Original Article
B7-H3 repression by miR-539 suppresses cell proliferation in human gliomas

Rong-Gang Li[1], Zhuo Gao[2], Yin Jiang[3]

[1]Department of Neurosurgery, Jinshan Hospital, Fudan University, Jinshan District, Shanghai, China; [2]Department of Clinical Laboratory, The Fourth Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang, China; [3]Department of Neurosurgery, Shanghai General Hospital, Shanghai, China

Abstract: Accumulating evidence has demonstrated that B7-H3 is deregulated in several cancers and is closely associated with malignant cell behavior. In the present study, we aimed to explore the expression pattern of B7-H3 and its functional relevance in glioma cells. Real-time PCR and western blotting were performed to detect the mRNA and protein expressions of B7-H3, respectively. Glioma cell growth was measured by CCK-8 and colony formation assays. A luciferase activity assay was used to validate the regulatory mechanism of B7-H3 by microRNA miR-539. CD276 (the gene encoding B7-H3) mRNA and protein levels were elevated significantly in glioma cell lines, including U87 and U251. Downregulation of CD276 mediated by a specific short interfering RNA led to decreased cell proliferation and colony formation in glioma cells. In addition, bioinformatic analysis revealed that CD276 was a potential target of miR-539; this was further validated by a luciferase activity assay. Furthermore, ectopic expression of miR-539 repressed the expression of CD276 and thus inhibited the growth of glioma cells. Taken together, the results showed that miR-539-mediated B7-H3 repression was implicated in glioma cell proliferation, suggesting that regulation of the miR-539/B7-H3 axis might represent a novel therapeutic strategy to treat glioma.

Keywords: Glioma, B7-H3, miR-539, cell proliferation

Introduction

Gliomas are among the most frequently occurring brain cancers; however, they show poor prognosis and low five-year survival rates [1]. Despite great improvements in therapeutic modalities, including surgery, radiotherapy, and chemotherapy, the overall survival and prognosis of gliomas remain unsatisfactory [2, 3]. Over recent decades, a large number of genes have been implicated in human cancers, including gliomas, breast cancer, colorectal cancer, and hepatocellular carcinoma. In particular, many genetic markers play critical roles in the malignant behavior of tumors, making them useful diagnostic tools and therapeutic targets.

B7-H3 belongs to the B7 superfamily, a molecule that inhibits or stimulates T-cell responses, and is encoded by the CD276 gene in humans. Originally, B7-H3 was identified in a dendritic cell cDNA library, with low expression levels in several normal lymphoid and peripheral tissues [4]. Initially, B7-H3 was shown to present stimulatory signals to T cells [5]. However, additional studies demonstrated a negative regulatory role for B7-H3 in T cell responses, suggesting a complex functional relevance in immune reactions [6]. Recently, several studies have suggested that B7-H3 is upregulated abnormally in a variety of cancers, such as non-small cell lung cancer, prostate cancer, and pancreatic cancer [7, 8]. Moreover, Sun et al. reported that B7-H3 was expressed abundantly in colorectal cancer, especially in patients with a more advanced tumor grade [9]. Chen et al. demonstrated that the ectopic expression of B7-H3 suppressed anti-tumor immune responses in lung cancer cells [10]. Functionally, inhibition of B7-H3 inhibits cell migration and invasion, and sensitizes tumor cells to chemotherapeutic drugs [11, 12]. However, the expression pattern and precise role of B7-H3 in gliomas are unknown. Therefore, the present study aimed to explore the expression profile of B7-H3 and its functions in gliomas.

Materials and methods

Cell culture

The human glioma cell lines, U87 and U251, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Normal human astrocytes were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in an atmosphere containing 5% CO_2 at 37°C.

Cell viability assay

Cells at a density of $5×10^4$/mL were seeded into a 96-well plate to determination their viability. The plate was incubated at 37°C for 48 h. Then, 10 μL/well of CCK8 was added to each well and incubated for 4 h. The absorbance of the reaction was detected at 490 nm using a spectrophotometer (BioRad, Hercules, CA, USA).

Real-time PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen, USA), according to the manufacturer's instructions. cDNA was synthesized from 2 μg of total RNA using a Prime-Script RT reagent Kit (Takara, Tokyo, Japan), according to the manufacturer's protocol. The PCR reaction was performed in a volume of 20 μl. The PCR program was as follows: 95°C for 1 min; 40 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 40 s; and 72°C for 7 min. The relative expression of each gene was calculated using the $2^{ΔΔCt}$ method.

