ORIGINAL ARTICLE

Prognostic significance of CDC25B expression in gliomas

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Background: CDC25B is a cell-cycle regulatory protein, which is considered to be related to tumorigenesis and progression of tumours.

Aims: To elucidate the role of CDC25B in glioma, the expression of CDC25B and the association of the CDC25B expression with the clinicopathological parameters were investigated.

Methods: Fifty seven gliomas, which included 21 low-grade astrocytomas, 17 anaplastic astrocytomas and 19 glioblastomas, were studied. Protein expressions of CDC25B were evaluated by immunohistochemical methods. Semiquantitative and real-time RT-PCR analyses for the expression of CDC25B mRNA were also carried out. Disease-free survival (DFS) data were analysed by using the Kaplan–Meier method.

Results: High expression of CDC25B was identified in 18 of the 19 glioblastomas, in 10 of the 17 anaplastic astrocytomas, but not in any of the 21 low-grade astrocytomas. The CDC25B mRNA expression increased with the rise in histological grade. Increased CDC25B expression was correlated significantly with a shorter period of DFS, as shown by multivariate analysis.

Conclusions: Patients with an unfavourable clinical outcome are characterised by the increased expression of CDC25B in their glioma samples. Useful clinical information, especially on its relevance as a prognostic indicator, is provided by the evaluation of CDC25B expression in gliomas.

Disorders of cell cycle regulation are reported to be deeply associated with tumorigenesis and progression of tumours. The cell cycle is a complex process with which many molecules are associated. The cell cycle regulatory protein, CDC25, has a critical role in the growth and arrest of the cell cycle. CDC25 is a family of protein phosphatase, which dephosphorylates and activates cyclin-dependent kinases. Three members of the CDC25 gene family have been identified in human cells. CDC25B promotes G₂/M transition by activating the CDC2/cyclin B complex, and its overexpression has been detected in various tumours. CDC25B expression in clinical samples from patients with glioma, however, has not been reported.

In this study, we investigated CDC25B expression in gliomas by using clinical samples and correlated the results with the clinicopathological parameters. Statistical analysis was also carried out to determine the prognostic effect of the CDC25B expression in gliomas on various clinical parameters.

METHODS

Patients and samples

A total of 57 gliomas of the cerebral hemisphere, resected between 1995 and 1998 at Osaka City University, Osaka, Japan, were studied. These tumours were from 40 men and 17 women, ranging in age from 21 to 83 (mean, 44.9) years at diagnosis. The tumours were classified according to the World Health Organization (WHO) criteria.¹ This series consisted of 21 low-grade astrocytomas (WHO grade II), 17 anaplastic astrocytomas (WHO grade III) and 19 glioblastomas (WHO grade IV). All patients underwent surgical intervention, with the maximum safe resection of the tumour. In most cases (53/57; 93%), the surgery was described as complete macroscopic or almost complete macroscopic resection of the tumour. In four cases (two patients with anaplastic astrocytoma and two patients with glioblastoma), surgery consisted of the subtotal removal of the tumour because of tumour invasion to vital structures. In anaplastic astrocytomas and glioblastomas, postoperative radiotherapy (60 Gy in standard) and adjuvant chemotherapy with interferon- β and advanced combination chemotherapy using nimustine hydrochloride were routinely added. In low-grade astrocytomas, only radiotherapy (40 Gy in standard) was

added. Tumour recurrence was seen in 35 cases (1 case of low-grade astrocytoma, 15 cases of anaplastic astrocytoma and all cases of glioblastoma). Evidence of recurrence was based on radiological findings.

Small parts of non-necrotic and non-haemorrhagic tumour tissues were snap frozen immediately in the operating room and stored in a deep freezer at -80°C before RNA isolation, and the rest of the tumour tissues were fixed in 10% buffered formalin and embedded in paraffin wax.

Immunohistochemical studies

CDC25B protein expression was evaluated by immunohistochemical methods. Immunostaining for CDC25B was carried out using the Dako EnVision kit (Dako, Copenhagen, Denmark). Four- μm paraffin wax sections were deparaffinised and rehydrated, and endogenous peroxidase was blocked by 0.3% hydrogen peroxidase in methanol. The sections were autoclaved for antigen retrieval (115°C , 10 min) and then incubated with 10% normal goat or rabbit serum for 20 min at room temperature. The sections were incubated for 1 h with the CDC25B monoclonal antibody (diluted 1:200; Transduction Laboratories, Lexington, Kentucky, USA). They were then incubated with the peroxidase-labelled polymer for 1 h. Finally, they were incubated with diaminobenzidine tetrahydrochloride, and the nuclei were counterstained with haematoxylin. The sections were examined by light microscopy at high-power magnification.

