Detection of CD133 expression in U87 glioblastoma cells using a novel anti-CD133 monoclonal antibody

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Abstract. In glioblastomas, the surface glycoprotein CD133 (prominin-1) indicates the presence of cancer stem cells (CSCs), which are able to initiate tumor growth and are highly resistant to conventional chemo/radiotherapy. However, a number of studies have reported that certain $CD133^-$ glioma cells are able to self-renew and retain tumorigenic potential. In addition, the reliability of CD133 as a CSC marker is controversial due to inconsistent findings with regard to the prognostic values and distribution of CD133. Such controversies may be due to the detection limits using currently available anti-CD133 antibodies. In the present study, novel anti-human CD133 monoclonal antibodies (mAbs) were generated using two recombinant extracellular domains of human CD133: CD133 ectodomain 1 (amino acids 171-420) and CD133 ectodomain 2 (amino acids 507-716). One of the antibodies produced against CD133 ectodomain 2, C2E1, detected high expression levels of CD133 protein in glioblastoma U87 cells, in contrast to previous studies which did not detect CD133 expression in these cells. The cells exhibited a cytoplasmic distribution pattern of CD133 and produced a 95 kDa band following western blot analysis. In addition, C2E1 was able to bind the full-length glycosylated CD133 on the cell surface and inhibit the proliferation of tumor cells. Therefore, this antibody may be a valuable tool to study CD133 as a CSC marker and may be significant in future cancer treatments.

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Introduction

Glioblastomas (GBMs) are the most common and aggressive malignant primary brain tumors in humans. GBM accounts for ~51% of all primary gliomas (1), with an annual incidence of 2-3 cases per 100,000 individuals in Europe and North America (2). GBMs are usually diagnosed by magnetic resonance imaging, and confirmed by biopsy (3). Certain genetic signatures, including TP53, IDH1 or TPEN, are hypothesized to present potential biomarkers for GBM (4). At present, the standard treatment for newly diagnosed glioblastoma patients is surgical resection, followed by a combination of chemotherapy and radiotherapy (5,6). The prognosis of patients with glioblastoma is poor, and the median survival time is 14.6 months (6). GBMs contain a subpopulation of cancer stem cells (CSCs) that are able to self-renew *in vitro* and initiate new tumors *in vivo* (7,8). CSCs may also mediate radio- and chemo-resistance in GBMs (7,8).

Previous studies have hypothesized that the transmembrane glycoprotein, CD133 (also known as prominin-1), is a CSC marker in malignant brain tumors (9,10). In addition, a number of studies have revealed that $CD133^+$ cells, but not $CD133^-$ cells, exhibit stem cell-like and tumor-initiating properties (9,10). In addition, a number of studies have shown that CD133 closely correlates with tumor size, a worse prognosis, higher rates of lymph node metastasis and resistance to adjuvant therapies (11-13). Therefore, decreasing the expression of CD133 or exposing the protein to certain antibodies, such as AC133, may inhibit tumor cell growth, cell motility, spheroid-forming capacity and tumorigenic ability (14,15). However, other studies have obtained contradictory results (16-20). Further controversial results include inconsistent findings with regard to the prognostic value and distribution patterns of CD133 (9,10,21-28). These controversies may be due to the detection limits of currently available anti-CD133 antibodies (20).

The aim of the present study was to advance understanding with regard to the significance of CD133 in GBM tumor biology. Thus, in the current study, novel anti-human CD133 monoclonal antibodies (mAbs) were generated using two recombinant extracellular domains of human CD133. In addition, the expression levels of CD133 protein in U87

glioblastoma cells was detected using the produced antibodies.

Materials and methods

Cell culture and transfection. Human colonic carcinoma Caco-2 cells, human glioblastoma U87 cells and human embryonic kidney (HEK) 293 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Grand Island, NY, USA) supplemented with 10% (vol/vol) fetal bovine serum (FBS; Gibco Life Technologies), 1% penicillin-streptomycin (MP Biomedicals, Santa Ana, CA, USA) and 1% L-glutamate (MP Biomedicals). In addition, mouse myeloma cells, SP2/0 (American Type Culture Collection), were cultured in RPMI 1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% FBS. The cell lines were maintained in a humidified atmosphere of 5% CO_2 at 37˚C. The standard calcium phosphate method (29) was used to transfect HEK 293 cells. The medium was replaced at 4 h post-transfection and the cells were analyzed at 24-48 h post-transfection.

