

## Downregulation of KIF23 suppresses glioma proliferation

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**Abstract** To identify therapeutic molecular targets for glioma, we performed modified serological identification of antigens by recombinant complementary DNA (cDNA) expression cloning using sera from a mouse glioma model. Two clones, kinesin family member 23 (Kif23) and structural maintenance of chromosomes 4 (Smc4), were identified as antigens through immunological reaction with sera from mice harboring syngenic GL261 mouse glioma and intratumoral inoculation with a mutant herpes simplex virus. The human Kif23 homolog KIF23 is a nuclear protein that localizes to the interzone of mitotic spindles, acting as a plus-end-directed motor enzyme that moves antiparallel microtubules in vitro. Expression analysis revealed a higher level of KIF23 expression in glioma tissues than in normal brain tissue. The introduction of small interfering RNA (siRNA) targeting KIF23 into two different glioma cell lines, U87MG and SF126, downregulated KIF23 expression, which significantly suppressed glioma cell proliferation in vitro. KIF23 siRNA-treated glioma cells exhibited larger cell bodies with two or more nuclei compared with control cells. In vivo analysis using mouse xenograft showed that KIF23 siRNA/DNA chimera-

treated tumors were significantly smaller than tumors treated with control siRNA/DNA chimera. Taken together, our results indicate that downregulation of KIF23 decreases proliferation of glioma cells and that KIF23 may be a novel therapeutic target in malignant glioma.

**Keywords** Glioma proliferation · KIF23 · MKLP1 · Microtubule

### Introduction

Despite the development of therapeutic modalities, including surgery, chemotherapy, and radiotherapy, limited progress has been achieved in the treatment of glioblastoma (GBM), the most malignant form of glioma. Most patients with GBM die within approximately 12 months of diagnosis [1–3]. GBM has a high rate of cellular proliferation and marked propensity to invade surrounding brain parenchyma. Such aggressive and invasive growth is the hallmark feature that gives rise to its high morbidity and mortality [4]. GBM can rarely be surgically resected. Therefore, the molecular mechanisms driving tumor proliferation must be understood to exploit them as targets for new glioma therapies.

In the present study, we conducted serological identification of antigens by recombinant cDNA expression (SEREX) in an attempt to discover novel tumor antigens, and identified six genes potentially coding candidate proteins for novel tumor antigens. Among these six genes, we focused on *Kif23* due to its specific immune reactivity toward serum from tumor-bearing, immune-precipitated mouse and also because of its preferable expression in glioma.

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KIF23, a human homolog of mouse Kif23, is a nuclear protein that localizes to the interzone of mitotic spindles and acts as a plus-end-directed motor enzyme that moves antiparallel microtubules in vitro [5]. Previous studies have reported that depletion of KIF23 in HeLa cells induces formation of multinucleate cells, likely because of a cytokinesis defect [6, 7]. Microtubules, for which KIF23 acts as the motor enzyme [5], have been known to control cellular shape and processes such as motility, mitosis, intracellular vesicle transport, organization, and positioning of membranous organelles [8–11]. Due to these molecular functions, microtubules have been adopted as one of the major targets in cancer chemotherapy [8]. Taking together the facts that KIF23 acts as a motor enzyme of microtubules, that KIF23 showed preferable expression in gliomas compared with normal brain controls, and that SEREX identified KIF23 as a candidate for novel glioma antigen, we considered that KIF23 might perform an important role in gliomagenesis. To date, no report on the relationship between KIF23 and glioma has appeared in the literature. In this study, we continued evaluating the expression of KIF23 in glioma cells and investigated the role of KIF23 in these cells by using an RNA interference (RNAi) technique.

## Materials and methods

### Tissue samples and cell lines

Brain tumor samples were obtained from the Department of Neurosurgery, Keio University, School of Medicine. Written informed consent for the study was obtained from all patients, and the study was approved by the ethical review board of Keio University (no. 12-21-2). Human adult brain tissue samples were obtained from Biochain (Hayward, CA). Normal human RNA samples were obtained from Biochain and Clontech (Palo Alto, CA). The human GBM cell lines SF126, KNS81, T98G, KNS42, and U87MG, human leukemia cell line K562, mouse brain neoplasm cell line GL261, and African green monkey kidney cell line Vero were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum and penicillin–streptomycin in a humidified incubator at 37°C with 5% CO<sub>2</sub>.

### Viruses

Herpes simplex virus-1 (HSV-1) mutant strain G207 was used in this study. The construction of G207 has been described elsewhere [12]. This mutant virus contains deletions in the *ICP34.5* and *ICP6* genes. Viruses were propagated and titered in Vero cells.

### Glioma model and HSV vaccination

Female C57BL/6 mice (6–8 weeks old) were purchased from Japan SLC, Inc. (Tokyo, Japan). The Animal Care and Use Committee of the School of Medicine, Keio University, approved all the animal procedures. GL261 tumor cells ( $1 \times 10^6$ ) were injected subcutaneously into both flanks of the mice. Once the subcutaneous tumors reached a maximum diameter of 5–7 mm, the tumors on the right flanks of the mice (day 0) were inoculated with either G207 ( $1.5 \times 10^7$  plaque-forming units) in 50  $\mu$ l virus buffer [150 mM NaCl and 20 mM Tris (pH 7.5)] and DMEM with 1% inactivated fetal calf serum (IFCS) (1:1) or 50  $\mu$ l of a “mock-infected extract” prepared from mock-infected cells created using the same procedure as that for the preparation of virus inocula. The right flank tumors were inoculated with G207 five times, once every 3 days, for a total of 15 days. Mice treated with G207 were killed 7 days after the last virus inoculation, and sera were prepared.

