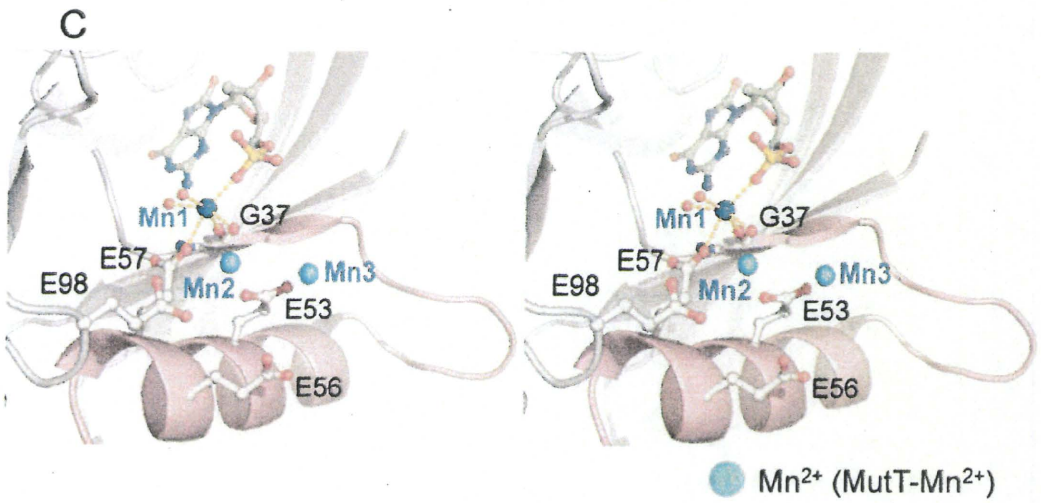
Fig. 4



J Neurooncol (2010) 100:43–49
DOI 10.1007/s11060-010-0147-3

CDC25A mRNA levels significantly correlate with Ki-67 expression in human glioma samples

Yoji Yamashita · Isao Kasugai · Masami Sato · Nobuhiro Tanuma · Ikuro Sato · Miyuki Nomura · Katsumi Yamashita · Yukihiko Sonoda · Toshihiro Kumabe · Teiji Tominaga · Ryuichi Katakura · Hiroshi Shima

Received: 4 December 2009 / Accepted: 15 February 2010 / Published online: 10 March 2010

Abstract Cell division cycle 25 (CDC25) phosphatases are cell-cycle regulatory proteins which are overexpressed in a significant number of human cancers. This study evaluated the role of CDC25 phosphatases in human glioma proliferation. Upregulation of CDC25A was observed in human glioma specimens and human glioma cell lines. Comparison of expression levels of CDC25A and CDC25B messenger ribonucleic acid (RNA) to Ki-67 labeling index in glioma tissues found that Ki-67 labeling index was significantly correlated with the expression of CDC25A, but not with that of CDC25B. Depletion of CDC25A by small interfering RNA and inhibition of CDC25 suppressed cell proliferation and induced apoptosis in glioma cell lines, indicating that CDC25A is a potential target for the development of new therapy for glioma.

Keywords CDC25A · Glioma · Ki-67 · Protein phosphatase

Yoji Yamashita and Isao Kasugai contributed equally to this work.

Y. Yamashita · I. Kasugai · M. Sato · N. Tanuma · M. Nomura · H. Shima (✉)
Division of Cancer Chemotherapy, Miyagi Cancer Center Research Institute, 47-1 Nodayama, Medeshima-Shiode, Natori, Miyagi 981-1293, Japan
e-mail: shima-hi632@pref.miyagi.jp

Y. Yamashita · R. Katakura
Department of Neurosurgery, Miyagi Cancer Center, Natori, Miyagi, Japan

I. Sato
Section of Clinical Research, Miyagi Cancer Center Research Institute, Natori, Miyagi, Japan

K. Yamashita
Division of Life Science, Graduate School of Natural Science and Technology, Kanazawa University, Kanazawa, Ishikawa, Japan