For the evaluation of the proliferative potential of tumours, the immunostaining for Ki-67 antigen was carried out by using the MIB1 antibody (Immunotech, Marseilles, France). After antigen retrieval, the MIB1 antibody was also seen with the Dako Envision system and diaminobenzidine tetrahydrochloride substrate.

Evaluation of immunostainings

One investigator (HN), who was blinded to the patient's history and clinical course, assessed the immunostainings. To assess the immunoreactivity of CDC25B, more than 1000 cells

Abbreviations: DFS, disease-free survival; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Figure 1 CDC25B immunohistochemical images of a low-grade astrocytoma (A, B) with low immunopositivity and a glioblastoma (C, D) with high immunopositivity. CDC25B was expressed in the cytoplasm of tumour cells. Original magnification: $\times 200$ (A, C); $\times 400$ (B, D).

were counted in each specimen. The CDC25B labelling index was expressed as the percentage of cells that showed positive staining among the total number of tumour cells counted. For statistical analysis, the immunoreactivity of CDC25B was classified according to the CDC25B labelling index as follows: low expression ($<20\%$ of CDC25B LI) and high expression ($\geq 20\%$ of CDC25B LI). The Ki-67 labelling index was calculated as the percentage of Ki-67-positive cells of all tumour cells counted.

RNA extraction and RT-PCR analysis

Semiquantitative RT-PCR analysis for CDC25B mRNA was carried out on the RNA extracts of 53 glioma samples (19 low-grade astrocytomas, 15 anaplastic astrocytomas and 19 glioblastomas) by the multiplex RT-PCR technique.² The RNA

samples of four gliomas were inadequately stored and so we could not carry out the RT-PCR analysis of these samples. For RNA preparation, snap-frozen tumour samples were broken down into small pieces, and total RNA was isolated with a guanidinium isothiocyanate-based system (RNeasy; Qiagen, Valencia, California, USA). A total of 1 μg of RNA from each sample was reverse-transcribed by using oligo (dT) primer and Moloney murine leukemia virus reverse transcriptase according to the protocol of the manufacturer (Clontech, Palo Alto, California, USA). The cDNA was diluted to give a total volume of 200 μl , and 5 μl of this dilution was used for each PCR reaction. For the detection of mRNA of CDC25B, PCR amplification was carried out with the following oligonucleotide primers: CDC25B forward primer, 5'-TCT CAT CTG AGC GTG GGC-3' and CDC25B reverse primer, 5'-CTT CAG GCC TCG AAA GGC-3'. PCR was carried out with the Takara Taq (Takara Shuzo, Otsu, Japan) in a thermal cycler (GeneAmp PCR System 9700, PE Applied Biosystems, Foster City, California, USA). As an internal control, a fragment of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified by PCR using the following primers: forward primer, 5'-CGG AGT CAA CGG ATT TGG TCG TAT-3' and reverse primer, 5'-AGC CTT CTC CAT GGT GGT GAA GAC-3'. The amplified products were separated by electrophoresis in a 2% agarose tris-borate ethylenediamine tetraacetic acid gel, and the bands were visualised by ethidium bromide.

Real-time PCR for CDC25B mRNA was carried out for 30 representative samples (10 samples from each histological grade, respectively). The sequences of the forward and reverse primers used to amplify the human CDC25B and GAPDH genes were designed by using LightCycler Probe Design Software V.1.0 (Roche Applied Science, Penzberg, Germany). The RT-PCR reactions were carried out in a LightCycler 3.5 Instrument (Roche Applied Science). The standard curve quantitation method was used, and the amount of transcript in each sample was calculated by interpolation, using the formula (threshold cycle $- y$ intercept)/S. The steady-state concentrations of mRNA for CDC25B in each glioma sample were normalised to the amount of GAPDH mRNA. PCR was carried out at least twice as an independent experiment.