### Immunoscreening and characterization of immunoreactive clones

Each serum was diluted 1:100 with 5% skim milk in Tris-buffered saline with 0.05% polyoxyethylene sorbitan monolaurate. Sera were pre-absorbed with transformed *Escherichia coli* lysates and *E. coli* infected with lambda ZAP phage (Stratagene, La Jolla, CA). Three sera samples were prepared, mixed, and diluted to 1:300. A mouse testis cDNA library (Stratagene) was expressed with BL21 (DE3) pLysE, and the colonies were transferred to nitrocellulose membranes (Hybond-C; Amersham Pharmacia, Buckinghamshire, England). The membranes were incubated with the diluted sera for 4 h at room temperature, followed by incubation in 1:4,000 diluted goat anti-mouse immunoglobulin G (IgG) antibody conjugated with alkaline phosphatase (Cappel, Aurora, OH). Nitro blue tetrazolium (Boehringer Mannheim, GmbH, Germany) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemical Co., St Louis, MO) were used for enzymatic detection of bound secondary antibodies. Positive plaques were picked from the plates and purified through secondary and tertiary rounds of additional screening. The purified cDNAs were amplified via polymerase chain reaction (PCR) using Ex Taq (Takara Shuzo, Shiga, Japan) and sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit and an ABI Prism automated sequencer (PerkinElmer, Branchburg, NJ). The sequenced DNAs were analyzed with a basic local alignment search tool (BLAST) search of genetic databases at the National Center for Biotechnology Information (NCBI).

## Detection of antibodies against candidate genes using SEREX

Monoclonal phages from each clone that was reacted with IgG antibodies in the serum were mixed with nonreactive phages of the cDNA library as internal negative controls at ratio of 1:10 and expressed with BL21 (DE3) pLysE. The colonies were transferred to nitrocellulose membranes. IgG antibodies in the diluted (1:100) pre-absorbed *E. coli* sera from HSV-treated mice, HSV-treated GL261-harboring mice, or GL261-harboring untreated mice were screened to determine antibody responses to candidate genes.

## Quantitative PCR analysis

cDNA was synthesized from 10 µg total RNA using reverse transcriptase XL (AMV) (Takara Bio, Tokyo, Japan). The primers were designed as follows: for KIF23, forward primer, 5'-CTGACCCAGAGCAAAGCTTTC-3', and reverse primer, 5'-GTTCTAAAGTGCATTCTTG-CAGC-3'; for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward primer, 5'-CCCACTCCTCCACCTT TGAC-3', and reverse primer, 5'-ATGAGGTCCACCA CCCTGTT-3'. Quantitative reverse-transcriptase polymerase chain reaction (PCR) analysis was performed using SYBR Green (PerkinElmer, Foster City, CA) and the ABI prism 7700 Sequence Detection System (PerkinElmer). The thermal cycler conditions were as follows: 10 min at 95.0°C, 50 cycles of 95.0°C for 15 s, and 1 min at 60.0°C. The threshold cycle value was defined as the value obtained in the PCR cycle when the fluorescence signal increased above the background threshold.

## Western blot analysis

Cell lysates were prepared using a radioimmunoprecipitation assay buffer (Thermo Scientific, Rockford, IL) containing protease inhibitors (Cocktail Tablet; Roche Diagnostics, Tokyo, Japan). Lysates were centrifuged at  $14,000 \times g$  for 10 min at 4°C, and the protein concentration of each sample was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA) with bovine serum albumin as the standard. Identical amounts of the proteins were electrophoresed in 4–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membranes. Blots were blocked with Blocking One™ (Nacalai Inc., Kyoto, Japan) at room temperature and incubated with either a rabbit anti-MKLP1 (KIF23) antibody (1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA) or a mouse anti-β-actin antibody (1:4,000; Sigma, St. Louis, MO) overnight at 4°C. After being washed three times in Tris-buffered saline Tween 20 (20 mM Tris-HCl, 150 mM NaCl, and 0.02% Tween 20; pH 7.4), the blots

were incubated with the secondary antibody conjugated with horseradish peroxidase (1:4,000; anti-rabbit, MBL International Co., Woburn, MA; anti-mouse, GE Health Care Biosciences, Waukesha, WI) for 1 h at room temperature. Signals were detected with an enhanced chemiluminescence system (GE Health Care Biosciences) and exposed to Hyperfilm (GE Health Care Biosciences).

## RNA interference

Two short interference RNA (siRNA) oligonucleotide sequences for KIF23 were used: siRNA1 (sense strand, 5'-GACUAUAUCUAGAUC AUGUCU-3'; antisense strand, 5'-ACAUGAUCUAGAU AUAGUCUU-3') and siRNA2 (sense strand, 5'-GAGUGUUGCAUAGAAGUGAAA-3'; antisense strand, 5'-UCACUUCUAUGCAACACUCA-3'). Predesigned RNAi (sense strand, 5'-GUACCGCACGUC AUUCGUAUC-3'; antisense strand, 5'-UACGAAUGAC GUGCGGUACGU-3') was used as the negative control. The final concentration of 10 nM siRNA was incubated with Lipofectamine RNAiMax (Invitrogen, CA) according to the manufacturer's instructions. Cells were maintained for 24–120 h before experimentation.

