Laboratory Study

Antigen p57/Kip2 as a potential negative regulator of human astrocytoma growth

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Abstract

This study was performed to determine the relationship between p57/Kip2 and the growth of human astrocytomas. Immunohistochemical staining for p57/Kip2, p53, p16, and Ki67 antigen was performed on paraffin-embedded tissue specimens obtained from 36 patients with astrocytoma. Expression of p57/Kip2, p53, p16, and Ki67 antigen was generally increased in association with the astrocytoma tumor grade. Expression of p16 was higher in patients whose tumors express p57/Kip2 in greater than 10% of tumor cells ($p < 0.05$). Expression of p53 also tended to be higher, but not to a statistically significant extent, in patients whose tumors express p57/Kip2 in greater than 10% of tumor cells. These findings suggest that p57/Kip2 inhibits the growth of human astrocytomas, and may function in parallel with p16 and p53. However, p57/Kip2 is, by itself, insufficient to arrest the cellular proliferation of human astrocytomas.

Keywords: Astrocytoma; Cell proliferation; p16; p57/Kip2

1. Introduction

Astrocytoma is the most common human primary brain tumor. Despite many efforts to effectively treat astrocytomas, malignant astrocytoma remains most incurable. As a result, many pathways have been targeted in an effort to find a treatment for astrocytoma and to improve overall patient survival. Although some treatments have been successful *in vitro*, *in vivo* experimentation has not yielded the same results.

We showed that p57/Kip2 inhibited cell proliferation, motility and invasion of astrocytoma cell lines *in vitro*.[1,2] Ectopic expression of p57/Kip2 reduced cell proliferation and induced changes in cell morphology.[1] The induced expression of p57/KIP2 in the U87 and U373 astrocytoma cell lines significantly reduced cell motility and invasion.[2]

In this study, we examined surgical specimens of astrocytoma to determine the relationships between p57/Kip2 and other cell cycle factors such as p53, p16, and Ki67 antigen in an effort to predict patient outcome after treatment. This is the first report on p57/Kip2 expression in clinical astrocytoma specimens.

2. Materials and methods

2.1. Tissue specimens and patients

Fifty patients with brain tumors were studied. All had undergone surgery at Shinshu University Hospital, Matsumoto, Japan, between 1997 and 2000. The present study focused on astrocytoma; eight patients with ependymoma, one with subependymoma,

and two with oligodendroastrocytoma were excluded from further analysis. Small specimens such as those received following stereotaxic biopsy were also excluded. Finally, specimens were removed from 36 patients with astrocytoma and examined using immunohistochemical staining with antibodies to p57/Kip2, p53, p16, and Ki67 antigens. There were 23 men and 13 women, with a mean age (± standard deviation, SD) of 43.7 ± 21.4 years (range 1–72 years) (men: mean 39.2 ± 22.1, range 1–71 years; women: mean 51.5 ± 18.5, range 6–72 years) at the time of surgery.

2.2. Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue specimens were cut and deparaffinized using xylene and ethanol. The sections were immersed in 1% citric acid buffer (pH 6.0) and microwave-irradiated at 500 W for 25 minutes. Background staining was blocked using 1% normal goat serum in phosphate buffered saline (PBS) for 15 minutes at room temperature.

The antibodies used for p57/Kip2, p53, p16, and Ki67 antigen (NeoMarkers, Westinghouse, CA, USA) were labeled with horseradish peroxidase. Antibodies were diluted 1:100 in 1% bovine serum albumin in PBS. These primary antibodies were allowed to react for 1 hour at room temperature, followed by three washes in PBS for 10 minutes each. Counterstaining was then performed with hematoxylin and eosin. The stained specimens were dehydrated, and mounted with coverslips. Specimens were observed using a microscope (Olympus, Tokyo, Japan).

Staining was considered positive when the nucleus and/or cytoplasm of tumor cells was clearly stained. Specimens were then scanned at low magnification (from × 40 to × 100) to locate areas of maximal staining intensity. Positively stained tumor cells were

counted under high magnification ($\times 400$). At least 200 cells were counted in each specimen and at least five different microscopic fields were counted. The values were then averaged to obtain a count for the entire specimen.

2.3 Statistical analysis

A one-way analysis of variance was used to compare two independent groups. A *p* values < 0.05 was considered significant.

