DNA hypomethylation of CD133 promoter is associated with recurrent glioma

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Abstract. Gliomas are the most common type of brain tumor in the central nervous system of adults, and are highly aggressive, resistant to treatment, and prone to recurrence. Brain tumor stem cells (BTSCs) are implicated in tumor initiation and recurrence. Cluster of differentiation (CD)133 is currently the most widely used BTSC marker; however, its role in glioma development and progression is largely unknown. In this study, we evaluated CD133 expression in pairs of primary and recurrent human glioma specimens from 24 patients. We found that recurrent gliomas have aberrantly upregulated CD133 levels. To clarify the mechanism underlying this observation, we assessed CD133 promoter (P)2 methylation status by bisulfite sequencing and found that P2 hypomethylation was associated with the increase in *CD133* expression and glioma recurrence. These results suggest that CD133 overexpression in BTSCs due to P2 hypomethylation underlies glioma recurrence, which may provide insight into the mechanism of glioma recurrence and provide a basis for novel therapies for glioma treatment.

Introduction

Gliomas are the most common type of intrinsic brain tumor of the central nervous system in adults, and are highly aggressive and insensitive to treatment. Current treatment strategies have not significantly improved long-term survival of patients (1); the median survival is just 12-15 months for patients with glioblastoma (World Health Organization (WHO) IV), 2-5 years for patients with anaplastic glioma (WHO III), and 6-8 years for patients with low-grade glioma (WHO II) (2). Surgery is the preferred treatment option, which is followed by observation of postoperative remission (3,4). However, even when surgery is combined with chemo- or radiotherapy, relapse is inevitable (5-7).

Human gliomas contain a small population of cells with stem cell-like features known as brain tumor stem cells (BTSCs) (8). These cells are hypothesized to initiate tumor growth and recurrence, since they are resistant to standard anticancer therapies (9). BTSCs may be difficult to remove surgically since they do not form an obvious tumor mass and may not manifest tumor-specific histological features (9).

The stem cell surface antigen cluster of differentiation (CD)133 (also known as AC133 or human prominin-1) is expressed in a subset of neural stem/precursor cells in the adult central nervous system and is a marker used to identify and isolate BTSCs (10). CD133 expression increases with tumor grade and is correlated with glioma patient survival (11); moreover, glioma recurrence can be predicted by an increase in the fraction of CD133-positive cells within the primary tumor (12). However, the regulation of *CD133* gene expression in gliomas is not well understood.

Promoter methylation is a mechanism underlying the downregulation of CD133 in colon and ovarian cancers and glioblastoma (13-15). DNA methylation can prevent binding of transcription factors to their target binding sites, thereby repressing transcription. *CD133* is transcribed in a tissue-specific manner from five alternative promoters (P1-P5) (16): liver, kidney, pancreas, placenta, lung, spleen, and colon express transcripts containing exons 1A and 1B. On the other hand, exon 1B transcripts are expressed in the brain and ovary, and exon 1A transcripts in the prostate, fetal liver, and small intestine. All CD133 promoters are TATA-less and P1, P2, and P3 are located in a CpG island (16). Recent evidence suggests that DNA methylation of P1 and P2 plays a role in the regulation of *CD133* expression. P1 methylation is not tissue-specific, and therefore is not likely to affect transcript levels; in contrast, the tissue-specific methylation of P2 is inversely correlated with *CD133* expression (17).

In this study, we analyzed CD133 expression in pairs of primary and recurrent human glioma specimens from 24 patients and found that recurrent gliomas exhibited aberrantly

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upregulated CD133 expression. To clarify the underlying mechanisms, we analyzed P2 of the *CD133* gene by bisulfite sequencing, and found that *CD133* P2 hypomethylation is associated with increased *CD133* gene expression and glioma recurrence.

Materials and methods

Glioma tissues. Surgical specimens of gliomas were obtained from 24 patients who had undergone tumor resection at Affiliated Hospital of Nantong University from January 2004 to December 2014. The study was approved by the local ethics committee, written informed consent was obtained from all the patients. Recurrence of glioma was defined as the presence of glioma at >3 months after surgery for primary glioma. All patients received chemoradiotherapy after the first surgical intervention. Pathological findings were determined by more than 2 pathologists and classified according to the WHO classification standard (Table I). The paired primary and recurrent glioma specimens were from the same patient. For immunohistochemistry, glioma tissue was fixed with 4% formalin, dehydrated, and then embedded in paraffin. Portions of the tumor tissues were rapidly frozen by liquid nitrogen and stored at -80˚C until RNA and DNA extraction for real-time PCR, DNA methylation and western blot analysis.