Colony formation

Cells were cultured in 6-wells plates for 48 h. The cells were then washed, fixed with methanol, and stained with a 5% Giemsa solution. The colonies were photographed (100× magnification) on five different fields and the relative colony number was calculated.

Western blotting

Cells were lysed in buffer containing 50 mM Tris, 150 mM NaCl, 1% NP-40, 10% glycerol, 10 mM Na_3VO_4, pH 7.5, 1 mM EDTA, and a Roche protease inhibitor cocktail. Proteins were extracted by centrifugation and were subject to SDS-PAGE followed by transfer onto polyvinyli-dene fluoride membranes. The membrane was incubated with anti-B7-H3 and anti-GAPDH antibodies (Santa Cruz, USA), followed by washing and incubation with the appropriate secondary antibodies. Protein signals were detected using an ECL kit (Pierce, Appleton, WI, USA) and quantified using a Bio-Rad imaging densitometer.

Luciferase assays

After transfection, cells were lysed and the extracts were centrifuged for 2 min at 13000× g. The luciferase activities were measured in the supernatants using the Dual-Luciferase Reporter Assay system (Promega, Madison, WI, USA), according to the manufacture's instruction.

Statistical analysis

Data are presented as means ± SD and were analyzed using SPSS 16.0 program (SPSS Inc., Chicago, USA). Comparison between groups was made using Student's t test or an ANOVA test. A difference with a *P*-value <0.05 was considered statistically significant.

Results

B7-H3 expression was increased in glioma cells

Several studies have revealed that the expression levels of B7-H3 are upregulated in cancer tissues, including lung cancer, pancreatic carcinoma, colorectal cancer, and gliomas [7, 9, 13]. Based on these observations, we measured the CD276 expression pattern in glioma cell lines using real-time PCR. Compared with the normal human astrocytes, CD276 mRNA expression was increased significantly in the glioma cell lines U87 and U251 (**Figure 1A**). Consistently, western blotting analysis also revealed that the protein levels of B7-H3 were obviously higher in U87 and U251 cells (**Figure 1B**). Taken together, these data showed that B7-H3 was overexpressed in glioma cell lines, suggesting the possible functional relevance of B7-H3 in the tumor's biological behavior.