Plasmid construction. The cDNA coding CD133 was isolated from the MegaMan Human Transcriptome Library (Agilent Technologies, Santa Clara, CA, USA) by polymerase chain reaction (PCR) using forward primer, 5'-aggatcc atggccctcgtactcggct-3', and reverse primer, 5'-tatcgatttaatgttgtgatgggcttg-3'.

The amino acid sequences of CD133 ectodomain 1 (amino acids 171-420) and CD133 ectodomain 2 (amino acids 507-716) were selected from the ectodomains of CD133 based on its reported structure (Fig. 1A) (30). CD133 ectodomains 1 and 2 were amplified using the following primers: CD133 ectodomain 1 forward, 5'-ccatcgatatgagtcggaaactggcagatag-3', and reverse, 5'-gctctagattactgaataggaagacgctgag-3'; CD133 ectodomain 2 forward, 5'-ccatcgatatgtgtgaaccttacacgagca-3', and reverse, 5'-gactagtttagttctgagcaaaatccagag-3'.

PCR was performed in a 50 μl reaction volume, consisting of 1 μl cDNA template, 10 mM dNTPS (Takara Biotechnology Co., Ltd., Dalian, China), and 1 U LA Taq DNA polymerase (Takara Biotechnology Co., Ltd.) under the following conditions: 30 cycles of 94˚C for 30 sec, 98˚C for 10 sec, 55˚C for 15 sec and 72˚C for 1 min, followed by a 10 min extension step at 72˚C. All the PCR products were cloned into a pGEM-T easy vector (Promega Corporation, Madison, WI, USA). The positive clones were identified by double restriction enzyme digestion with BamHI/ClaI for full length CD133, ClaI/XbaI for CD133 ectodomain 1 and ClaI/SpeI for CD133 ectodomain 2, and sequencing was performed by the Beijing Genomics Institute (Shenzhen, China). All restriction enzymes were obtained from New England Biolabs (Ipswich, MA, USA). The DNA sequences of CD133 ectodomain 1 and CD133 ectodomain 2 were subsequently inserted into a prokaryotic expression vector, pRSET, including an N-terminal 6xHis-tag.

Establishment of a stable U87 cell line expressing full-length CD133. The CD133 full-length cDNA was inserted into a *Xho*I/*Not*I-digested, modified lentiviral vector with an mCherry reporter gene and a hygromycin resistance gene (designated as L-mCherry-Hygro-CD133; Addgene Inc., Cambridge, MA, USA). 293T cells were co-transfected, using the calcium phosphate method, with the L-mCherry-Hygro-CD133 plasmid, pVPack-vesicular stomatitis virus G (VSV-G) vector (Addgene, Cambridge, MA, USA) encoding the VSV-G surface gene, and the psPAX2 vector (Addgene), which provides the env and gag genes. At 4 h after transfection, the medium was replaced by fresh DMEM. Next, lentiviral supernatants were enriched to transduce the U87 cells in the presence of 10 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO, USA). At 24 h after transfection, the transduced cells were selected using 750 μg/ml hygromycin and red fluorescence from the mCherry reporter gene. The resultant stable cell line was designated as U87-CD133 overexpression.

CD133 antigen generation. The pRSET vector expressing CD133 ectodomain 1 or 2 was transformed into BL21 (DE3) *E. coli*. The transformed bacteria were grown in lysogeny broth medium and were induced by 0.25 mM isopropyl β-D-1-thiogalactopyranoside (MP Biomedicals) for 4 h. Subsequently, the cells were precipitated, resuspended in cell lysis buffer (50 mM NaH_2PO_4, 10 mM Tris-HCl, 250 mM NaCl, 5 mg/ml lysozyme; pH 8.0; Takara Biotechnology Co., Ltd.) and disrupted by two cycles of sonication on ice at 900 W. The protein expression was analyzed by 12% SDS-PAGE using Coomasie blue (MP Biomedicals) staining and confirmed by western blot analysis. Recombinant proteins with the 6xHis-tag were purified using nickel-nitrilotriacetic acid beads.