Cell culture. The U251MG, U87MG, and A172MG glioblastoma cell lines were purchased from the Shanghai Cell Institution of Chinese Academic Sciences, which were cultured as described previously (18). They were maintained in Dulbecco's modified Eagle's medium: nutrient mixture F-12 (MDEM/F12) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). Cells were incubated at 37˚C in a humidified 5% CO_2 air atmosphere. When cell culture reached 50% confluence, U87MG, U251MG and A172MG cells were treated with 5-Aza-2'-deoxycytidine (5-Aza-dc, A3656; Sigma-Aldrich, St. Louis, MO, USA) at the final concentration of 10 nM for 72 h, respectively.

Western blot analysis. Cells or tissues were washed with cold PBS and lysed in ice-cold RIPA buffer containing protein inhibitors. Cell lysates were incubated on ice for 30 min and then centrifuged at 4˚C for 10 min. Supernatants were collected, and protein concentrations were measured. Equivalent amounts of protein in each sample were separated on a 10% SDS-PAGE gel for separation and then electrotransferred to PVDF membrane. Membranes were blocked and then probed with primary CD133 antibodies (2 μg/ml, Abcam) followed by the horseradish peroxidase (HRP)-conjugated goat anti-mouse or rabbit IgG antibodies. Mouse monoclonal anti-β-actin antibody (1:5,000, Sigma) was used as an internal control. The membranes were developed using an ECL detection system (Pierce, Rockford, IL, USA). The intensity of bands was determined using the Image-Pro Plus 6.0 software. The western blot experiments were repeated at least three times.

RNA extraction and real-time PCR. RNA expression levels of CD133 were determined using quantitative real-time PCR with GAPDH as positive controls. Total mRNA was isolated from glioma specimens and cell lines using mRNA isolation kit (Roche, UK) following the manufacturer's instructions. The concentration and purity of mRNA was determined by ultraviolet spectrophotometry. Isolated mRNA (100 ng) from each sample was transcribed to complementary DNA (cDNA) using a First-Strand cDNA Synthesis kit Roche (Roche), which was then used as a template for quantitative real-time PCR. Primers used for CD133 were 5'-GCACTCTATACCAAAGC GTCAA-3' (sense) and 5'-CTCCCATACTTCTTAGTTTCC TCA-3' (antisense); and for GAPDH primers were 5'-GGAA AGCTGTGGCGTGAT-3' (sense) and 5'-AAGGTGGAAGA ATGGGAGTT-3' (antisense). The primers were designed using Primer 5.0 software and manufactured by TIBmolbiol. After an initial denaturation at 95˚C for 5 min, the samples were subjected to 40 cycles of RT-PCR (95˚C for 10 sec, annealing temperature 59˚C for 15 sec, and 72˚C for 20 sec). At the end of each cycle, the fluorescence emitted was measured in a single step in channel F1 (gain 1). After the 40th cycle, the specimens were heated to 95˚C and rapidly cooled to 59˚C for 15 sec. All heating and cooling steps were performed with a slope of 20˚C/sec. The temperature was subsequently raised with a slope of 12˚C/sec and fluorescence was measured continuously (channel F1, gain 1) to obtain data for the melting curve analysis. The PCR reaction was subjected to a melting curve analysis to verify the presence of a single amplicon before the PCR products were visualized on agarose

Table I. WHO grades of glioma patients.

	WHO grade	
Patient	Primary	Recurrent
1	II	II
2	IV	IV
3	II	IV
4	III	III
5	III	IV
6	II	IV
7	III	IV
8	II	II
9	III	IV
10	IV	IV
11	IV	IV
12	III	III
13	II	II
14	IV	IV
15	IV	IV
16	IV	IV
17	III	III
18	II	IV
19	II	III
20	II	IV
21	IV	IV
22	IV	IV
23	IV	IV
24	IV	IV

gels using a gel analyser (SynGene, UK). All PCR reactions were performed in triplicate and a negative control was included that contained primers without DNA.