## Cell viability assay

Cell viability was evaluated using the CellTiter Glo® luminescent cell viability assay (Promega, Madison, WI) according to the manufacturer's instructions, using a luminometer (Wallac ARVO 1420 multilabel counter; WALLAC OY, Truku, Finland). Each experiment was performed in triplicate.

## Immunofluorescence

SF126 cells were seeded on chamber slides (Nunc, Kamstrup, Denmark) in DMEM containing 10% fetal bovine serum and placed in eight-well plates at density of  $1 \times 10^4$ /well. Twelve hours later, KIF23 and control siRNAs were transfected, and the cells were cultured for 48 h. The cells were then fixed, permeabilized, blocked, and incubated with 1:50 diluted anti-α/β-tubulin antibody (Cell Signaling Technology, Beverly, MA). The cells were then incubated with Alexa-Fluor® anti-rabbit IgG. Finally, they were treated with 4',6-diamidino-2-phenylindole (DAPI) for 10 min for nuclear staining. The slides were washed, mounted, and imaged using fluorescence microscopy (Axioplan 2 imaging; Carl Zeiss, Japan).

## In vivo analyses of tumors administered siRNA/DNA

A total of  $2.5 \times 10^6$  U87MG cells suspended in 0.2 ml saline were subcutaneously inoculated into the lower flank

of 7-week-old nude mice [BALB/c (nu/nu)] (CLEA, Tokyo, Japan). When the tumors reached approximately 5 mm in size (day 0), the mice were randomly divided into two groups and treated with either KIF23-targeting or control siRNA/DNA (2.5 nM each). The same amount of siRNA/DNA was injected into the same region 7 and 14 days after the first injection. The modified siRNAs (DNA-RNA chimeras) were designed according to the method of Ui-Tei et al. [13]. Each target sequence was as follows: KIF23, 5'-rGrArGUrGUUrGrCrAUrArGAAGT GAAA; control, 5'-rGUrArCrGrCrArCrGUrCrATTTCGT ATC. Tumor diameters were measured at regular intervals with digital calipers, and the tumor volume in cubic mm<sup>3</sup> was calculated according to the following formula: volume =  $4/3\pi(\text{width}/2)^2 \times \text{length}/2$ . The tumors were removed surgically at 3 weeks after the first injection of siRNA/DNA and were embedded in Tissue-Tek OCT compound (Sakura Finetechnical Corp., Tokyo, Japan) and then frozen. Cryostat sections (20  $\mu\text{m}$ ) were prepared for hematoxylin and eosin (H&E) staining.

#### In silico REMBRANDT analysis

The relationship between patients' prognosis and expression intensity of the target gene was analyzed in silico using National Cancer Institute's (NCI's) REMBRANDT database [14] (<http://rembrandt.nci.nih.gov>, accessed 9 July 2011).

#### Statistical analysis

Data are expressed as mean  $\pm$  standard error (SE). Statistical significance was determined with one-way analysis of variance and a Tukey post-test method or Student's *t*-test using Prism software (GraphPad, San Diego, CA). *P* values lower than 0.05 were regarded as statistically significant.

## Results

#### Immunoreactive clones

Approximately  $1 \times 10^6$  clones from the mouse testis cDNA library were screened with SEREX, using mixed sera samples of three GL261-implanted mice after intratumoral inoculation with HSV. After subsequent sequencing of positive clones, six clones that encoded an open reading frame were identified using the BLAST database (NCBI; Table 1). We further evaluated the immune response of the clones to the following mouse sera: normal male or female mice, mice with subcutaneously implanted GL261, and mice with subcutaneously implanted GL261 and HSV treatment. The screening showed that two clones,

**Table 1** List of isolated genes generated with SEREX screening

Identified clones	UniGene accession number	Human homolog	UniGene accession number
<i>Spag6</i>	Mm.31701	<i>SPAG6</i>	Hs.655170
<i>Lrig2</i>	Mm.34855	<i>LRIG2</i>	Hs.448972
<i>Protamine 1</i>	Mm.42733	<i>Protamine 1</i>	Hs.2909
<i>Kif23</i>	Mm.259374	<i>KIF23</i>	Hs.270845
<i>Sycp1</i>	Mm.243849	<i>SYCP1</i>	Hs.112743
<i>Smc4</i>	Mm.206841	<i>SMC4</i>	Hs.58992

SEREX serological identification of antigens by recombinant cDNA expression

Kif23 (gene bank accession number Mm.259374) and Smc4 (Mm.206841), reacted exclusively with sera from GL261-bearing mice treated with HSV (Table 2). The expression of SMC4, a human homolog of mouse SMC4, was ubiquitous in the NCBI expressed sequence tags database (<http://www.ncbi.nlm.nih.gov/UniGene>, accessed 25 March 2011). In contrast, the expression of KIF23, a human homolog of mouse Kif23, in the NCBI database was higher in glioma tissue (9 transcripts/million) than in normal brain tissue (1 transcript/million) (<http://www.ncbi.nlm.nih.gov/UniGene>, accessed 25 March 2011). Previous reports have indicated the functional relation of KIF23 to cytokinesis [7, 15–19]. Therefore, we focused on further analysis of KIF23 in this study.