Y. Sonoda · T. Kumabe · T. Tominaga
Department of Neurosurgery, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan

Introduction

Glioblastomas are the most common and lethal type of malignant brain tumor. Median survival from the time of diagnosis is less than a year, with fewer than 5% of patients surviving 5 years. Glioblastoma is characterized by highly proliferative and invasive activity, and widespread infiltration of tumor cells into the surrounding brain tissue [1]. Recent standard therapy for glioblastomas includes surgical resection, radiotherapy, and adjuvant temozolomide chemotherapy administered both during and after radiotherapy. However, most patients develop tumor recurrence or progression after this multimodality treatment. There is clearly an urgent need to develop new classes of treatment modalities, such as molecular target-directed therapies [2–4]. Understanding the molecular pathogenesis of glioma may allow the rational development of new therapy approaches.

The rate of cell proliferation in glioma tissues as assessed by Ki-67 immunoreactivity has been studied as a prognostic indicator, and correlates with tumor grade and clinical course [5, 6]. Ki-67 detected by MIB-1 antibody is a core antigen present in proliferating cells and absent in quiescent cells. This antigen is expressed in all phases of the cell cycle except for G0 and the early parts of G1. The precise function of the Ki-67 protein is still unclear. Therefore, identification of molecules involved in the upregulation of Ki-67 antigen may help to understand the

malignant phenotype of glioma, and may become a candidate target for treatment.

Regular control of cell cycle progression requires correct function of a small family of phosphatases termed cell division cycle 25 (CDC25), which contain highly conserved domains for dual specificity phosphatases [7]. The CDC25 family is fundamental in transitions between cell cycle phases during normal cell division through the activation of cyclin-dependent kinase (CDK)/cyclin complexes. Three genes code for the CDC25A, B, and C proteins with both different and redundant specificities and regulations in humans. In particular, the CDC25A and B phosphatases have oncogenic properties and are overexpressed singly in some types of cancers and together in others [8]. Therefore, CDC25s are promising targets for the development of new anticancer therapeutic strategies. Overexpression of CDC25 is linked to clinicopathological features such as tumor grade, recurrent disease, or disease-free survival [9, 10].

The present study examined whether CDC25 isotype-specific linkage is present in human glioblastoma samples.

Materials and methods

Patients and glioma samples

Newly diagnosed human glioma tissues were obtained from 25 consecutive patients (15 males and 10 females) who underwent surgery (14 surgical resections and 11 stereotactic biopsies) at the Department of Neurosurgery, Miyagi Cancer Center, from July 2008 onwards. Their median age was 63 years (range 21–83). Without regard to tumor volumes or tumor malignancies, small samples weighing from 10 to 30 mg were collected for this study from all surgical specimens and serial numbers were added in the order of surgery. Each sample was immediately divided in two. One was frozen for ribonucleic acid (RNA) preparation, the other was formalin-fixed and paraffin-embedded for conventional histopathological evaluation and counting of Ki-67 labeling index (Ki-67LI). Histological diagnoses were made by a neuropathologist, based on the World Health Organization criteria as glioblastoma (16 cases; nos. 1–5, 7–12, 14, 18–21), anaplastic astrocytoma (6 cases; nos. 6, 13, 17, 23–25), and diffuse astrocytoma (3 cases; nos. 15, 16, 22). RNA analysis was approved by the Ethics Committee of the Miyagi Cancer Center.