Immunohistochemical analyses. Serial sections measuring 5 μm thick were cut from paraffin blocks and mounted on glass slides coated with 10% polylysine. Sections were dewaxed in xylene and rehydrated in graded ethanols. Endogenous peroxidase activity was blocked by immersion in 0.3% methanolic peroxide for 30 min. Immuno-reactivity was enhanced by microwaving and incubating the tissue sections for 10 min in 0.1 mol/l citrate buffer. Tissues were then incubated with an anti-CD133 antibody (1:200, Abcam) overnight at 4˚C. Immuno-staining was performed using the avidin-biotin peroxidase complex method, and antigen-antibody reactions were visualized with chromogen diaminobenzadine. Appropriate positive and negative controls were used. Ten high-power fields were randomly chosen, and ≥300 tumor cells were counted per field. Percentage of cells showing positive staining in cytoplasm/membrane was designated as the CD133 labeling index, as a percentage (%). The staining procedures were repeated at least three times.

DNA isolation and bisulfite sequencing. With the proteinase K digestion and phenole-chloroform method, genomic DNA was extracted from frozen tissues (19). Sodium bisulfite treatment of the extracted DNA was performed as previously described (20). In brief, 10 μg DNA in 50 μl TE was incubated with 5.5 μl of 0.3 M NaOH at 37˚C for 15 min and 95˚C for 2 min, and subjected to sodium bisulfite chemical treatment (2.4 M sodium metabisulfite; 0.5 mM hydroquinone, pH 5.0, both from Sigma). Following incubation at 55˚C for 4 h, the treated DNA was purified using the SK1261 kit (Shenggong, China), desulfonated in 0.3 M NaOH, neutralized to pH 7.0 using 3 M sodium acetate (pH 5.2). The neutralized DNA was purified using SK1261 purification kit again, dissolved in TE buffer (pH 8.0).

The primers (fwd: 5'-TYGYGGTGAGTATGTTTAAGG-3', rev: 5'-ACCCAACTACTCACCRTACACC-3') were designed to amplify the promoter 2 from -237 to +52 for bisulfite genomic sequencing. An initial denaturation at 98˚C for 4 min was followed by five PCR cycles of 94˚C for 45 sec, 68˚C for 45 sec and 72˚C for 1 min. The PCR was then completed with 35 cycles of 45 sec at 95˚C, 45 sec at 58˚C. The amplified products were gel-purified using the SK1261 kit and subjected to TA-cloning using pUC18-T vector (Shenggong, Biotechnology Co.). Ten clones for each case were selected for sequencing using BigDye version 3.1, and analyzed on automated DNA sequence analyzer (ABI PRISM 3730; Applied Biosystems, Inc., Foster City, CA, USA). The cytosine or thymine residues at the CpG sites represented methylated or unmethylated status, respectively.

Statistical analyses. Statistical analysis was performed using SPSS 13.0 for Windows. Data are expressed as median and 25-75 percentiles [median (25th percentile, 75th percentile)]. Mann-Whitney U test was used to determine the differences of CD133 expression between primary and recurrent tumor. Paired t-test was used to analyze the differences of CD133 expression between the treated glioma cell lines. The correlation between CD133 DNA methylation and mRNA expression was analyzed by Pearson's correlation test. All statistical tests were calculated two-sided and values of P<0.05 was considered to be statistically significant.