Table 1 Relationships of CDC25 B protein expression to other clinical data

	CDC25B protein expression		p Value
	Low	High	
Total	29	28	
Age in years (SD)	41.0 (18.2)	48.7 (17.3)	0.106
≥ 55	5	9	
< 55	24	19	0.312
Sex			
Male	21	19	
Female	8	9	0.931
WHO grade			
II	21	0	
III	7	10	
IV	1	18	< 0.0001
Ki-67 labelling index (%)	3.27 (2.76)	13.8 (7.15)	< 0.0001
≥ 10	1	21	
< 10	28	7	< 0.0001
Tumour recurrence			
Positive	7	28	
Negative	22	0	< 0.0001

High expression, $\geq 20\%$ of positive cells; low expression, $< 20\%$ of positive cells.

Statistical analysis

Statistical analysis was carried out with StatView J-V.5.0 statistical software. The χ^2 test and Student's *t* test were used to analyse the association between two categorical variables. Disease-free survival (DFS) was obtained by using the Kaplan–Meier method and was calculated from the date of surgery. Survival curves were compared with the log-rank test. In multivariate analysis, independent prognostic factors were determined by Cox's proportional hazards model. In all tests, results with a *p* value < 0.05 were considered to be significant.

RESULTS

Immunohistochemical expression of CDC25B

In CDC25B immunostaining, CDC25B was expressed in the cytoplasm of tumour cells (fig 1). CDC25B expression was not apparent in the nuclei of tumour cells. Normal brain tissues were not stained. The immunoreactivity of CDC25B showed appreciable variation in staining intensity among the different areas of each tumour. Histologically, high-grade gliomas tended to show high CDC25B protein expression. High expression of CDC25B was identified in 18 of the 19 grade IV tumours and in 10 of the 19 grade III tumours, but not in any of the 21 grade II tumours (table 1).

RT-PCR analysis for CDC25B mRNA expression

Figure 2 shows the results of semiquantitative RT-PCR analysis of representative patients. The CDC25B mRNA expression increased with increasing histological grade.

In real-time RT-PCR analysis for CDC25B mRNA, the CDC25B/GAPDH mRNA level was 0.75 (SD 0.24) for low-grade astrocytomas, 1.54 (SD 0.38) for anaplastic astrocytomas and 2.38 (SD 0.72) for glioblastomas. The CDC25B/GAPDH mRNA level increased with increasing histological grade (fig 3). Significant differences were observed in CDC25B mRNA transcript ratio among these groups (*p* < 0.05). The mRNA data were compatible with the protein data.

Correlation of CDC25B expression and clinicopathological parameters

Table 1 summarises the correlations of the immunohistochemical expression for CDC25B with the clinicopathological parameters. CDC25B expression was associated significantly with the histological grade (WHO grade) of the tumour (*p* < 0.0001). The group with high CDC25B expression had significantly higher frequency of high-grade glioma. Furthermore, high CDC25B expression significantly correlated with high Ki-67 labelling index and tumour recurrence (*p* < 0.0001). The mean Ki-67 labelling index of the group with high CDC25B expression was significantly higher than that of the group with low CDC25B expression (*p* < 0.0001). CDC25B expression did not associate significantly with age and sex.

Figure 2 Semiquantitative RT-PCR analysis for CDC25B mRNA. RT-PCR analysis was carried out for CDC25B mRNA in glioma tissues. The CDC25B mRNA expression increased with increasing histological grade. AA, anaplastic astrocytoma; GBM, glioblastoma; LGA, low-grade astrocytoma.

Figure 3 Real-time RT-PCR analysis for CDC25B mRNA. The CDC25B/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level was 0.75 (SD 0.24) for low-grade astrocytomas (LGA), 1.54 (SD 0.38) for anaplastic astrocytomas (AA) and 2.38 (SD 0.72) for glioblastomas (GBM). The CDC25B/GAPDH mRNA level increased with increase in histological grade. Significant differences were shown in the CDC25B mRNA transcript ratio of these groups (*p* < 0.05).

Survival analysis

DFS rate was assessed by the Kaplan–Meier survival analysis in all patients in this series, including the three histological grades. The group with high CDC25B expression had significantly shorter DFS than that with low CDC25B expression (*p* < 0.0001; fig 4).

Significant prognostic factors for DFS

Multivariate analysis by Cox's proportional hazards model showed that high CDC25B expression, high Ki-67 labelling index and high histological grade were independent prognostic factors for DFS (*p* < 0.0001; log-rank test; table 2).