3. Results

3.1. Patient characteristics

The total number of patients was 36, all of whom had primary astrocytoma. No patient had undergone chemotherapy or irradiation before surgery. All patients with high-grade tumors were followed with adjuvant therapy after surgery.

Two patients, whose average age was 6, had grade 1 disease. Both had pilocytic astrocytoma. Interestingly, negative cell-cycle regulator antigens such as p57/Kip2 and p16 were not expressed in their grade 1 tumors.

Six patients, with an average age of 39.8 years, had grade 2 tumors. Two of them had fibrillary astrocytoma and four had gemistocytic tumors.

Seventeen patients, with an average age of 52.8 years, had grade 3 tumors, including two patients with primitive neuroectodermal tumor and 15 with anaplastic astrocytoma.

Eleven patients, with an average age of 52.8 years, had grade 4 tumors; all had glioblastoma.

Staining for p57/Kip2 was identified in both the nucleus and cytoplasm, while other cell-cycle related molecules were confined to the nucleus. Representative cases with mild, moderate, and high percentages of cells expressing of p57/Kip2 are presented in Figure 1.

3.2. Astrocytoma grade and labeling index

In general, the labeling indices of p57/Kip2, p53, p16, and Ki67 were higher in high-grade astrocytomas, although not to a significant extent (Fig. 2). The labeling index of Ki67 antigen was higher in high-grade (grades 3 and 4) than low-grade astrocytomas (grades 1 and 2) ($p < 0.05$). Other labeling indices for antigens including p16, p57/Kip2, and p53 tended to be higher in high-grade rather than than low-grade astrocytomas (Fig. 3).

3.3. Relationships between p57/Kip2 expression and expression of the other cell cycle factors p16, p53, and Ki67 antigen

In those patients who expressed p57/Kip2 in $\geq 10.0\%$ of tumor cells, expression of p16 was higher than in patients whose tumors expressed p57/Kip2 in $< 10.0\%$ of tumor cells ($p < 0.05$) (Fig. 4). The labeling index of p53 expression tended to be higher in patients whose tumors express p57/Kip2 in $> 10.0\%$ of tumor cells (($p > 0.05$) (Fig. 4). In the group where $\geq 10.0\%$ of tumor cells express p57/Kip2, the labeling index for Ki67 tended to be lower ($p > 0.05$) (Fig. 4).

3.4. Relationship between incidence of p57/Kip2 and patient survival

The group with high-grade astrocytomas was followed with adjuvant therapy (chemotherapy and/or irradiation). In this group, no significant relationships were observed among age, sex, mean duration of survival, and p57/Kip2 staining (data not shown).

4. Discussion

To improve the prognosis of patients with malignant astrocytoma, regulators of cell cycle control (including cyclins, cyclin-dependent kinases, pRB, and cyclin-dependent kinase inhibitors) have been extensively studied over the past two decades.[3–8] In particular, expression of p57/Kip2 has been reported to inhibit the growth of astrocytoma cells and result in a reduction of tumor size.[1,2] In other neoplasms, p57/Kip2 has also been shown to negatively affect cell-cycle progression *in vivo*.[9–23]

Our study shows, for the first time, the expression of p57/Kip2 in human astrocytoma specimens. Our findings suggest that an inverse correlation exists between expression of p57/Kip2 and Ki67 antigen. Recently, Martha et al. reported that loss of p57/Kip2 expression was dependent upon expression of p53 inhibitor.[24] In addition, our study also suggested a positive correlation between p57/Kip2 and p16 expression, though no clear correlation between p57/Kip2 and p53 expression was found. Taken together, these findings suggest that p57/Kip2 may be one of the cell-cycle family members that negatively regulates the growth of astrocytomas, in parallel with p16 expression.

We previously showed that p57/Kip2 inhibited cell proliferation of astrocytomas *in*

vitro.[1] However, it is also true that factors other than p57/Kip2 control the cell cycle and proliferation of astrocytoma cells. For example, in malignant primary intestinal large B-cell lymphoma, expression of p57/Kip2 was more strongly suppressed than in its benign counterpart, reactive lymphoid hyperplasia.[12] As shown in the present study, low-grade astrocytoma less frequently expressed p57/Kip2, whereas some glioblastomas also expressed p57/Kip2 in the cytoplasm and nucleus. These findings suggest that astrocytoma cells may have multiple pathways working in concert to control the cell cycle. This may also help to explain why it is difficult to control cell proliferation in astrocytomas by the targeting of just one cell-cycle regulator.