Quantitative real-time polymerase chain reaction

Total RNA was prepared from the specimens with the RNeasy Lipid Mini kit (Qiagen). Complementary deoxyribonucleic acid (cDNA) was synthesized using an oligo-d(T)12-18 primer with Superscript III reverse transcriptase (Invitrogen) and applied to quantitative real-time polymerase chain reaction (qPCR) using the LightCycler 480 and the probes master kit (Roche Diagnostics). The PCR primers and the probes were designed and selected for the intron spanning condition according to the online software (Roche Applied Science). The PCR reaction was performed in 20 μl containing 10 μl of Probes Master (Roche), 0.5 μM of each primer, 0.1 μM of probe, and 5 μl of cDNA solution. The protocol of PCR involved initial denaturation at 95°C for 5 min, followed by 55 cycles of 95°C for 10 s, then 60°C for 25 s. Threshold cycle values (Second Derivative Maximum method) were normalized to the housekeeping gene, porphobilinogen deaminase (PBGD). Human brain (frontal lobe) total RNA from a pool of four different donors and from single donor were obtained from Clontech (Palo Alto, CA, USA) and BioChain Institute (Hayward, CA), respectively, were also subjected to cDNA synthesis and subsequent qPCR. The levels of CDC25A and CDC25B messenger RNA (mRNA) in the gliomas were expressed as ratios to that of the mixed human brain RNA sample (Clontech). The following probes were used: no. 17 (CDC25A), no. 68 (CDC25B), no. 2 (CDC25C), and no. 25 (PBGD) (Roche Universal Probe Library). The primer sequences were as follows: CDC25A 5′-TCTGAAGAATGAGGAGGAGACC-3′ and 5′-AAACAGCTTGCATCGGTTGT-3′; CDC25B 5′-ACG CCCGTGCAGAATAAG-3′ and 5′-AGTGATTTTGAG CGGAGGAC-3′; CDC25C 5′-GAGGCCATGTCGGAA GAAG-3′ and 5′-GCTTCCTTCCTCTCTTGTGG-3′; PBGD 5′-AGCTATGAAGGATGGGCAAC-3′ and 5′-TTGTATG CTATCTGAGCCGTCTA-3′.

Ki-67LI

Ki-67LI was measured. Fields with the highest number of Ki-67-labeled cells were initially selected through generalized survey, and then the percentage of positive-labeled cells was determined by counting more than 1,000 tumor nuclei of more than three fields of a specimen at ×400 magnification without knowing any clinical information. Only strong nuclear staining was regarded as positive, and weak nuclear or cytoplasmic staining was regarded as negative.

Statistical analysis

Statistical analysis used the software Statview 5.0 (SAS Institute, Cary, NC). The expression level of CDC25A mRNA was compared with the Ki-67LI in human primary glioblastoma samples using simple linear regression analysis, and a P value of less than 0.05 was considered to indicate statistical significance.

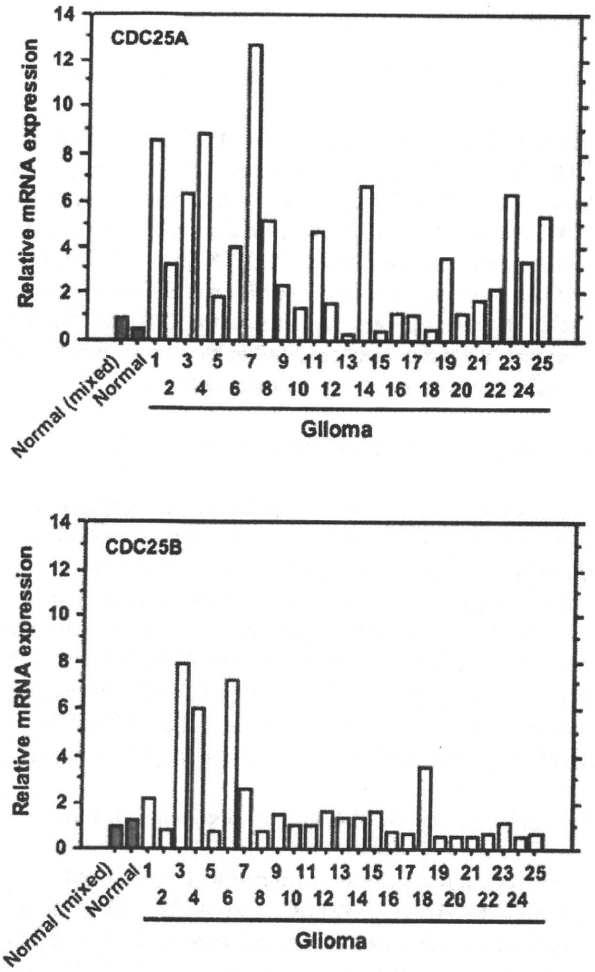


Fig. 1 Analysis of *CDC25A* and *CDC25B* mRNA levels in human glioma samples. Expression levels of *CDC25A* (*upper*) or *CDC25B* (*lower*) mRNA in normal brain and primary glioblastoma samples were estimated by qPCR. Results were normalized to the mRNA levels of the housekeeping gene porphobilinogen deaminase (PBGD) and shown relative to mRNA levels seen in the normal brain (four mixed samples), which was set as 1.0