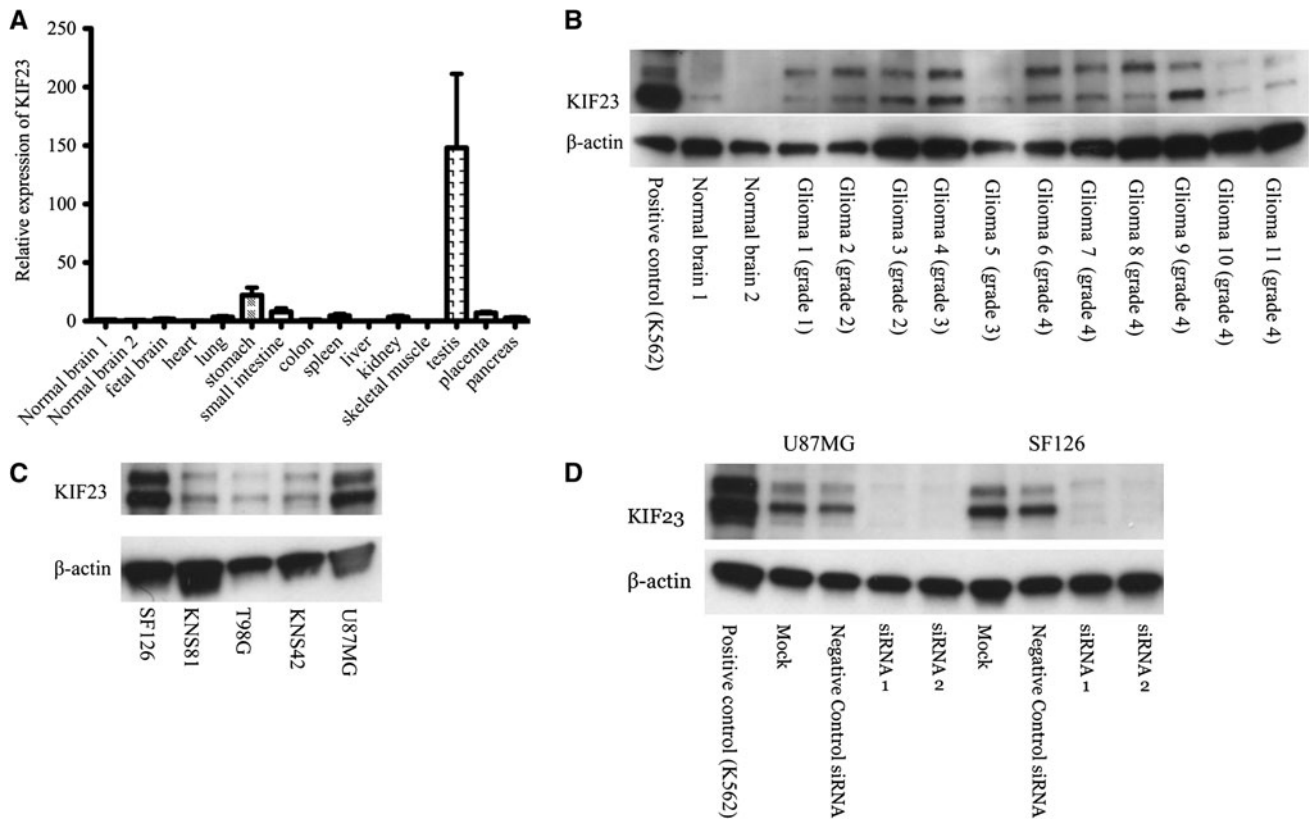
#### Expression pattern of KIF23

Quantitative PCR analysis using a cDNA panel containing normal human tissue (Fig. 1a) showed that KIF23 was more highly expressed in the testis and stomach than in normal brain. Western blot analysis was performed using lysate from K562 human leukemia cells as a positive control. KIF23 has two isoforms: a 110-kDa isoform 1 and a 98-kDa isoform 2 (<http://www.uniprot.org/uniprot/>, accessed 11 February 2011) [20, 21]. According to the manufacturer of the antibody for KIF23 used in this study, both isoforms can be detected. Our results showed two clear bands, corresponding to the two isoforms. KIF23 was expressed in all 11 glioma tissue samples evaluated, but was expressed to a lesser extent in control samples from normal brain tissue (Fig. 1b). Although it cannot be statistically evaluated, no correlation is apparent between the World Health Organization tumor grading and level of expression. We also conducted a Western blot analysis using five glioma cell lines (U87MG, KNS42, T98G, KNS81, and SF126) and confirmed the expression of KIF23 in all five glioma cell line samples evaluated (Fig. 1c). Among the five cell line samples evaluated, the expression of KIF23 was especially high in samples from

**Table 2** Immunoreactions against cloned proteins in mouse sera

Gene	Female mouse serum	Male mouse serum	Serum from GL261-bearing mouse	Serum from GL261-bearing mouse given HSV treatment
Kif23	0/5	0/5	0/5	5/5
Smc4	0/5	0/5	0/5	2/5

HSV herpes simplex virus



**Fig. 1** Expression of KIF23 in gliomas. Quantitative PCR analysis of the KIF23 gene in normal human tissues (**a**). The relative KIF23 expression level was normalized to the GAPDH level in each sample and calculated as the threshold cycle (CT) value in each sample divided by the CT value in normal brain. Mean  $\pm$  standard error of the mean (SEM) for at least three experiments are shown. Western

blot analysis of the KIF23 protein in normal brain, glioma tissues (**b**), and glioma cell lines (**c**) with  $\beta$ -actin as the control. **d** Effects of KIF23 short interfering RNA (siRNA) on KIF23 expression. Western blot analysis of KIF23 protein expression in U87MG and SF126 cells treated with mock, negative control siRNA, and two KIF23 siRNAs. Cell lysate of K562 cells was used as a positive control

SF126 and U87MG, and we conducted functional analyses of KIF23 in glioma using those two cell lines.