Knockdown of B7-H3 inhibited glioma cell proliferation and colony formation

Previous studies have shown that B7-H3 might function as an oncogenic factor in tumor cells [8]. Thus, we silenced the expression of CD276 mRNA in glioma cells and investigated the role
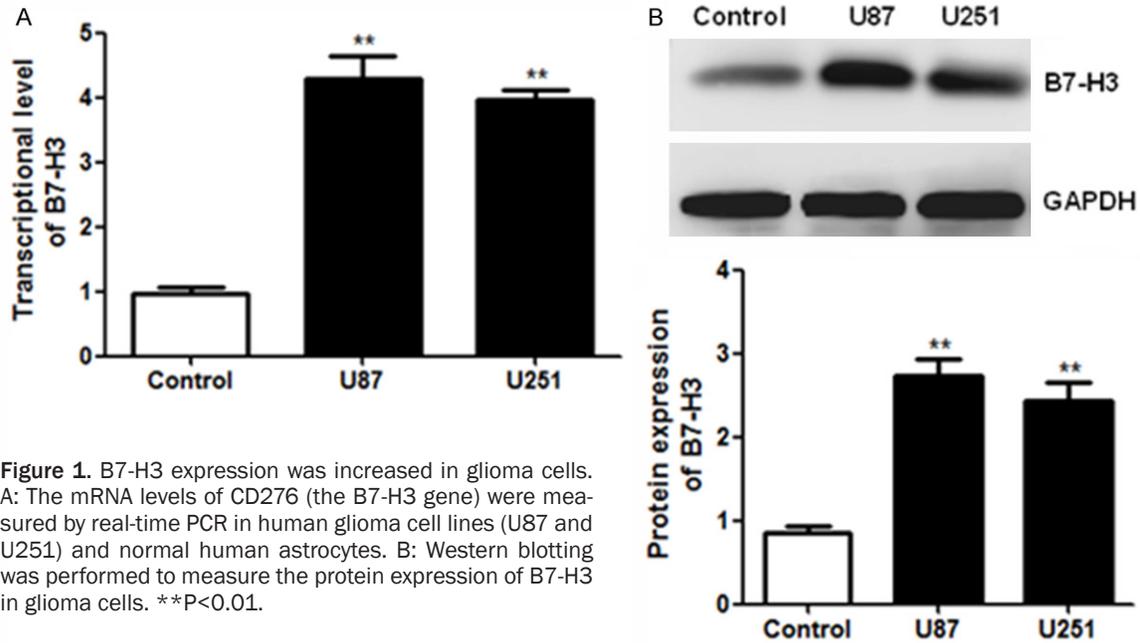
Figure 1. B7-H3 expression was increased in glioma cells. A: The mRNA levels of CD276 (the B7-H3 gene) were measured by real-time PCR in human glioma cell lines (U87 and U251) and normal human astrocytes. B: Western blotting was performed to measure the protein expression of B7-H3 in glioma cells. **P<0.01.
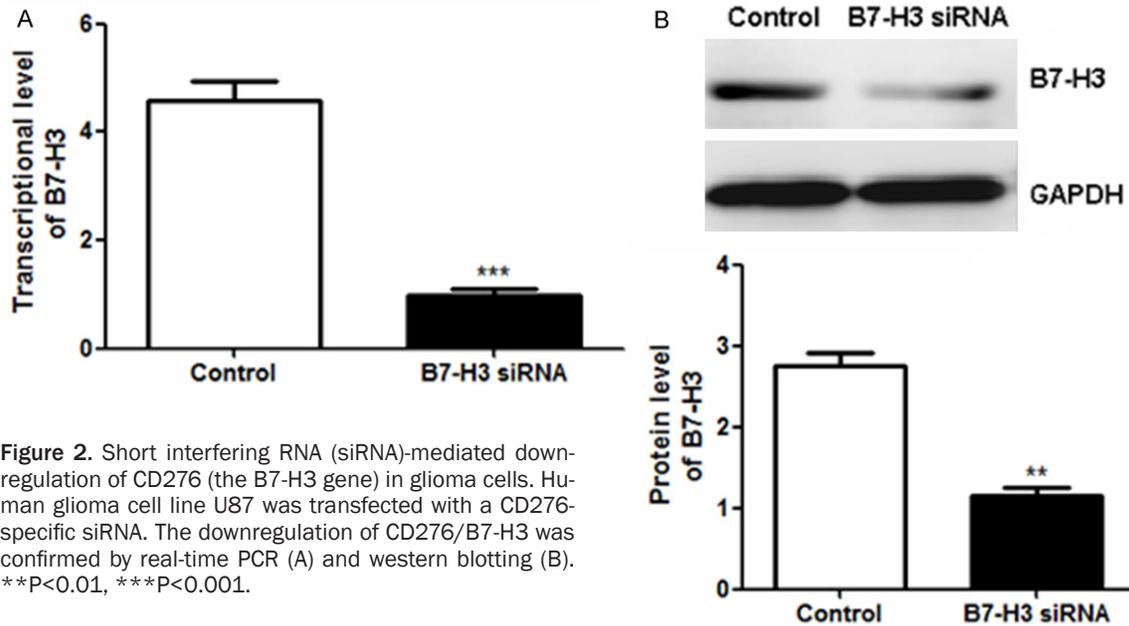
Figure 2. Short interfering RNA (siRNA)-mediated downregulation of CD276 (the B7-H3 gene) in glioma cells. Human glioma cell line U87 was transfected with a CD276-specific siRNA. The downregulation of CD276/B7-H3 was confirmed by real-time PCR (A) and western blotting (B). **P<0.01, ***P<0.001.

of B7-H3 inhibition in the regulation of tumor cell behavior. After transfection with a CD276 specific short interfering RNA (siRNA), we found that CD276 transcripts were downregulated significantly in CD276 siRNA-transfected U87 cells (**Figure 2A**). Moreover, the protein levels of B7-H3 were also decreased by more than two-fold in U87 cells after transfection with the CD276-specific siRNA (**Figure 2B**). Consequently, downregulation of B7-H3 dramatically inhibited the proliferation and colony formation

of glioma cells (**Figure 3A** and **3B**). Collectively, these results suggested that inhibition of B7-H3 had anti-proliferative effects in glioma cells.