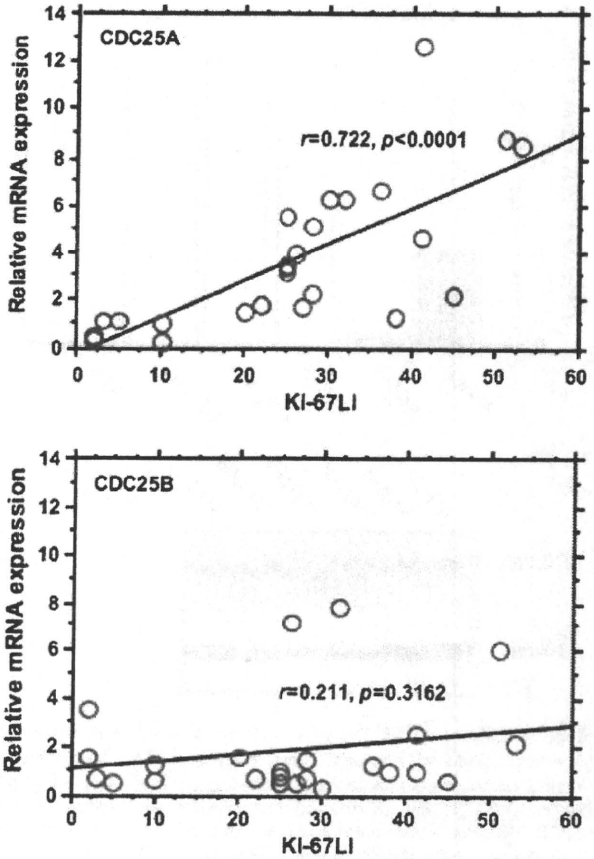


Fig. 2 Statistical comparison of the expression levels of *CDC25A* and *CDC25B* mRNA with *Ki-67LI* in human primary glioma samples. Simple linear regression analysis showed the relationship between the expression of *CDC25A* (*upper*) or *CDC25B* (*lower*) mRNA and *Ki-67LI* in human primary glioblastoma samples

Cell culture

Human glioblastoma cell line A172 was obtained from the RIKEN BRC (Tsukuba, Ibaraki, Japan), and U87, U251, and U373 were obtained from DS PHARMA Biomedical (Osaka, Japan), Health Science Research Resources Bank (Osaka, Japan), and ATCC, respectively. A172, U87, U251, and U373 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). A172 cells were cultured in RPMI-1640 (Gibco) supplemented with 10% FBS. Normal human astrocytes (NHAs) (Lonza, Basel, Switzerland) were cultured in Astrocyte Basal Medium supplemented with AGM SingleQuots according to the manufacturer's instructions. NHA proliferation was arrested after 21 days culture, and used as quiescent cells.

Small interfering RNA transfection

Small interfering RNA (siRNA) duplexes against human CDC25A (Stealth RNAi), HSS101654 (siRNA1-CDC25A), and HSS10165 (siRNA2-CDC25A) were purchased from Invitrogen. Stealth RNAi Negative Control Medium GC duplex (Invitrogen) was used as the control. siRNA transfection was undertaken using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instruction at a final siRNA concentration of 5 nM in the culture.