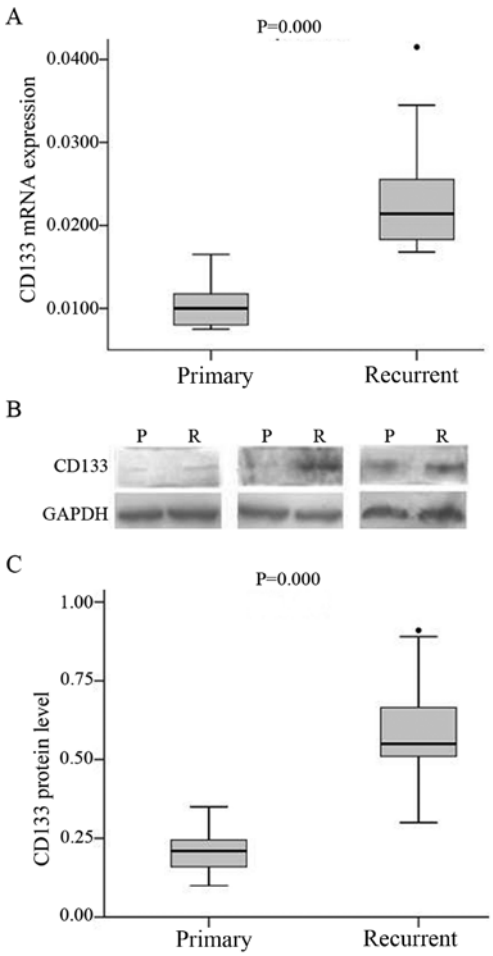

Figure 1. *CD133* expression in pairs of primary and recurrent glioma. (A) *CD133* mRNA relative levels by real-time PCR. The value was quantified compared with GAPDH. (B) CD133 protein levels by western blotting. The blot shows representative results from the pairs of primary and recurrent gliomas. GAPDH was used as a loading control. P, primary gliomas; R, recurrent gliomas. (C) Statistical analysis of CD133 protein in primary and recurrent gliomas. Data are presented as box plots, where the lines inside the boxes represent the medians, the boxes represent the 25th and 75th percentiles, and the lines outside the boxes represent the 10th and 90th percentiles.

Results

CD133 expression is upregulated in recurrent glioma. CD133 mRNA and protein expression in primary and recurrent glioma specimens was assessed by real-time PCR and western blotting, respectively. While *CD133* transcript was detected in all specimens, the levels were higher in recurrent (median 0.0214; range, 0.0182-0.0249) than in primary glioma (median 0.1,000; range, 0.0080-0.0119, P<0.001) (Fig. 1A). A similar trend was observed for CD133 protein level (Fig. 1B and C).

CD133-positive cell number is increased in recurrent as compared to primary glioma. CD133 protein expression and localization in glioma specimens were evaluated by immunohistochemistry. CD133-positive cells were detected in all specimens, and expression was mainly observed in the cytoplasm and plasma membrane (Fig. 2A). The percentage

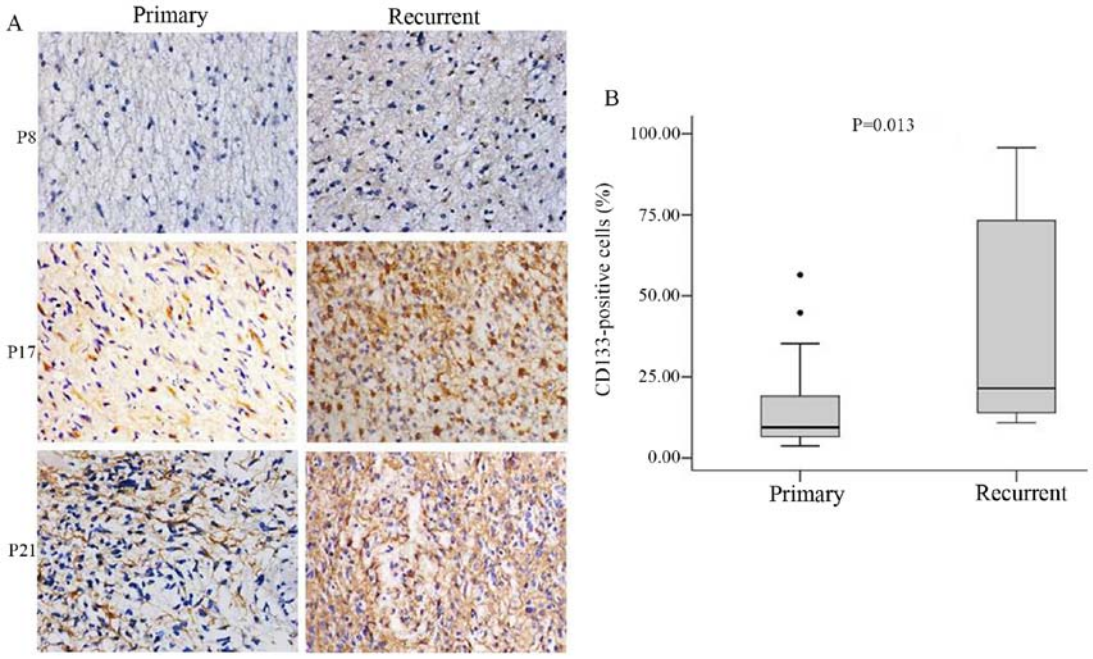

Figure 2. CD133-positive cell number is increased in recurrent glioma by IHC. (A) CD133 expression in paraffin-embedded sections from representative 3 pairs of primary and recurrent gliomas. CD133 expression was primarily localized in the cytoplasma and the membrane of tumor cells (brown). (B) Statistical analysis of the percentage of CD133-positive cell number in primary and recurrent gliomas. Data are presented as box plots, where the lines inside the boxes represent the medians, the boxes represent the 25th and 75th percentiles, and the lines outside the boxes represent the 10th and 90th percentiles.

