 LI group ($n = 31$). The 50% survival

\[ \text{Fig. 3 Kaplan-Meier survival curves show the association of survival with expression of Ki-67 and p27 for the group of all patients with primary astrocytomas (A and B), those with primary high-grade astrocytomas (C and D), and those with primary grades 3 and 4 astrocytomas (E and F), comparing patients with $\leq 10\%$ Ki-67 LI (low level) to those with $\geq 10\%$ Ki-67 LI (high level) and patients with $\leq 50\%$ p27 LI (low level) to those with $\geq 50\%$ p27 LI (high level). A, Ki-67 for all patients; B, p27 for all patients; C, Ki-67 in high-grade group; D, p27 in high-grade group; E, p27 in the grade 3 group; F, p27 in the grade 4 group.} \]
times for patients with low p27Kip1 LI and high LI were 13.1 and 33.7 months. The 1-, 2-, and 5-year survival rates for the low LI group were 53.6, 25.8, and 11.1%, and those for the high LI group were 79.3, 62.1, and 28.4%, respectively. Moreover, we considered whether p27 expression was prognostically significant within an individual (grades 3 and 4) malignancy group. In grade 3 group (n = 39), the 50% survival times for patients with low p27Kip1 LI and high LI were 16.5 and 50.5 months (Fig. 3E). In the grade 4 group (n = 31), the 50% survival times for patients with low p27Kip1 LI and high LI were 10.3 and 24.9 months (Fig. 3F). These findings indicate that the correlation between p27Kip1 expression and survival was highly significant, although our survey was focused on patients with high-grade astrocytoma (P = 0.0042; log-rank). Multivariate analysis of high-grade astrocytomas (n = 70) identified low expression of p27Kip1 as a novel independent prognostic factor for survival time (relative risk = 2.2, P = 0.0139; Table 2); however, Ki-67 was not an independent prognostic factor for this patient group (P = 0.2940).

**DISCUSSION**

The aim of this investigation was to identify a biological marker that is potentially useful in classifying astrocytomas by morphological malignancy and in predicting the prognosis of patients with astrocytomas. Recently, the incorporation of bromodeoxyuridine (27) and immunohistochemical reaction into monoclonal antibody Ki-67 (28) has yielded reliable markers for histological and clinical malignancy (29, 30), but there are still some discrepancies between the immunoreactivity of Ki-67 and prognosis (31–33). p27Kip1 is a CKI inhibitor that negatively regulates cell proliferation by mediating cell cycle arrest in G1 (3, 12). Functional activities of cell cycle regulators are decreased by various mechanisms, such as point mutation in p53 and homozygous deletion in or methylation of p16INK4A, as we reported previously (6, 7, 34). Although disruption of p27Kip1 in mice leads to multiple organ hyperplasia and pituitary tumors (35–37), the mRNA levels remain constant, whereas p27Kip1 protein level increases, suggesting that p27Kip1 is primarily posttranslationally regulated (38). The loss of p27Kip1 appears to occur through accelerated degradation by the ubiquitin-proteosome pathway (14, 16, 23). Clinically, a relationship between low p27Kip1 expression and aggressive behavior has recently been demonstrated for various malignancies. Decreased p27Kip1 protein has potential importance as a prognostic factor in breast (13–15), colon (16), and gastric carcinomas (17) and prostate adenocarcinoma (18), and a decrease in p27Kip1 level may be associated with tumor progression.

Here, we examined the expression of p27Kip1 and the proliferation marker Ki-67 antigen in paraffin sections of 130 histologically verified cerebral astrocytomas. All astrocytomas examined expressed Ki-67 antigen and p27Kip1 to various degrees. No relationship was observed between expression of p27Kip1 and sex or age of patients. Decrease of p27Kip1 LI tended to correlate with increase in the histological grade of malignancy. p27Kip1 expression was significantly correlated with good prognosis, and p27Kip1 LI remained a significant independent predictor of survival in multivariate analysis for the group of all primary astrocytomas and for high-grade astrocytomas.

Piva et al. (39) reported an inverse relationship between MIB-1 (antibody for Ki-67) LI and p27Kip1 LI counted in the same areas of 50 gliomas, and they reported that absence of p27Kip1 was independent of all histological features of differentiation or anaplasia in malignant gliomas. However, they did not determine the correlation between p27Kip1 LI and survival of patients with gliomas. Compared with low-grade astrocytomas, high-grade astrocytomas are more aggressive and have a poorer prognosis. Therefore, we performed survival analysis for the group of all patients and for the high-grade group. On Kaplan-Meier analysis, level of expression of p27Kip1 was significantly associated with survival time for both groups. These findings suggested that p27Kip1 is an important prognostic factor for astrocytoma, as for some other malignancies (13–18).

Many analyses have been reported for prediction of prognosis for patients with astrocytomas. Increased Ki-67 immunoreactivity, usually associated with increase in the grade of astrocytomas, has rarely been significantly related to poorer patient survival (29, 40, 41). However, several reports have suggested that Ki-67 LI is inversely related to survival on univariate analysis (31, 33). Using multivariate analysis, Sallinen et al. (31) reported that Ki-67 was a better predictor of prognosis than histological grade, although Cunningham et al. (33) insisted that patient age and tumor grade remained the most significant independent predictors of survival, as has been described by others (19, 42). Here, Ki-67, WHO grade, and p27Kip1 were related to survival and were considered candidates for prognostic factors.

In conclusion, p27Kip1 is a novel prognostic indicator, the expression of which is related to better outcome for patients with primary astrocytoma. In particular, p27Kip1 was one of the most significant predictors of survival of patients with high-grade astrocytoma.

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