Immunization and mAb production. All the animal experiments used in the study were approved by the Institutional Animal Care and Use Committee of Shaanxi Normal University (Xi'an, China). In total, 20 female Balb/c mice (age, 6 weeks) were injected subcutaneously with recombinant protein CD133 ectodomain 1-6xHis or CD133 ectodomain 2-6xHis, which were emulsified in complete Freund's adjuvant (Sigma-Aldrich). Next, the mice were immunized with the same recombinant protein three times until a sufficient serum titer against CD133 was obtained, as analyzed by enzyme-linked immunosorbent assay (ELISA). Mice with a high serum titer were selected and boosted a fourth time prior to cell fusion.

The spleen cells of the immunized Balb/c mice were isolated and fused with SP2/0 myeloma cells using polyethylene glycol. Following fusion, the cells were cultured in hypoxanthine-aminopterin-thymidine (HAT) selection medium (Gibco Life Technologies). The HAT selection medium was refreshed every 2-3 days, and the selection medium was omitted after 6 days. Following a further 5 days, culture supernatants were screened for the production of antibodies against CD133 ectodomains 1 and 2 using ELISA.

Western blot analysis. Caco-2, U87 and U87-CD133 overexpression cells were lysed in radioimmunoprecipitation assay lysis buffer (Shaanxi Pioneer Biotech Co., Ltd., Xi'an, China). The samples were subjected to 12% SDS-PAGE and then blotted onto methanol-pretreated polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were incubated with mouse anti-human CD133 ectodomain

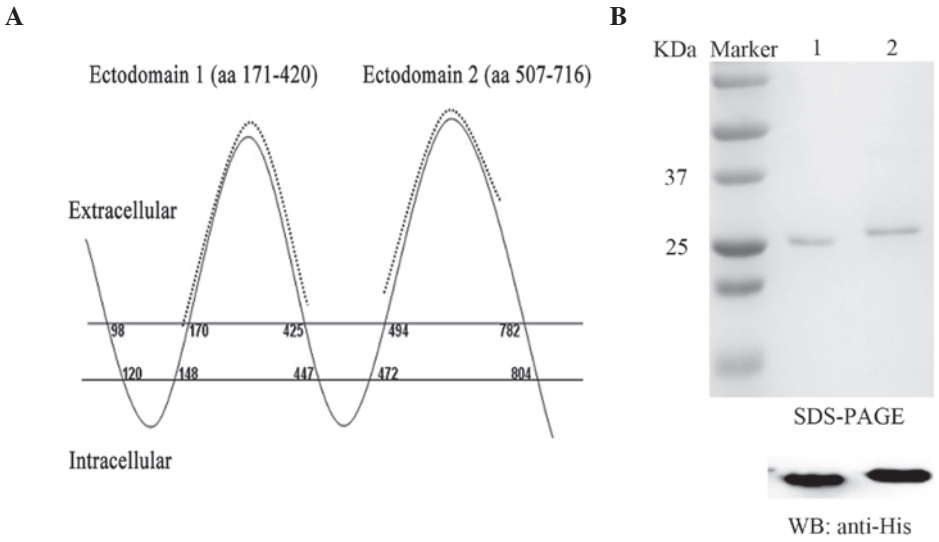

Figure 1. CD133 antigens used for mAb production. (A) Topological map of CD133 protein. Recombinant chimeric CD133 antigens, consisting of aa residues 171-420 and 507-716 (dotted line), were generated. (B) The two antigens, each tagged by an N-terminal 6xHis-tag, were expressed in *E. coli* and purified. The recombinant antigens were further verified by WB analysis with mouse anti-His mAb. Lane 1, ectodomain 1, Lane 2, ectodomain 2. CD133, prominin-1; mAb, monoclonal antibody; aa, amino acid; WB, Western blot.

mAbs (dilution, 1:300) for 90 min at room temperature. The rabbit anti-human CD133 mAb C24B9 (dilution, 1:1000; cat. no. 3663; Cell Signaling Technology, Danvers, MA, USA), which detects the full-length CD133 protein in Caco-2 cells (20), was used as a positive control. Next, the membranes were washed using phosphate-buffered saline (PBS)/Tween-20 (PBST) and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) polyclonal antibody (dilution, 1:10,000; cat. no. ZB-2305; Beijing Zhongshan Jinqiao Biological Technology Ltd., Beijing, China) or HRP-conjugated goat anti-rabbit IgG polyclonal antibody (dilution, 1:10,000; cat. no. ZB-2301; Beijing Zhongshan Jinqiao Biological Technology Ltd.). The membranes were further washed with PBST and visualized using the SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific, Hudson, NH, USA).