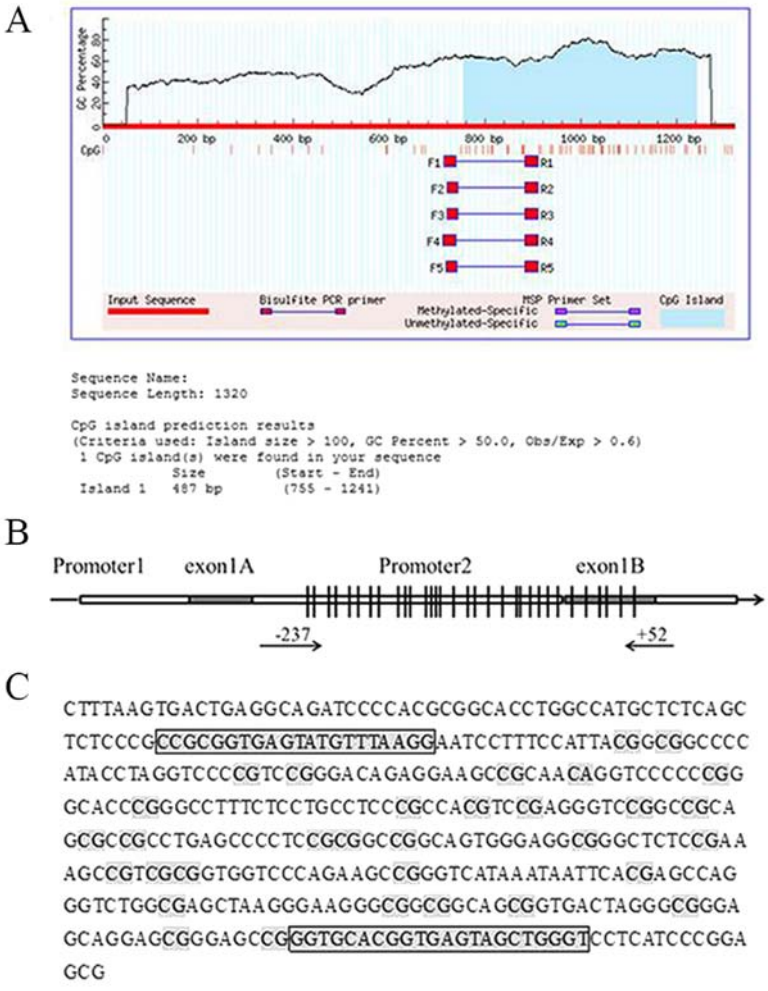

Figure 3. The diagram of CpG islands in CD133. (A) Structure of CD133 promoter, showing one CpG island (blue-shaded region). (B) The sketch of detection region. (C) Sequence of CpG island. The bisulfite sequencing PCR primers are boxed. Thirty-two CpG sites are labeled with gray background.