Effect of KIF23 siRNA on KIF23 expression in glioma cells

To evaluate the function of KIF23 protein in glioma cells, we designed two different siRNAs to downregulate the gene expression of KIF23. To confirm the effect of KIF23 siRNA on KIF23 protein expression in the glioma cell lines U87MG and SF126, we performed Western blot analysis (Fig. 1d). Transfection with two independent siRNAs targeting KIF23 was capable of downregulating the expression of KIF23 in these glioma cells 72 h after transfection in both glioma cell lines evaluated.

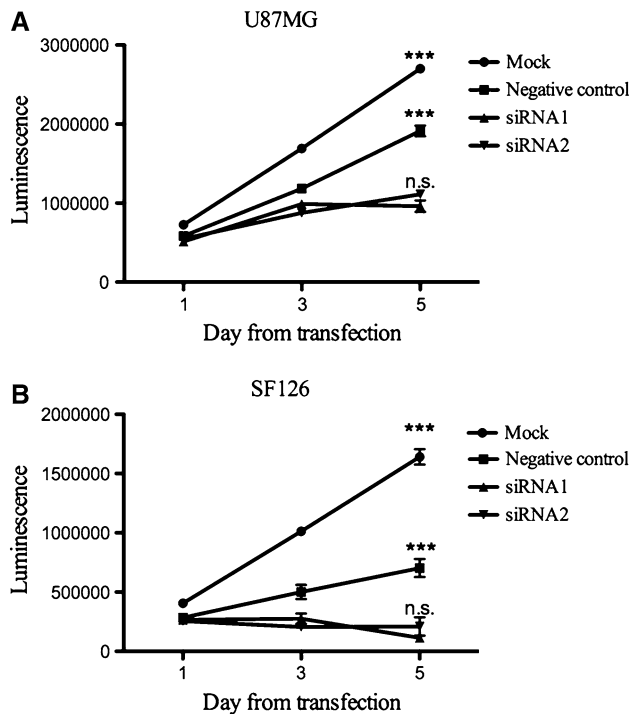
Inhibition of glioma cell proliferation by siRNA-mediated downregulation of KIF23 expression

Cell number was observed after siRNA transfection into SF126 and U87MG cells. There seemed to be fewer cells present after transfection with either of the two KIF23 siRNAs compared with their control counterpart (data not shown). So, to evaluate the relationship between cell proliferation and KIF23 expression, we conducted a cell viability assay using the CellTiter Glo<sup>®</sup> luminescent cell viability assay. After KIF23 siRNA treatment, the proliferation of both glioma cell lines, U87MG and SF126, was significantly suppressed compared with control cells at 5 days after transfection (Fig. 2a, b).

## Change in glioma cell morphology by downregulation of KIF23

Previous reports showed that KIF23 is a motor enzyme that moves antiparallel microtubules [5] and depletion of KIF23 in HeLa cells induces the formation of multinucleate cells, likely because of a cytokinesis defect [6, 7]. To identify the function of KIF23 in glioma cells, we examined the effect

of downregulation of KIF23 on cellular shapes in SF126 cells by staining microtubule with  $\alpha/\beta$ -tubulin and nucleus by DAPI. Typical fluorescent images of the stained cells (blue, nucleus; green,  $\alpha/\beta$ -tubulin) are shown in Fig. 3. More KIF23 siRNA-treated glioma cells than control cells exhibited large cell bodies with two or more nuclei. KIF23 siRNA-treated glioma cells can be seen as not able to complete cytokinesis properly and enlarged.



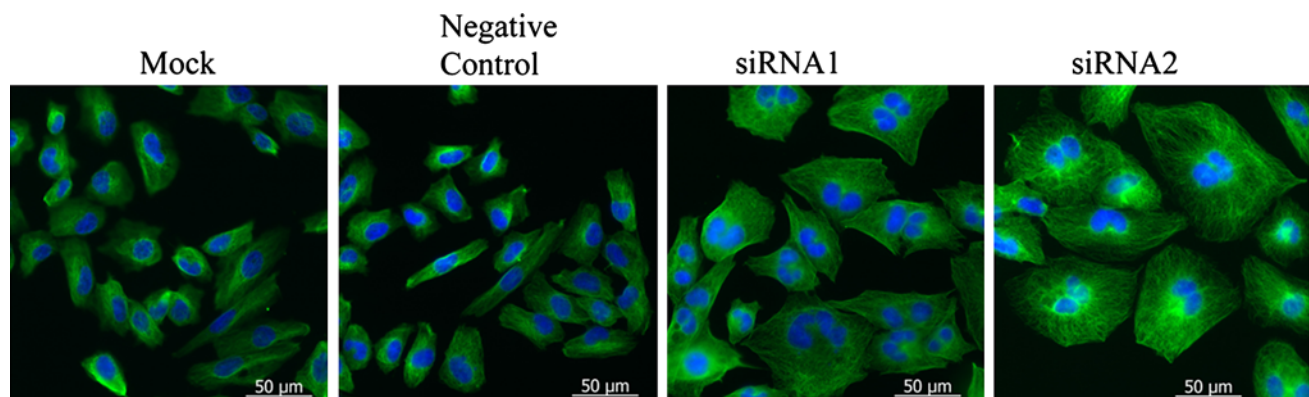
**Fig. 2** Effect of KIF23 siRNAs on glioma cell proliferation in vitro: **a** U87MG cells and **b** SF126 cells. Cells were treated with siRNAs for 24–120 h, and cell viability was determined using a CellTiter Glo luminescent cell viability assay. Results shown are mean  $\pm$  SEM (bars) of three experiments. *n.s.* not significant, \*\*\* $P < 0.001$  using one-way analysis of variance with a Tukey post-test method