DISCUSSION

In eukaryotes, the entry into mitosis is regulated by the activation of CDC2–cyclin B. The CDC2–cyclin B complex is inactivated by phosphorylation of the threonine 14 and

Figure 4 Kaplan–Meier survival curves. Curves defined by CDC25B expression. Patients with low CDC25B expression had significantly longer disease-free survival times.

Table 2 Results of multivariate analysis of factors predicting recurrence using Cox's proportional hazards model

Variable	Relative risk (95% CI)	p Value
Age (years)		
≥55 v <55	1.60 (0.75 to 3.53)	0.225
Sex		
Male v female	0.79 (0.38 to 1.62)	0.513
WHO grade		
IV v II	1.50 (18.2 to 1000)	<0.0001
IV v III	6.06 (1.91 to 19.2)	0.0002
Ki-67 labelling index (%)		
≥10 v <10	7.98 (3.73 to 17.1)	<0.0001
CDC25B protein High v low expression	22.5 (7.56 to 66.7)	<0.0001

tyrosine 15 residues of CDC2 by Wee-1 or Mik 1 until G₂-M transition,^{3,4} and is activated on dephosphorylation by CDC25. CDC25 is a family of cyclin-dependent kinase-activating phosphatases. Three different members, CDC25A, CDC25B and CDC25C, are known. They seem to act at different points of the cell cycle.⁵⁻⁸ CDC25A regulates the G₁-S complex, and CDC25B and CDC25C are reported to be responsible for the activation of CDC2-cyclin B in mitosis.⁹⁻¹¹ CDC25C works at the G₂-M transition, whereas CDC25B works at the G₂ phase.^{7,8,12} CDC25B in its original form is able to activate the CDC2-cyclin B complex, whereas CDC25C is able to catalyse CDC2-cyclin B complex activation in only its phosphorylated form.⁹ In short, CDC25B is more effective than CDC25C at prompting mitosis.^{7,8,12} The role of CDC25B in the late G₂ phase as a regulator of centrosomal microtubule nucleation⁵ and as a starter of mitosis^{11,13} was shown. Overexpression of dominant-negative mutants of CDC25B and CDC25C results in a G₂ phase arrest. Cells overexpressing CDC25B dominant-negative mutants block all events of mitosis, whereas cells overexpressing CDC25C dominant-negative mutants often show evidence of mitotic events in the cytoplasm but a complete block in a nuclear mitotic event. These findings also suggest a role for CDC25B as the initiator of mitosis and the role for CDC25C in ensuring the full activation of CDC2-cyclin B and a rapid entry into mitosis. Recent studies have shown that serine 146 phosphorylation is a key event in the regulation of CDC25B function in the initiation of mammalian mitosis.¹⁴ Thus, CDC25B has critical roles in the regulation of mitosis and may be related to cell proliferation.

CDC25B is reported to interact with Raf-1¹² to cooperate with Ras and loss of Rb to transform cells.¹⁵ Furthermore, CDC25A and B, but not C, have been identified as direct transcriptional targets of c-myc, and c-myc/max binding sites have been found in the CDC25A and B regulatory regions.¹⁶ These findings suggest the possible association of CDC25B with tumorigenesis.

Overexpression of CDC25B is reported in various human malignancies. A significantly higher expression of CDC25B was observed in aggressive non-Hodgkin's lymphomas¹⁷ than in indolent non-Hodgkin's lymphomas. Overexpression of CDC25B in breast cancer was reported to be associated with poor prognosis.¹⁵ CDC25B protein was shown to be overexpressed in 43% of colorectal carcinomas and as an independent prognostic factor in patients with colorectal carcinoma.¹⁸ Broggini *et al*¹⁹ showed that high CDC25B expression was related to a worse prognosis in patients with ovarian cancer.

To our knowledge, there are no reports concerning expression of CDC25B in clinical samples of glioma. This is the first study to examine the expression of CDC25B in glioma samples. In this

Take-home messages

- The expression of CDC25B increases with the increase in the degree of malignancy of gliomas.
- Multivariate analysis showed that overexpression of CDC25B is an unfavourable prognostic factor in patients with glioma.
- CDC25B is suggested to be a predictor of aggressive phenotype of gliomas and to be a possible target molecule for the treatment of gliomas.

study, we focused on the expression level of CDC25B in gliomas and its significance in clinical outcome. Our results showed that the expression of CDC25B increases with the increase in the degree of malignancy of gliomas. Furthermore, multivariate analysis showed that overexpression of CDC25B is an unfavourable prognostic factor in patients with glioma.

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