Cell proliferation assay

Cells transfected with siRNA or small compounds were plated on a 96-well plate in octuplicate wells. Cell

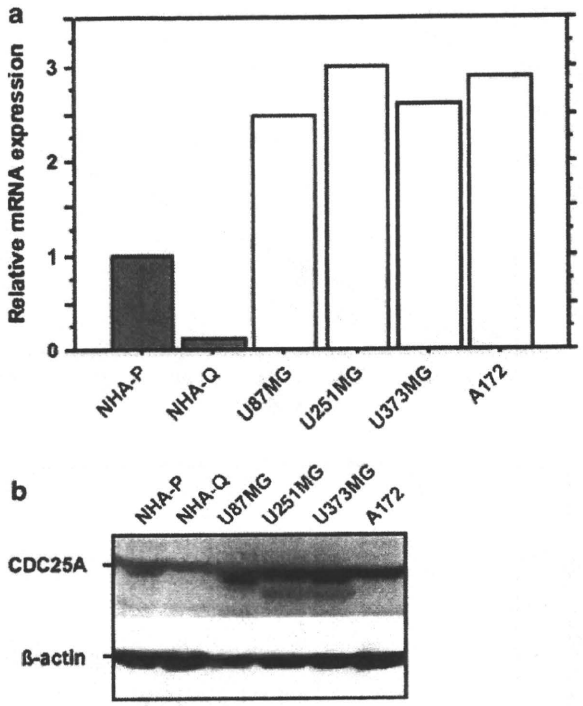


Fig. 3 Analyses of CDC25A mRNA levels (**a**) and protein levels (**b**) in human glioma cell lines. **a** Expression levels of CDC25A mRNA in normal human astrocytes in the proliferating (*NHA-P*) and quiescent stages (*NHA-Q*) and glioma cell line samples were estimated by qPCR. Results were normalized to the mRNA levels of the housekeeping gene porphobilinogen deaminase (PBGD) and shown relative to the mRNA levels seen in normal brain (four mixed samples), which was set as 1.0. **b** Immunoblot analysis of *CDC25A* in normal human astrocytes and glioma cell lines

proliferation was assessed as incorporation of 1-methoxy PMS by the DOJINDO cell counting Kit-8 according to the manufacture's protocol (DOJINDO, Kumamoto, Japan). Optical density was read at 450 nm at various time points using a microplate reader (TECAN, Research Triangle Park). The corresponding background value was subtracted from the reading obtained from each well. To estimate half maximal inhibitory concentration (IC50) for each compound, values at 96 h of treatment were used for linear regression analysis.

Caspase-3 and -7 activity

To measure apoptosis in glioma cell lines treated with siRNAs or small compounds, the cells were plated on a 96-well plate in triplicate wells. The caspase-3 and -7 enzyme activities were measured by the Caspase-Glo 3/7 Assay (Promega) according to the manufacturer's protocol.

Results

Upregulation of CDC25A in human glioma samples

The expression level of the three CDC25 family members (CDC25A, B, and C) was examined in 25 human glioma samples by qPCR. Figure 1 shows the mRNA levels of CDC25A and CDC25B in the glioma samples compared to that in normal (mixed) brain. The CDC25A and CDC25B mRNAs were elevated in 17 (68%) and 6 (24%), respectively, of these samples. In contrast, the CDC25C mRNA level was under the detection limit in the normal brain, and no induction was observed in tumor samples.

Correlation of CDC25A expression with Ki-67LI

To examine the possible involvement of CDC25A and CDC25B in cellular proliferation, the expression of Ki-67, a marker for cell proliferation, was examined by immunohistochemistry with MIB-1 antibody. Figure 2 shows that Ki-67LI levels were significantly correlated with the level of CDC25A ($r = 0.722$, $P < 0.0001$), but not with that of CDC25B ($r = 0.211$, $P = 0.3162$). Therefore, overexpression of CDC25A rather than CDC25B is involved in increased cell proliferation in glioma tissues.

Upregulation of CDC25A in human glioma cell lines

CDC25A expression was examined in 4 glioma cell lines, U87MG, U251MG, U373MG, and A172. NHAs in the proliferating and quiescent stages were used as controls. Figure 3a shows the relative expression of CDC25A in these cells. The level of CDC25A mRNA was reduced to 20% after the end of proliferation in NHAs, supporting the importance of CDC25A expression in cell proliferation. Compared to proliferating NHAs, glioma cell lines such as U87MG, U251MG, U373MG, and A172 showed 2- to 3-fold increase in CDC25A mRNA expression. Analysis of the CDC25A protein levels in these cells showed almost similar levels to those of CDC25A mRNA (Fig. 3b). Therefore, CDC25A is overexpressed in glioma cell lines.