## Effect of KIF23 siRNA/DNA chimeras on the growth of a U87MG mouse xenograft tumor model

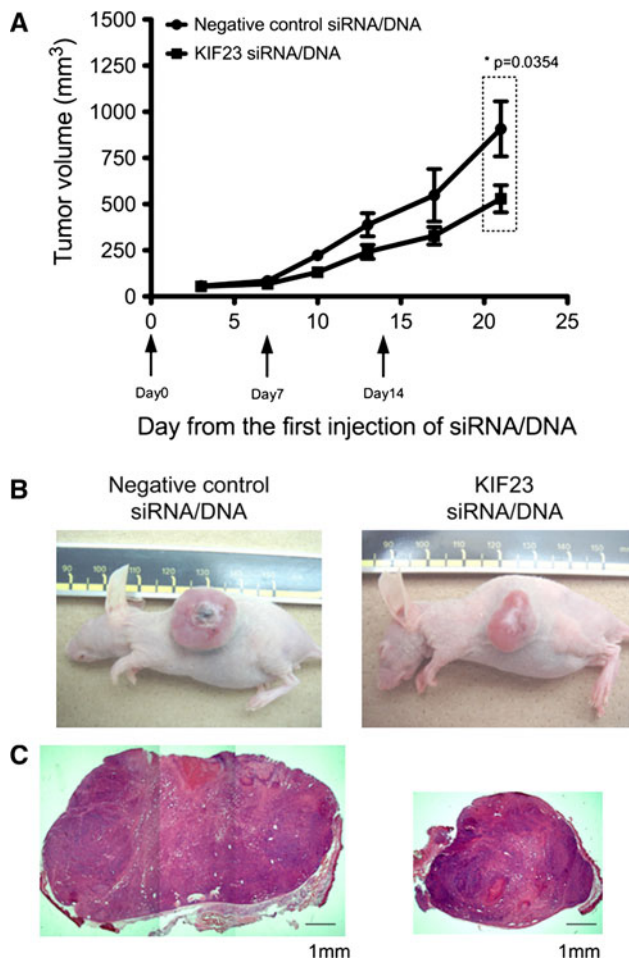
To examine the effect of KIF23 depletion on tumor growth in vivo, we used a xenograft model in which the U87MG human glioma cells were subcutaneously implanted into nude mice. When the average tumor size reached approximately 5 mm in size, KIF23 siRNA/DNA or control siRNA/DNA was injected into the tumors weekly for 3 weeks, and the tumor size was measured periodically (Fig. 4a). Under control siRNA/DNA treatment, U87MG cells developed into prominent tumors, and the average tumor volume at 3 weeks from initial injection was 908 mm<sup>3</sup>. The administration of KIF23 siRNA/DNA statistically significantly inhibited the growth of U87MG tumors (Student *t*-test;  $P = 0.0354$ ). The average tumor volume at 3 weeks was 529 mm<sup>3</sup> (Fig. 4).

## Relationship between clinical prognosis of glioma patients and expression value of KIF23

To examine the relationship between clinical prognosis of glioma patients and KIF23 expression value, we used the NCI REMBRANDT database [14]. According to the result obtained from the REMBRANDT database, patients with highly KIF23 messenger RNA (mRNA)-expressing glioma ( $n = 171$ ) tend to show statistically poorer prognosis compared with patients with intermediately KIF23



**Fig. 3** Effect of KIF23 siRNA on glioma morphology (SF126 glioma cell line). Representative micrographs 48 h after KIF23 siRNA treatment. Scale bars 50  $\mu$ m



**Fig. 4** Effect of KIF23 siRNA/DNA chimeras on the growth of a U87MG mouse xenograft tumor model. **a** Tumor volume measurement. Results shown are mean  $\pm$  SEM (bars) (control,  $n = 4$ ; KIF23 siRNA/DNA-chimeras treated,  $n = 6$ ). Arrows: KIF23 siRNA/DNA chimera injection. After implantation, one set of mice was injected with control siRNA/DNA and another set with KIF23 siRNA/DNA, at day 0, 7, and 14. \* $P < 0.05$  using Student's  $t$ -test. **b**  $2.5 \times 10^6$  U87MG cells were subcutaneously inoculated in nude mice (3 weeks after initial injection). **c** Representative H&E staining of tumor developed in nude mice from each set. Scale bar 1 mm

mRNA-expression glioma ( $n = 165$ ,  $P = 0.00321$ ). Patients with highly KIF23 mRNA-expressing glioma also tend to show poorer prognosis compared with patients with low KIF23 mRNA-expressing glioma; however, this was not statistically significant ( $P = 0.357$ ), probably due to small sample size of patients with low KIF23 mRNA expression ( $n = 7$ ) (Fig. 5).