Fluorescent immunocytochemistry. HEK 293 cells transfected with CD133 full-length cDNA were fixed with 4% paraformaldehyde in PBS for 15 min, followed by washing with PBS. The fixed cells were then incubated with mouse anti-human CD133 ectodomain mAbs (dilution, 1:50) for 2 h at room temperature. Following three washes with PBS, the cells were incubated with biotin-conjugated goat anti-mouse IgG polyclonal antibody (dilution, 1:100; cat. no. SA1072; Wuhan Boster Biological Technology, Ltd., Wuhan, China) for 30 min at 37˚C. The cells were further washed and incubated with streptavidin biotin complex (SABC)-Cy3 (dilution, 1:100; cat. no. SA1072; Wuhan Boster Biological Technology, Ltd.) for 15 min at 37˚C. Finally, the cells were briefly washed with PBS and visualized under a Zeiss Axio Observer Z1 inverted fluorescence microscope (Zeiss, Oberkochen, Germany).

Immunohistochemistry. U87 cells were fixed with 4% paraformaldehyde in PBS for 15 min. The cells were subsequently washed using PBS, blocked in normal horse serum (dilution, 1:200; cat. no. PK-6102; Vector Laboratories, Burlingame, CA, USA) and incubated with mouse anti-human CD133 ectodomain mAbs (dilution, 1:50) at 4˚C overnight. After washing with PBS, the cells were incubated with biotin-conjugated horse anti-mouse IgG polyclonal antibody (dilution, 1:100; cat. no. PK-6102; Vector Laboratories) for 30 min at 37˚C. The cells were rinsed with PBS prior to incubation with HRP-conjugated SABC (dilution, 1:100; cat. no. PK-6102; Vector Laboratories) for 30 min at 37˚C. The 3,3'-diaminobenzidine chromogen (cat. no. ZLI-9017; Beijing Zhongshan Jinqiao Biological Technology Ltd.) was used for visualization under an inverted fluorescence microscope (DMIL LED; Leica, Wetzlar, Germany).

Cell proliferation assay. An MTT assay was performed according to a previously reported procedure (31), with minor modifications. Briefly, cells in 100 μl medium were seeded in 96-well flat-bottom plates and supplemented with 50 μl mAbs at various dilutions. Following a three-day incubation period, 15 μl MTT solution (5 mg MTT/ml of distilled water) were added to each well, followed by incubation for a further 4 h. Following removal of the supernatant, 150 μl dimethyl sulfoxide was used to dissolve the MTT crystals in the wells. Finally, the plates were subjected to cell viability detection using an ELISA reader (Multiskan EX; Thermo Fisher Scientific) at a wavelength of 570 nm. Optical density (OD) at 570 nm [absorbance value at 570nm (OD570)] was examined to analyze cell proliferation.

Statistical analysis. All data are presented as the mean + standard deviation (SD) from at least three different experiments. One way analysis of variance was used to investigate differences between the experimental and control groups. All statistical analyses were performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA).

Results

Generation and purification of CD133 antigens. CD133 amino acid fragments 171-420 (CD133 ectodomain 1) and 507-716 (CD133 ectodomain 2) were selected as the CD133