It appears that p57/Kip2 functions cooperatively with p16 and probably other cell-cycle regulators as well as modulating the growth of human astrocytomas. This observation can now be tested in an experimental system to determine whether targeting of multiple members of the cell-cycle control pathway will more strongly inhibit cell growth than targeting of a single factor.

Acknowledgement

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Figure legends

Fig. 1. Immunohistochemical staining of astrocytoma tissue showing positive staining for p57/Kip2, p53, p16, and Ki67 antigen. Upper, middle, and lower rows of panels indicate astrocytomas of grades 2, 3, and 4, respectively. Antigen p57/Kip2 was detected in both the nucleus and cytoplasm. (Scale bar = 100 μm; HE = hematoxylin and eosin staining).

Fig. 2. Incidences (percentages) of tumor cells expressing p57/Kip2, p53, p16, and Ki67 antigen in astrocytomas showing that tumors of a higher grade tended to have higher labeling indices.

Fig. 3. Relationships between tumor grade (high-grade [grade 3 or 4] or low-grade [grade 1 or 2]) and labeling indices. Expression of Ki67 antigen was significantly higher

in high-grade than in low-grade astrocytomas ($p < 0.05$). Expression of other cell-cycle related molecules tended to be higher in high-grade astrocytomas.

Fig. 4. Relationships between p57/Kip2 expression and expression of p16, p53, and Ki67 antigen. In the group expressing p57/Kip2 in ≥ 10.0% of tumor cells, the labeling index for p16 was higher ($p <0.05$). The labeling index for p53 tended to be higher in the group expressing p57/Kip2 in ≥ 10.0% of tumor cells. By contrast, in the group expressing p57/Kip2 ≥ 10.0% of tumor cells, labeling index for Ki67 tended to be lower.
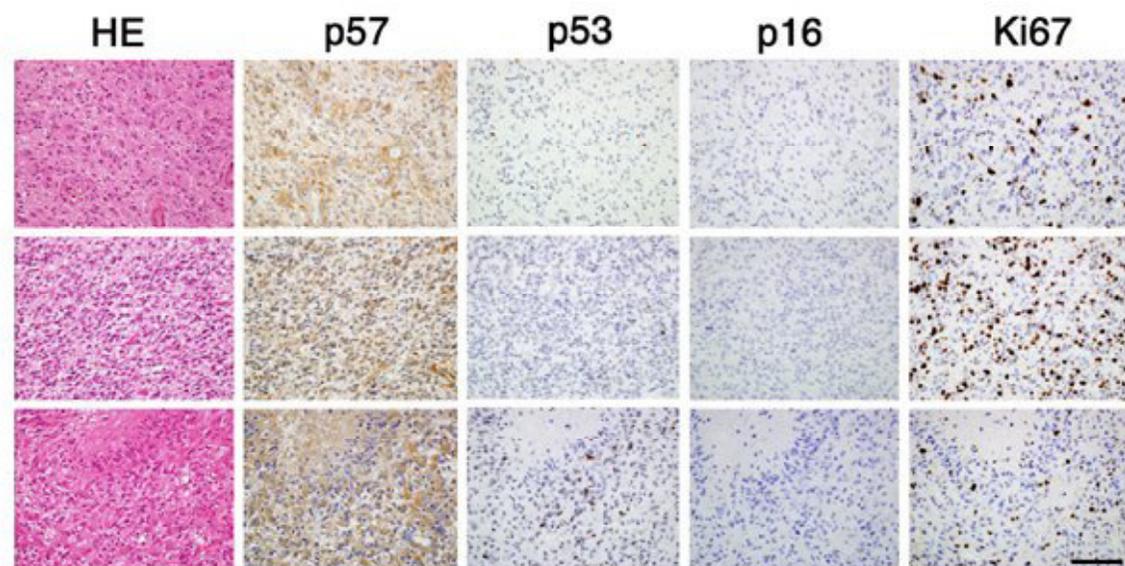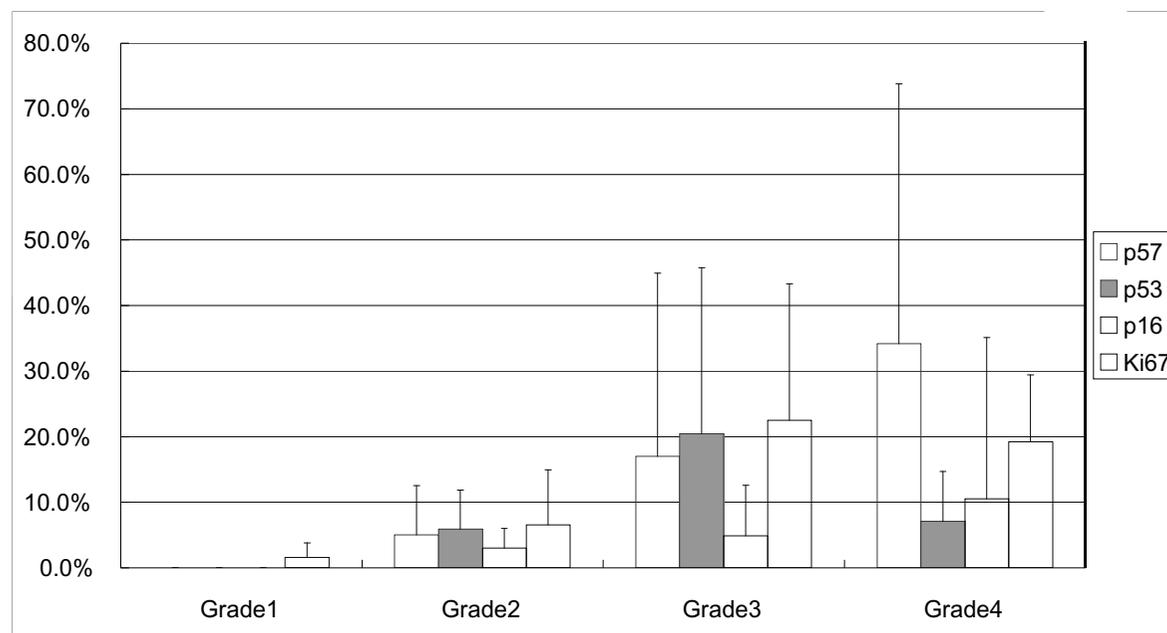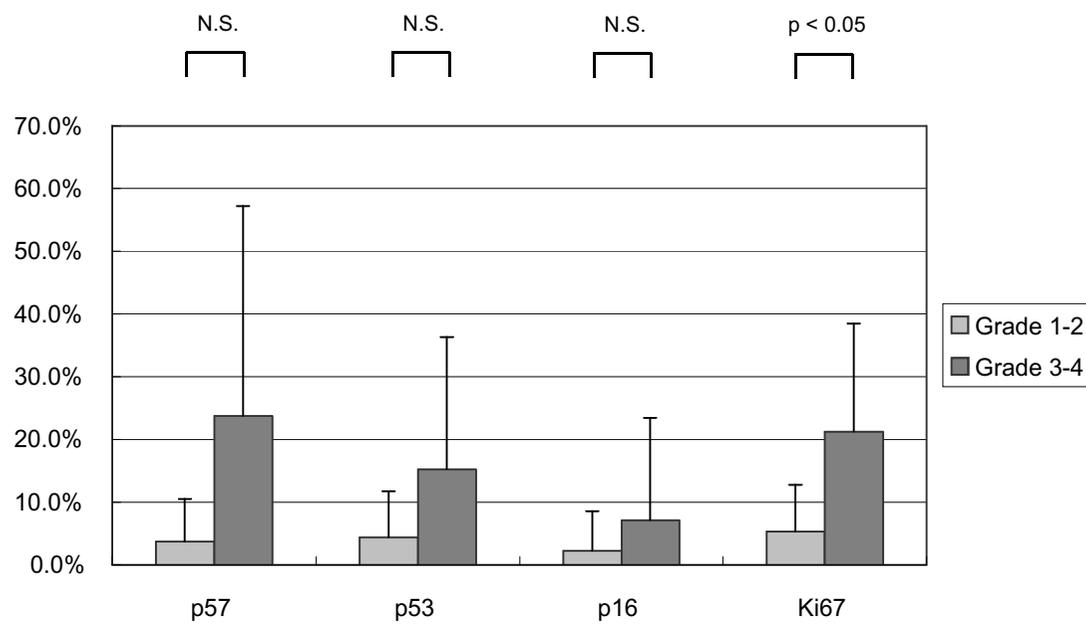
Figure 1

Figure 1

| HE | p57 | p53 | p16 | Ki67 |

Figure 2

Figure 2

Figure 3

Figure 3

Figure 4

Figure 4