## Discussion

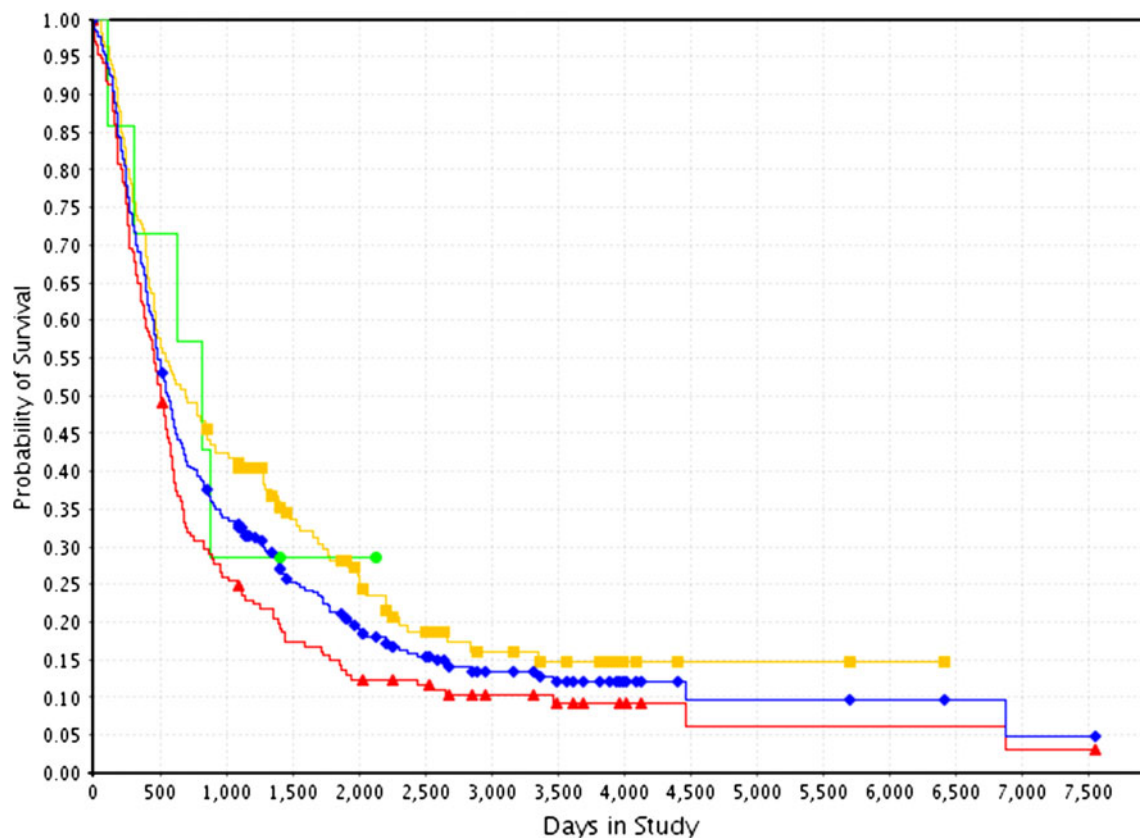
Serological identification of antigens by recombinant expression cloning (SEREX) has been developed for the discovery of novel tumor antigens [22]. SEREX can

isolate antigens recognized by both B and T cells; thus, it can be used to identify antigenic targets [22]. For example, NY-ESO-1, a tumor-specific antigen in the cancer/testis antigen group that is expressed in various types of tumors, was identified using SEREX [23]. Several candidate anticancer vaccines using NY-ESO-1-based immunogens are now under clinical trial [24–26]. Several candidate glioma antigens including Sox6 [27] have also been isolated using SEREX. Thus, SEREX is an effective method for the identification of useful tumor antigens.

Several lytic viruses have been used in an attempt to induce antitumor immunoreactions by modifying tumor cells [28]. Most of these cancer vaccines have included oncolysates or membranes from tumor cells infected with the vaccinia virus [29], parainfluenza virus [30], Newcastle disease virus [31], etc. Accumulating evidence suggests, however, that the immunogenicity of cellular material such as membranes is lower than that of whole tumor cells [31, 32]. To induce a strong antitumor response, we have used a replication-conditional herpes simplex virus (HSV) mutant, G207, to modify tumor cells directly in situ [33–37]. We have demonstrated that intratumoral inoculation with G207 elicits strong immune responses not only to HSV but also to the tumor antigen [34, 35].

In this study, we used intratumoral administration of G207 to vaccinate mice harboring a syngenic mouse glioma cell line, GL261, which is widely used for the study of immunotherapy against brain tumors [38–41]. We applied a modified SEREX method to the mouse glioma GL261 model in an attempt to identify molecular therapeutic targets for glioma.