Inhibitor of CDC25 suppresses cell proliferation

To examine the role of CDC25s in glioblastoma, the effects of two quinone-based CDC25 inhibitors, BN82002 [11] and BN82685 [12], were analyzed on cell proliferation in U87MG and A172 cells (Fig. 4a). Both compounds inhibited CDC25A and CDC25B, and they were already reported to be active not only in vitro but also in vivo [11, 12]. U87MG and A172 cells were treated with increasing concentrations of the inhibitors until 96 h. The IC50s of

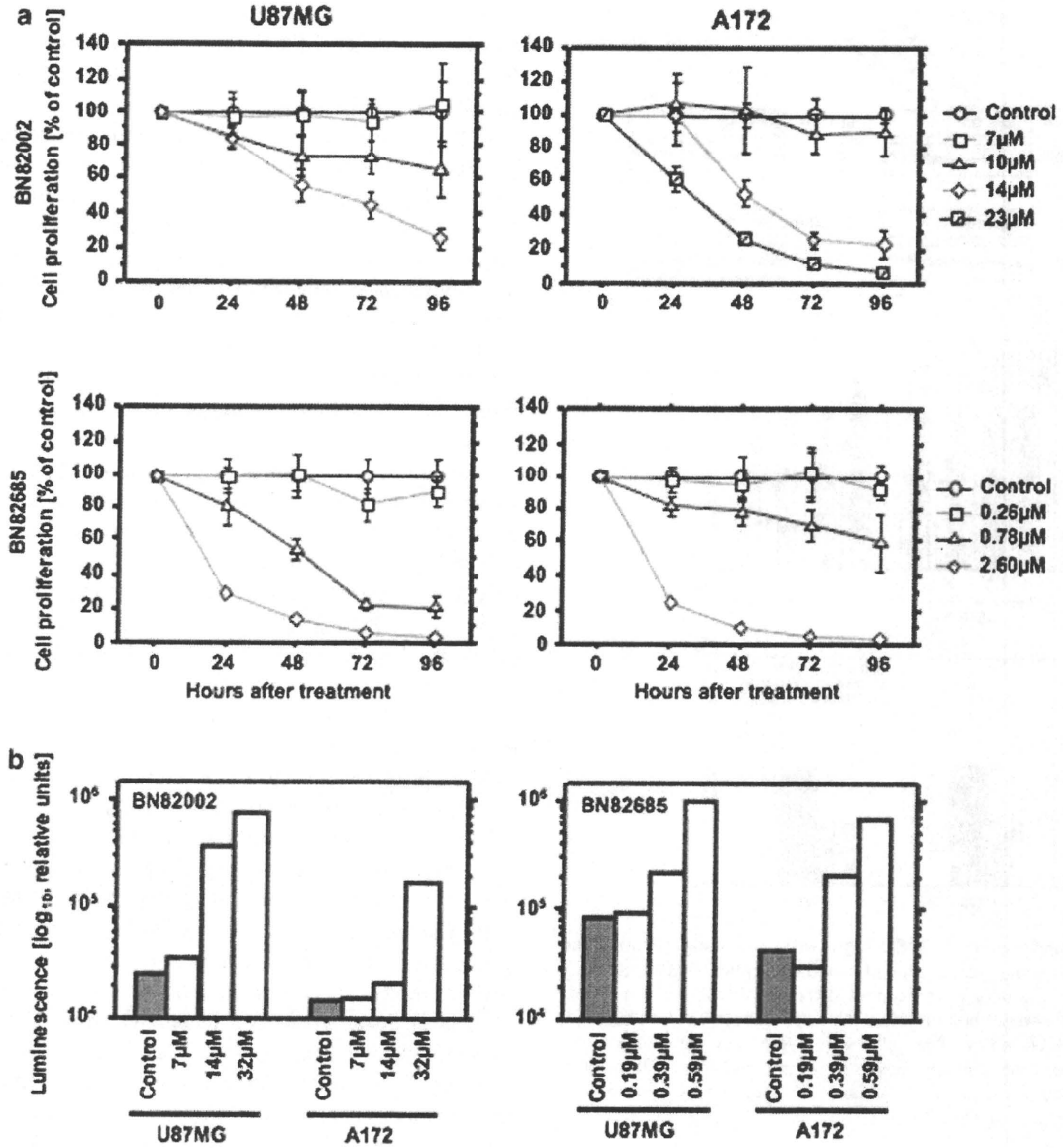


Fig. 4 Inhibition of CDC25 reduces cell proliferation (**a**) and induces cell apoptosis (**b**). *U87MG* and *A172* cells were grown in 98-well plates and treated with dimethyl sulfoxide (*control*) or CDC25 inhibitors, *BN82002* or *BN82685*. Cell proliferation assay (**a**) and caspase-3 and -7 assay (**b**) as described in "Materials and methods"

BN82002 for U87MG and A172 were 18 and 12 μM, respectively, and the IC50s of BN82685 for U87MG and A172 were 0.54 and 0.90 μM, respectively. In addition, BN82002 and BN82685 dose-dependently induced apoptosis, as assessed by the caspase-3 and -7 activities (Fig. 4b).

Silencing of CDC25A inhibits cell growth