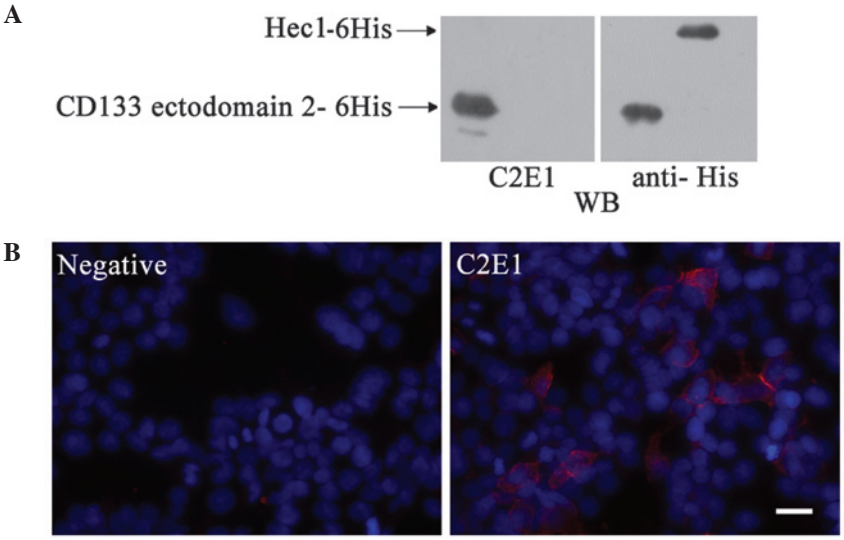

Figure 2. Validation of CD133 hybridoma cell lines. (A) The purified CD133 ectodomain 2-His and control Hec1-6xHis fusion protein were blotted from the SDS-PAGE gel onto a polyvinylidene difluoride and probed with C2E1 and control anti-His monoclonal antibodies. (B) Immunofluorescence staining with C2E1 of HEK 293 cells transfected with a plasmid expressing CD133. Pre-immunized serum served as a negative control. Scale bar, 20 μm. CD133, prominin-1; WB, western blot.

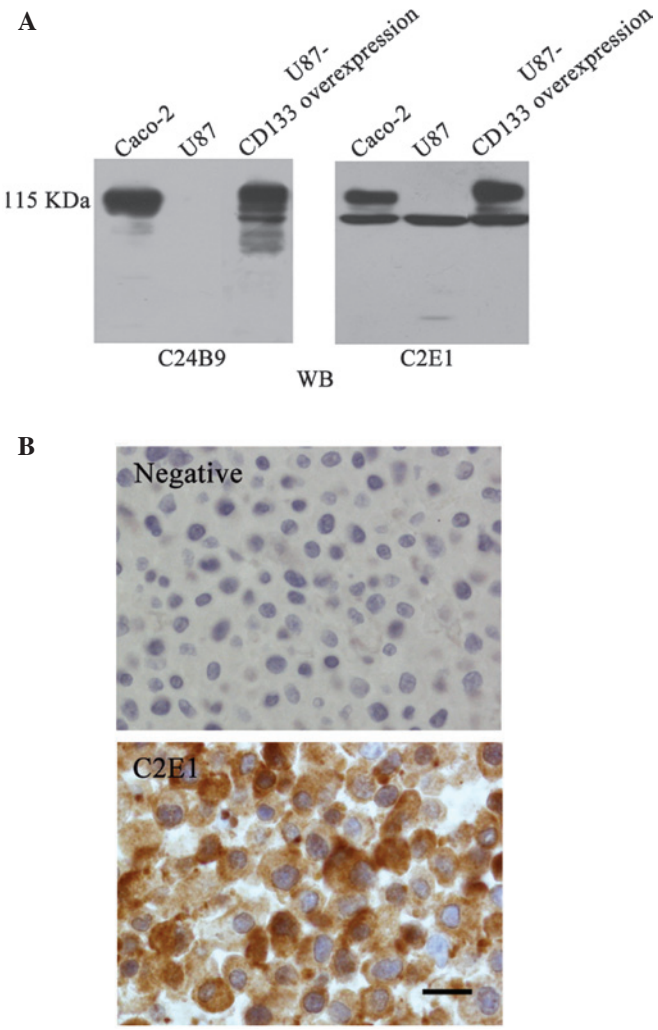

Figure 3. CD133 expression in glioblastoma U87 cells. (A) The cell lysates of U87, U87-CD133 overexpression and the control cell line Caco-2 were analyzed by WB analysis using C2E1 and the commercially-available antibody, C24B9. (B) Immunohistochemical staining with C2E1 revealed endogenous CD133 expression in the U87 cells. Pre-immunized serum served as a negative control. Scale bar, 20 μm. CD133, prominin-1; WB, western blot.