Using SEREX, we identified six clones that encode an open reading frame confirmed by BLAST analysis (NCBI; Table 1). We further evaluated the specific immunoreactivity of these clones against glioma by using sera from normal male and female mice, GL261-implanted mice, and mice harboring GL261 treated with HSV. Two of the six clones (Kif23 and Smc4) reacted with only the sera from GL261-harboring mice treated with G207. The fact that we found no positive reaction to the two clones with sera from GL261-implanted mice indicates that GL261 glioma cells may not be immunogenic themselves and that HSV treatment could activate an antitumor response against GL261 in mice. Of the two clones identified, we focused on Kif23, since immunoreaction against Kif23 was detected in all five sera from GL261-bearing mice given HSV treatment, while immunoreaction against Smc4 was detected in two out of five sera from GL261-bearing mice given HSV treatment, and since KIF23, a human homolog of mouse Kif23, showed higher expression in glioma compared with normal brain according to NCBI database (<http://www.ncbi.nlm.nih.gov/dbEST>, accessed 25 March 2011).



**Fig. 5** Correlation between glioma patient survival and KIF23 mRNA expression, *red* indicates high KIF23 expression, *yellow* indicates intermediate KIF23 expression, *green* indicates low KIF23

expression, and *blue* indicates all patients (<http://rembrandt.nci.nih.gov>, accessed 2011 July 9)

Human Kif23 homolog, KIF23, was firstly identified by Nislow et al. [5] in 1992 as a plus-end-directed motor enzyme that moves antiparallel microtubules in vitro. After this first report, others were published, and KIF23 has been identified as a key regulator of cytokinesis [7, 15–19]. In this study, the KIF23 protein was expressed in all 11 glioma tissue samples evaluated but not in the normal brain controls. We also showed that KIF23 was expressed in five glioma cell lines (U87MG, KNS42, T98, KNS81, and SF126) but not in normal control brains. These findings demonstrate high KIF23 expression in glioma compared with that in the normal brain. The recruitment of KIF23 to the spindle midzone/midbody by a chromosomal passenger protein is essential for midbody formation and completion of cytokinesis in human cells [7]. Zhu et al. [7] have shown that downregulation of KIF23 abrogated midbody formation and completion of cytokinesis. Liu and Erikson have reported that a mutant Kif23 without nuclear localization signals leads to cell cycle arrest, further demonstrating that Kif23 is essential for cytokinesis [42]. These studies suggest that KIF23 is essential for cytokinesis. In addition, microtubules, for which KIF23 acts as the motor enzyme, have been known to control cellular shape and processes

such as motility, mitosis, intracellular vesicle transport, organization, and positioning of membranous organelles [8–11], and they have been one of the major targets in cancer chemotherapy [8]. Natural microtubule-targeting agents such as paclitaxel, vinblastine, or vincristine [43–46] have been used in clinical practice for years. In this study, the results of the cell viability assay using CellTiter Glo<sup>®</sup> luminescent and evaluation of in vivo tumor growth analysis as well showed that downregulation of KIF23 suppressed the growth of glioma cells both in vitro and in vivo. In addition, KIF23 siRNA-treated glioma cells showed larger cell bodies, and were binuclear/multinuclear, likely because of a cytokinesis defect as demonstrated in HeLa cells by others in previous reports [6, 7]. Taken together, downregulation of KIF23 may inhibit glioma proliferation by interrupting cytokinesis via modification of microtubules. Targeting microtubules for treating cancer has been shown to be a promising strategy [43–46]; however, clinical use is limited by hematological and neurological toxicities and by tumor resistance that leads to tumor recurrence [47]. Although further evaluation is required, molecular therapy targeting KIF23 may overcome this problem.



In the present *in vivo* tumor growth analysis, we used siRNA/DNA, since it has been reported that siRNA/DNA can overcome the disadvantages of usual siRNAs such as the problems of instability [48], induction of silencing of unintended genes (off-target effects) [49], or induction of immune response *in vivo* [50]. On the other hand, it has previously been reported that gene silencing induced by siRNA/DNA is likely to be less effective than that induced by its unmodified counterparts, because siRNA/DNA exhibits weaker activity for formation of an RNA-induced silencing complex [13, 51]. Considering these facts, we adopted a multiple siRNA/DNA injection protocol (in the present study, thrice, at days 0, 7, and 14) as described elsewhere [51, 52].

As the next step of *in vivo* study, we also should conduct histological analysis of *in vivo* experiments in an attempt to identify if there is any morphological or immunohistological difference between KIF23-knockdown tumors and control tumors as shown at the cellular level in *in vitro* experiments. KIF23 siRNA-treated glioma cells more frequently exhibited large cell bodies with two or more nuclei when compared to control cells. For now, it is not clear whether cells in the KIF23-knockdown tumors also present similar morphological change observed in the *in vitro* analysis or not. We will conduct further research to reveal the point.

The results of this study show that downregulation of KIF23 by specific RNAi could inhibit glioma proliferation *in vitro* and *in vivo*. Although RNAi rescue experiments could facilitate the establishment of a direct link between KIF23 expression and the cellular functions demonstrated in the present study, we designed two siRNA sequences for KIF23 instead and proved that their effects were similar. The present study suggests that KIF23 is indispensable for glioma cytokinesis and, consequently, glioma growth. The expression analysis demonstrated high expression of KIF23 in glioma cells compared with normal brain cells. Therefore, KIF23 may be a novel therapeutic target for GBM.

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