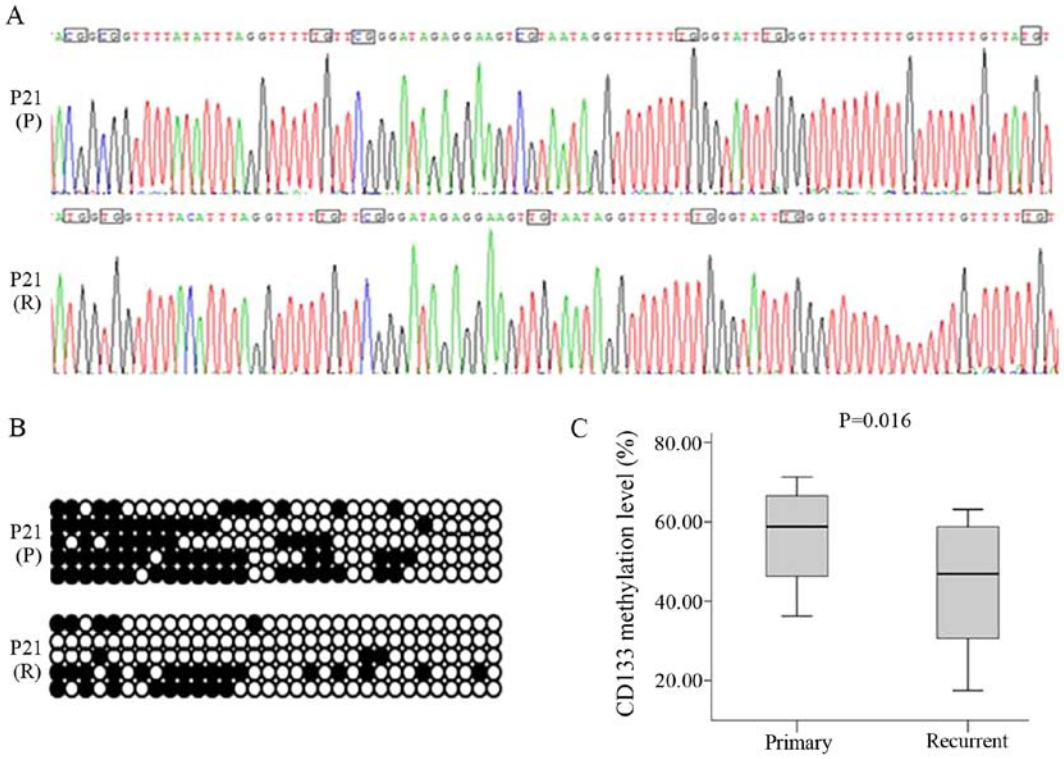

Figure 4. Hypomethylation of the CD133 promoter in recurrent glioma. (A) Sequencing of CpG island. CG represents methylated, TG represents unmethylated. (B) Methylation patterns of CpG island. Black and white circles represent methylated and unmethylated sites, respectively. (C) Statistical analysis of CD133 promoter methylation in primary and recurrent gliomas. Data are presented as box plots, where the lines inside the boxes represent the medians, the boxes represent the 25th and 75th percentiles, and the lines outside the boxes represent the 10th and 90th percentiles.

of CD133-positive cells was significantly higher in recurrent (median 12.99%; range, 3.94-58.23%) as compared to primary glioma (median 4.35%; range, 2.43-14.35%, P<0.05) (Fig. 2B).

Hypomethylation of the CD133 promoter increases CD133 expression in recurrent glioma. To clarify the mechanism underlying the upregulation of CD133 level in recurrent glioma, we analyzed the methylation status of CpG islands in the *CD133* promoter. A sequence analysis revealed that the *CD133* P2 promoter contains a 487-bp CpG island, with a GC content >50% and a CpG ratio (Obs/Exp) >0.6 (Fig. 3A). The P2 promoter region (–237 to +52) contained 32 CpG sites, as determined by bisulfite genomic sequencing (Fig. 3B and C). The 332-bp CD133 target fragment was cloned and sequenced, revealing unmethylated C residues that were completely converted to U by bisulfite treatment; these were replaced by T following PCR amplification. In contrast, methylated C residues persisted after bisulfite treatment, indicating that CpG islands of the *CD133* promoter were methylated (Fig. 4A and B). However, DNA methylation levels were lower in recurrent (median, 46.88%; range, 26.56-59.69%) as compared to primary glioma (median, 58.76%; range, 42.19-67.35%, P<0.05) (Fig. 4C). Correlation analysis showed a negative relationship between DNA methylation and CD133 expression levels (r = -0.715, P<0.05).

Promoter demethylation leads to upregulation of CD133 expression. To assess the effects of demethylation *in vitro*, we treated U87, U251, and A172 glioma cells with the demethylating agent 5-aza-2'-deoxycytidine (10 μM for 72 h). *CD133* mRNA expression was upregulated in these cells by 2.15, 2.56, and 3.03-fold, respectively (Fig. 5A). A similar trend was observed for CD133 protein expression (Fig. 5B). These results indicate that CD133 demethylation increases CD133 level in glioma.