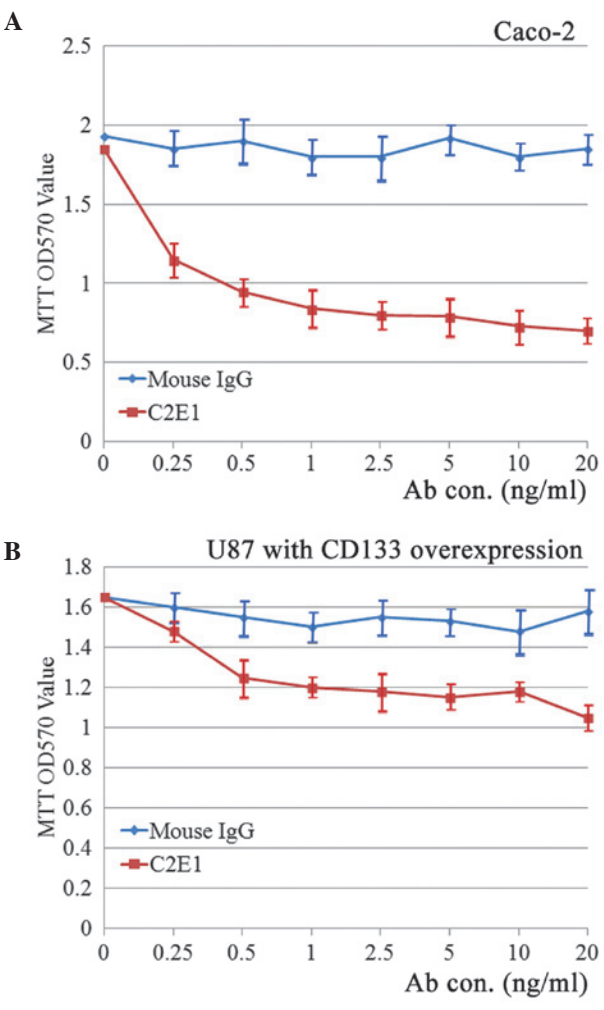

Figure 4. The effect of C2E1 on the proliferation of (A) Caco-2 and (B) U87-CD133 overexpression cells. Proliferation curves were determined using an MTT assay. Values are expressed as the mean ± standard error of four treatments. The highest inhibitory rates were 63.3±2.93% ($P<0.01$) for the Caco-2 cells and 33.3±4.73% ($P<0.05$) for the U87-CD133 overexpression cells. CD133, prominin-1; Ab con, antibody concentration; IgG, immunoglobulin G.

antigens. The locations of these two amino acid fragments in the CD133 protein are illustrated in Fig. 1A. The two CD133 ectodomain antigens, which were tagged by an N-terminal 6xHis-tag, were expressed in *E. coli*, purified and analyzed by SDS-PAGE (Fig. 1B). The purified recombinant proteins were further verified using western blot analysis with mouse anti-His mAb (Fig. 1B).

Generation and validation of CD133 mAbs. The purified antigens were injected into Balb/c mice to generate monoclonal anti-CD133 antibodies using standard hybridoma technology. ELISA was used to screen cell culture supernatants from the resulting hybridoma clones for the production of antibodies against recombinant CD133. At least six positive stable clones secreting anti-CD133 antibodies were obtained (data not shown). The positive hybridoma clones were validated by western blot analysis using prokaryotically-expressed CD133 ectodomain 1 and CD133 ectodomain 2 recombinant proteins. Four hybridoma clones against CD133 ectodomain 1 and two clones against ectodomain 2 were identified. HEK 293 cells were transfected with an expression plasmid encoding the human CD133 full-length cDNA and stained immunohistochemically with different hybridoma clones. One of the hybridoma clones, C2E1, which recognized the recombinant CD133 ectodomain 2 specifically, exhibited cell membrane and cytoplasmic staining in the transfected HEK 293 cells (Fig. 2).

Novel anti-CD133 mAb detects CD133 expression in the glioblastoma U87 cell line. CD133 expression in U87 cells was screened with each of the obtained anti-CD133 mAbs. The $CD133^+$ colorectal adenocarcinoma cell line, Caco-2, was used as a positive control and the commercially available anti-CD133 mAb, C24B9, was used as a positive control. Similar to C24B9, all the hybridoma clones detected a 115 kDa glycosylated full-length CD133 protein in Caco-2 cells, but were unable to detect a considerable CD133 expression in U87 cells, with the exception of C2E1 (Fig. 3A). In the C2E1 clones, a 95 kDa band was revealed in Caco-2 and U87 cells, in addition to the 115 kDa band in Caco-2 cells (Fig. 3A). A stable U87 cell line expressing full-length CD133 (termed as U87-CD133 overexpression) was established by infecting native U87 cells with CD133-expressing lentivirus, followed by selection. Western blot analysis with C2E1 and C24B9 demonstrated glycosylated full-length CD133 bands in the U87-CD133 overexpression cell line (Fig. 3A). Cellular distribution of endogenous CD133 expression in the native U87 cells was evaluated by immunohistochemical staining with C2E1, which revealed a cytoplasmic distribution pattern (Fig. 3B).