To evaluate the CDC25A specific role in proliferation and survival in glioma cells, the siRNA approach was used to deplete CDC25A in the glioma cell lines. Two CDC25A-specific siRNA (siRNA1-CDC25A and siRNA2-CDC25A) and negative control siRNA were transfected into U87MG and A172 cells, both of which overexpressed CDC25A. Immunoblot analysis confirmed suppression of CDC25A expression in siRNA1-CDC25A and siRNA2-CDC25A at days 2 and 3 after transfection (Fig. 5a).

The effects of CDC25A suppression on the cell proliferation of the glioma cells were analyzed. U87MG and A172 were transfected with siRNA1-CDC25A, siRNA2-CDC25A, or control siRNA, and cell proliferation was assessed daily over 5 days (Fig. 5b). Both cell lines

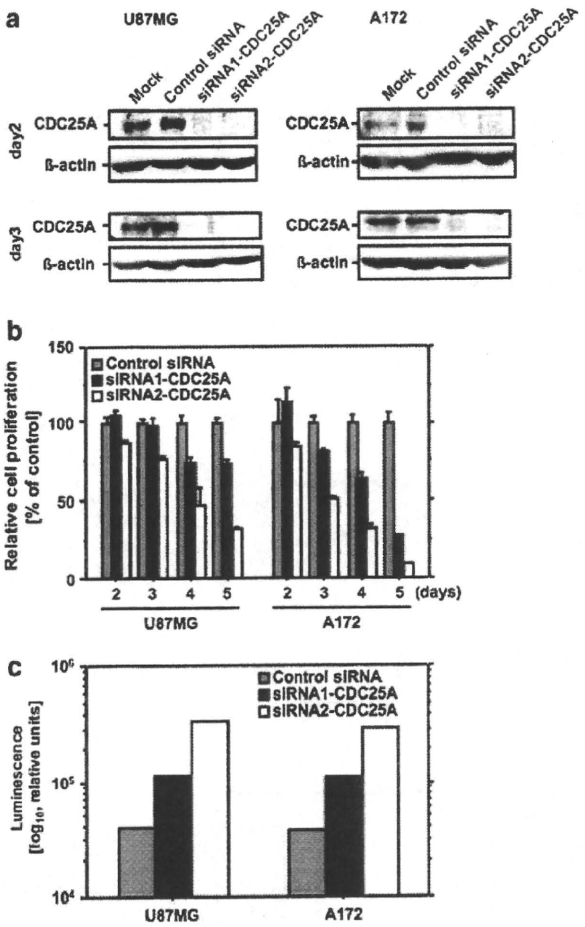


Fig. 5 Suppression of CDC25A by siRNA treatment inhibits cell proliferation and induces apoptosis in glioma cells. *U87MG* and *A172* cells were transfected with control siRNA or siRNAs against human CDC25A as described in "Materials and methods" and cultured for the indicated times. The cells were lysed and immunoblot was performed using antibody for CDC25A or β-actin (**a**). Cell proliferation assay (**b**) and caspase-3 and -7 assay (**c**) as described in "Materials and methods"