B7-H3 expression was regulated by miR-539 in glioma cells

MicroRNAs (miRNAs), a group of small non-coding RNAs, are ultimately transformed into RNA-induced silencing complex after interaction with RNA Pol II and Dicer [14]. Via binding
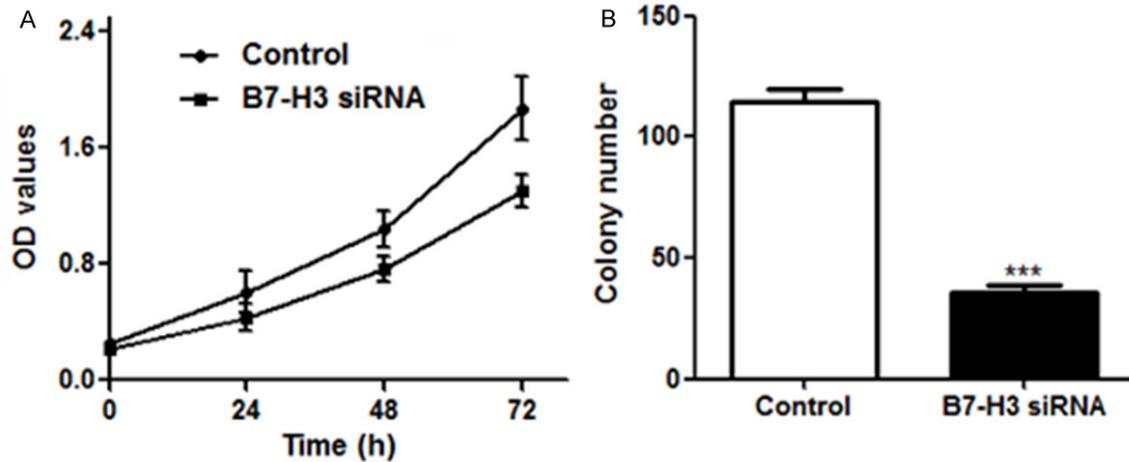
Figure 3. Effects of CD276 (the B7-H3 gene) knockdown on glioma cell growth and colony formation. After transfection with a CD276-specific siRNA, the cell proliferation (A) and colony formation (B) of U87 cells were determined. ***P<0.001.
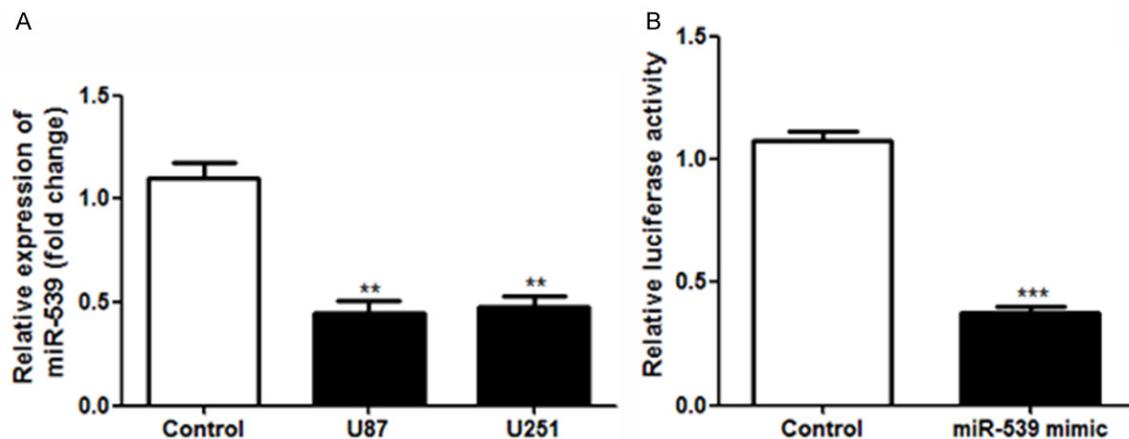

Figure 4. MicroRNA miR-539 regulated the expression of CD276 (the B7-H3 gene) in glioma cells. The expression of miR-539 was detected in human glioma cell lines (U87 and U251) and normal human astrocytes (A). Luciferase activity was measured in U87 cells after transfection with an miR-539 mimic (B). **P<0.01, ***P<0.001.