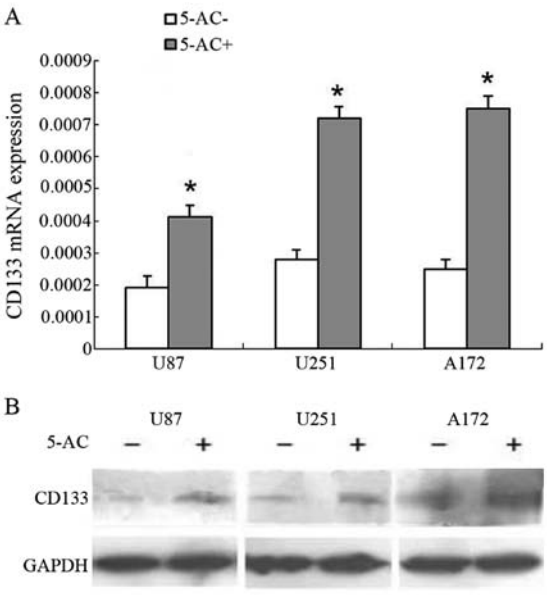

Figure 5. CD133 expression in glioma cell lines after treatmeat with 5-AC. (A) Real-time PCR analysis of *CD133* mRNA expression. Error bars represent the standard deviation. Compared with untreated group *P<0.05. (B) Western blot analysis of CD133 protein levels. GAPDH was used as a loading control.

Discussion

BTSCs are thought to be the main determinants of the occurrence, development, metastasis, recurrence, and treatment sensitivity of malignant glioma (21). BTSCs can form the same pathological type of tumor as the parent tumor both *in vitro* and *in vivo* (22,23). However, the direct study of BTSCs is challenging due to the lack of specific markers. In theory, expression levels of stem cell-related genes represent changes in the BTSC population of gliomas. The most common stem cell-related genes are nestin, *CD133, ATP-binding cassette superfamily G member 2, SRY box-containing gene 2, POU class 5 homeobox 1/OCT4*, and *musashi-1*. In our previous study we reported the expression and methylation status of *OCT4* in glioma (18). Based on these findings and other previous reports, we hypothesized that BTSCs initiate gliomas and are responsible for their recurrence.

CD133 is a cell membrane glycoprotein that has been identified as a marker of a subset of BTSCs in the adult central nervous system and in glioblastoma stem-like cells (10,11). CD133-positive cells exhibit stem cell-like qualities *in vitro* and are capable of tumor formation *in vivo* (24). CD133-positive BTSCs in an animal model showed an unlimited capacity for self-renewal and for inducing tumor initiation and progression (25). CD133 has been isolated from hematopoietic stem cells using an antibody that recognizes the AC133 epitope, and is used as a marker for the isolation of brain cancer stem cells (26).

In this study, we determined that CD133 expression was upregulated in recurrent as compared to newly diagnosed glioma specimens. This suggests that there were more residual BTSCs in recurrent than in primary glioma. Surgery is an invasive procedure that is accompanied by tissue injury, the production of inflammatory cytokines, angiogenesis, and glioma cell proliferation, which can lead to changes in the local microenvironment and the recruitment of residual BTSCs to the surgery site. The proliferation of BTSCs and their differentiation into glioma cells (27,28) result in the upregulation of CD133 expression and glioma recurrence.

Changes in DNA methylation patterns are an important hallmark of tumor development and progression (29). CD133 contains five alternative promoters, three of which are partly regulated by methylation (16). AC133 promoters are TATA-less and three (P1-P3) are located within a CpG island. *In vitro* methylation suppressed the activity of P1 and P2, and recent studies have shown that DNA methylation is inversely correlated with *CD133* transcription (13,30,31). Here, we found that *CD133* transcription in glioblastoma is dependent on DNA hypomethylation, which has been shown to promote tumorigenesis by inducing the activation of oncogenes and/or causing genomic instability (32). Thus, *CD133* promoter hypomethylation may be associated with the maintenance of BTSCs in brain tumors. It has been proposed that a reduction in overall genomic methylation, an important feature of tumor cells, is responsible for decreased methylation of specific genes (33).

Treatment of glioma cell lines with a demethylating agent increased *CD133* mRNA and protein expression, which was consistent with changes reported for some oncogenes (34). DNA methylation status regulates the transition from CD133 transcriptional activation to repression in glioma. DNA methylation is reversible; as such, methylation status and consequent dysregulation of target gene expression can be altered by drug treatment (35). In conclusion, the results of this study provide insight into the mechanism of glioma recurrence and provide a basis for novel therapies for glioma treatment.

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