transfected with siRNA1-CDC25A and siRNA2-CDC25A showed slower growth rates than cells transfected with control siRNA. Additionally, caspase activity was measured in the CDC25A-depleted cells. The U87MG and A172 cells transfected with the CDC25A siRNAs showed increased levels of caspase, demonstrating that apoptosis was induced in the CDC25A-depleted cells (Fig. 5c). Therefore, CDC25A-mediated cell proliferation and survival in glioma cells, and suppression resulted in growth inhibition.

Discussion

The present study investigated expression levels of CDC25s in human gliomas, and found that CDC25A is overexpressed, and that its expression level is closely correlated with Ki-67LI in gliomas. This may be the first functional molecule with expression well correlated with Ki-67LI in glioma tissues.

CDC25 was overexpressed and contributed to tumorigenesis in various patients with high-grade malignant tumor [8]. Both CDC25 A and B have prognostic value. Overexpression of CDC25B and significant correlation of expression with shorter periods of disease-free survival were found in glioma samples, but the level of CDC25A expression was not defined [13]. CDC25 overexpression within each cancer subtype tends to occur in an isoform-specific manner, and the overexpression of multiple isoforms in the same cancer subtypes probably occurs through independent pathways [8].

This study showed that expression of CDC25A was more prominent and correlated better with Ki-67LI than expression of CDC25B in glioma tissues. These findings indicate that overexpression of CDC25A rather than CDC25B is involved in increased cell proliferation in glioma tissues. The majority of studies have clearly shown the prognostic importance of Ki-67LI in glioma, both regarding survival and recurrence [5, 6], so CDC25A should be considered the marker for prognosis and/or target for treatment rather than CDC25B.

The present study showed overexpression of CDC25A in both surgical specimens and glioma cell lines. CDC25A expression was 2–3 times higher in glioma cell lines than the proliferating NHAs, suggesting overexpression of CDC25A characterizes the malignant phenotype of glioma cells. The mechanisms by which CDC25 isoforms become deregulated during tumorigenesis remains unclear. Several positive and/or negative regulators of CDC25A transcription have been described, including c-myc, hypoxia-inducible factor-1 alpha, p53, p21, and E2F, but there is no evidence that CDC25 overexpression resulted from gene amplification or rearrangements, or any other specific genetic mutations that may be responsible for deregulating CDC25 activities in cancer [8]. The development of CDC25A targeting therapy will require further studies to reveal the CDC25 biology in glioma.

Recently, CDKs are crucial in the control of the cell cycle, so are attractive pharmacological targets for the development of antiproliferative agents. Inhibitors of these enzymes are currently in clinical trials in patients with various malignant tumors. CDC25s are activators of CDKs, so are particularly attractive target candidates for the development of anticancer agents. Various classes of CDC25 inhibitors have been identified, and the specificities were examined in vitro. We selected BN82002 and BN82685 because of their relatively higher selectivity for CDC25A. The IC50s of BN82002 towards CDC25A, CDC25B2, and CDC25B3 are 2.4, 3.9, and 6.3 μM, and

those of BN82685 are 109, 160, and 249 nM, respectively, in vitro [11, 12]. In this study, these inhibitors showed remarkable growth suppression and induction of apoptosis in glioma cells, and similar effects were observed after selective depletion of CDC25A from glioma cells by siRNA. These results suggest that CDC25A is important in glioma cell proliferation and survival, and CDC25A targeted therapy using isoform specific inhibitor is a potential approach to glioma therapy.

Acknowledgments This work was supported in part by Grants-in-aid for Scientific Research (C) to Y.Y. and H.S. and for Exploratory Research to R.K. provided by the Japan Society for the Promotion of Science.

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