to target mRNAs, miRNAs regulate the expression of target genes and thus affect diverse biological processes [15]. The deregulation of B7-H3 in several types of cancers led us to hypothesize the regulatory mechanism of B7-H3 was mediated by miRNAs. Interestingly, bioinformatic analysis showed that CD276 was a potential target of miR-539, which has been reported to exhibit tumor suppressor properties in prostate and thyroid cancer [16, 17]. In contrast to CD276, the expression of miR-539 was downregulated significantly in glioma cell lines compared with normal human astrocytes (**Figure 4A**). Additionally, co-transfection with an miR-539 mimic suppressed the luciferase

activity of B7-H3 in U87 cells substantially (**Figure 4B**). Taken together, these data suggested that miR-539 targets CD276 directly in glioma cells.

CD276 repression by miR-539 mediated the proliferation of glioma cells

Given that CD276 is a direct target of miR-539, we further examined the effect of CD276 repression by miR-539 on tumor cell behavior. We transfected the miR-539 mimic into U87 cells and found that ectopic expression of miR-539 decreased the mRNA expression of CD276 in tumor cells significantly (**Figure 5A**).
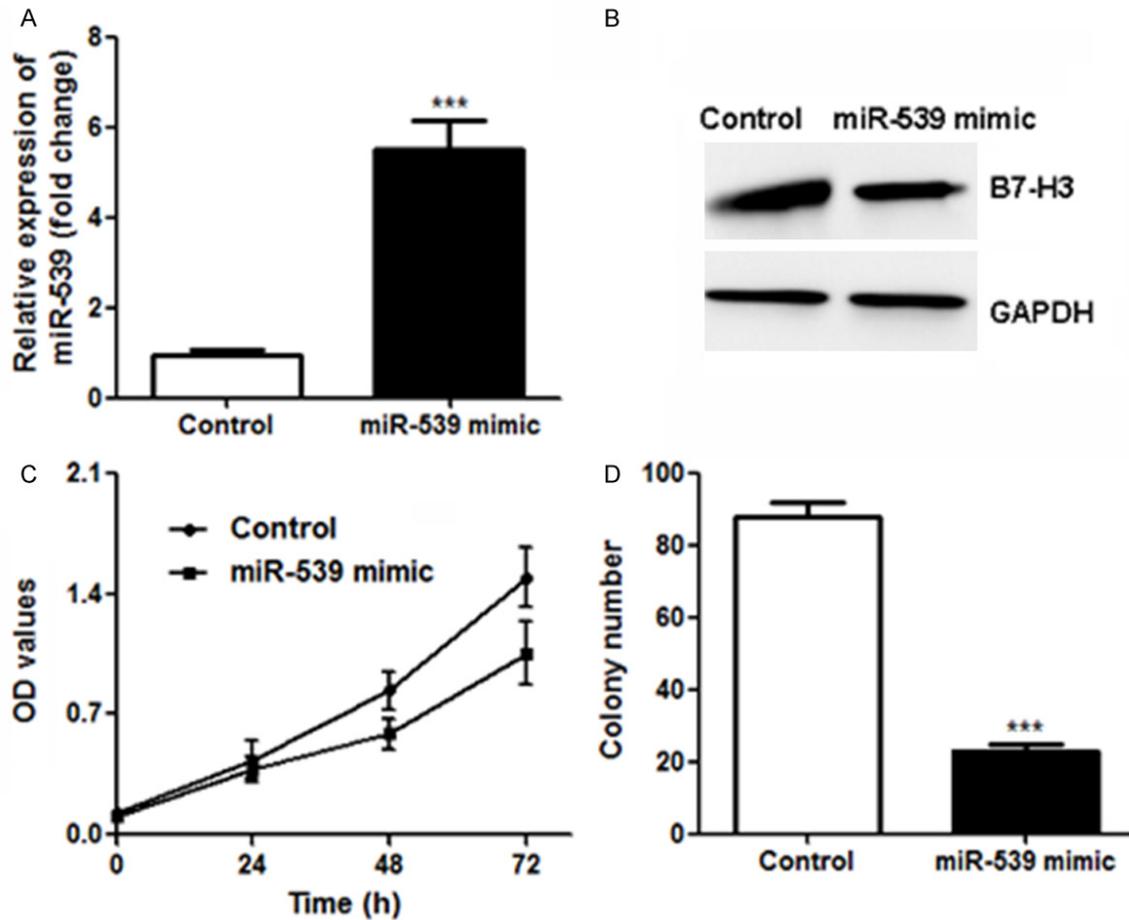
Figure 5. Effects of CD276 (the B7-H3 gene) repression by microRNA miR-539 on glioma cell proliferation and colony formation. (A) U87 cells were transfected an miR-539 mimic and the upregulation of miR-539 was confirmed by real-time PCR. (B) Western blotting was performed to analyze the protein level of B7-H3 in U87 cells transfecting with the miR-539 mimic. Determination of cell proliferation (C) and colony formation (D) in U87 cells transfecting with the miR-539 mimic. ***$P<0.001$.