In vitro biological effects of the novel anti-CD133 mAb on cancer cells. To investigate whether the C2E1 antibody exhibits a biological activity (for instance, neutralization of the membranous CD133 protein), the Caco-2, native U87 and U87-CD133 overexpression cells were incubated with increasing concentrations of C2E1 for 72 h. An MTT assay revealed that incubation with C2E1 inhibited the proliferation of Caco-2 cells in a dose-dependent manner (Fig. 4A). The inhibitory rate was calculated using the following formula: Inhibitory rate (%) = (OD570 value of normal mouse IgG - OD570 value of C2E1)/(OD570 value of normal mouse IgG) x 100. The highest inhibitory rate was 63.3±2.93% ($P<0.01$), which was observed when the antibody concentration reached 20 ng/ml. However, C2E1 had no effect on the proliferation of U87 cells (data not shown), which may be due to the predominantly cytoplasmic distribution of CD133 protein in native U87 cells. As predicted, U87-CD133 overexpression cells responded to the cell growth inhibitory effect of C2E1 (Fig. 4B). The highest inhibitory rate was 33.3±4.73% ($P<0.05$), which was observed when the antibody concentration reached 20 ng/ml. Notably, the cell proliferation rate of untreated U87-CD133 overexpression cells was similar to that of native U87 cells.

Discussion

U87, a commonly-used commercial cell line for glioblastoma research, exhibits the behaviors and features of CSCs (32,33). However, previous studies have reported that the U87 cell line does not express the CSC marker, CD133, upon employing standard anti-CD133 antibodies, including AC133, 293C3 (34) or W6B3C1 (35). To the best of our knowledge, in the current study, an anti-human CD133 mAb was constructed that detected a 95 kDa endogenous CD133 protein with a cytoplasmic distribution pattern in U87 cells for the first time.

A number of studies have reported inconsistent immunolabeling of CSCs upon using different CD133 antibodies. This inconsistency may be associated with the glycosylation status and variants of the CD133 glycoprotein (36,37). Tumor stem cells may change the glycosylation status of CD133 upon differentiation (28,36,38). Antibodies that recognize the glycosylated epitopes may therefore detect only a limited subset of $CD133^+$ cells. In addition, ≥28 alternatively spliced CD133 variants have been identified (39-41). As different CD133 antibodies do not recognize all the splice variants, this may explain the inconsistencies in immunolabeling and varying protein sizes in different studies (20).

In the present study, the novel mAb C2E1 recognized a CD133 protein product of U87 cells with a molecular weight of 95 kDa, which has a similar size to the non-glycosylated full-length CD133. However, the 95 kDa CD133 in U87 cells may be a CD133 variant rather than the non-glycosylated full-length CD133, since the latter should be detected by the 'pan'-C24B9 antibody (20). Furthermore, the cytoplasmic distribution of CD133 in U87 cells revealed by C2E1 immunostaining indicated that this protein is not likely to be glycosylated.

Whether this novel CD133 antigen plays a role in the stem cell behavior of U87 cells, or whether it is present in other CSCs, remains unclear. CD133 analysis must not focus on specific glycosylated epitopes, but instead cover all the CD133 variants. The inclusion of different CD133 variants may produce more consistent results in the study of the CD133 biology in CSCs (20). C2E1, the novel anti-CD133 mAb that was produced in the current study, is able to recognize the 95 kDa CD133 variant and also bind full-length glycosylated CD133. Therefore, this novel mAb may be a valuable tool for the study of CD133 as a CSC marker.

An important advance in cancer therapy is the development of targeted agents that are able to neutralize the CSC response to circulating growth factors. The results of the current study indicated that incubation with C2E1 may inhibit proliferation in Caco-2 and U87-CD133 overexpression cells, indicating

that C2E1 exerts a neutralizing effect on $CD133^+$ cells; this may be of significance in the development of cancer therapies.

In conclusion, the novel anti-CD133 mAb, C2E1, indicated that endogenous CD133 expression is present in glioblastoma U87 cells. This mAb is able to bind cell surface CD133 and inhibit the proliferation of tumor cells.

Acknowledgements

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