Moreover, western blotting analysis revealed that the protein level of B7-H3 was reduced dramatically in U87 cells transfected with the miR-539 mimic (**Figure 5B**). Consequently, a CCK-8 assay showed that cell proliferation was inhibited substantially after transfection with the miR-539 mimic (**Figure 5C**). In addition, CD276 repression by miR-539 also led to a reduction in the number of cell clones (**Figure 5D**). Taken together, these data revealed that CD276 repression by miR-539 mediated glioma cell proliferation.

Discussion

Malignant gliomas are the most common primary brain tumors derived from the central nervous system. Although significant improvements have been made in therapeutic modali-

ties, the prognosis of patients with gliomas remains unsatisfactory [1, 2]. In the present study, we examined the expression pattern of B7-H3 and its regulation by an miRNA in glioma cells.

Human B7-H3 belongs to the immunoglobulin family and is usually expressed at a low level under physiological conditions [4]. Nevertheless, elevated expression of B7-H3 has been observed in several different types of cancers, including breast cancer, neuroblastoma, non-small cell lung cancer, and clear cell renal cell carcinoma [18-20]. Liu et al. reported that the expression of B7-H3 correlates positively with advanced tumor stage and lymph node metastasis in breast cancer [21]. Another study suggested that B7-H3 levels are associated significantly with tumor size and might serve as a

novel biomarker in patients diagnosed with cervical cancer [22]. The functional relevance of B7-H3 in a broad range of biological processes has been investigated extensively. For example, ectopic expression of B7-H3 increased the proliferation and invasion of hepatocellular carcinoma cells via regulation of the epithelial-to-mesenchymal transition [23]. Downregulation of CD276/B7-H3 mediated by siRNAs suppressed cell growth and colony formation, and increased chemosensitivity in acute monocytic leukemia [12]. However, the precise role of B7-H3 in malignant gliomas has not been elucidated. Our study found that CD276/B7-H3 mRNA and protein levels were upregulated significantly in glioma cell lines. Additionally, functional investigation revealed that siRNA-mediated knockdown of CD276 led to a reduction of cell growth and colony-forming abilities in glioma cells.

MiRNAs are a group of small noncoding RNAs of 18-22 nt in length. They modulate the expression of target genes by binding to the 3' untranslated regions of specific mRNAs. Subsequently, miRNAs promote the degradation of the target mRNAs or suppress protein translation [15]. Accumulating evidence has demonstrated a critical role of miRNAs in cancer cell growth, migration, and invasion [24, 25]. In the present study, we found that CD276 was a potential target of miR-539 in glioma cells. Moreover, we identified that the expression of miR-539 was obviously decreased in glioma cells. MiR-539 has been reported in several types of cancer cells, including prostate cancer, thyroid cancer, and osteosarcoma [16, 17, 26]. Upregulation of miR-539 inhibited the malignant behavior of cancer cells by targeting a variety of genes, such as SPAG5, CARMA1, and the gene encoding matrix metallopeptidase-8 [16, 17, 27]. These studies suggested the anti-tumor properties of miR-539. Consistently, our study found that CD276 repression by miR-539 led to decreased cell proliferation and colony number in glioma cells, suggesting that miR-539 exerted its anti-cancer effects by regulating CD276 expression in glioma cells.

In conclusion, our current study demonstrated that miR-539-mediated downregulation of CD276/B7-H3 was implicated in glioma cell proliferation. These findings might provide a novel therapeutic target for glioma treatment.

Disclosure of conflict of interest

None.

Address correspondence to: Yin Jiang, Department of Neurosurgery, Shanghai General Hospital, Shanghai 200080, China. Tel: +86-21-377985-66; Fax: +86-21-37798631; E-mail: jiangyin2410@126.com

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