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MiR-29a-Mediated CD133 Expression Contributes to Cisplatin Resistance in CD133$^+$ Glioblastoma Stem Cells

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Abstract

CD133 positive (CD133$^+$) cells are cancer stem cells in glioblastoma that are associated with poor prognosis and resistance to radiotherapy. However, the role of CD133 in chemoresistance is inconclusive, although recent studies suggest that increased CD133 expression may lead to increased cisplatin resistance under certain circumstances. In this study, we further explored the mechanism underlying CD133-mediated cisplatin resistance in glioblastoma stem cells. We sorted human glioblastoma T98G and U87MG cells into CD133$^+$ and CD133$^-$ pools and measured apoptosis and CD133 expression levels in response to cisplatin treatment. We predicted candidate microRNAs that might target CD133 and assessed their levels in cisplatin-treated CD133$^+$ cells. Finally, we overexpressed miR-29a in CD133$^+$ cells and tested its effects in cisplatin-mediated apoptosis and survival of CD133$^+$ tumor bearing mice receiving cisplatin treatment. We found that CD133$^+$ glioblastoma stem cells showed more resistance to cisplatin treatment. Cisplatin increased CD133 expression by suppressing miR-29a levels. MiR-29a overexpression improved sensitivity of cisplatin in CD133$^+$ cells and significantly suppressed tumor growth in CD133$^+$ tumor bearing mice in response to cisplatin treatment. Our data show that miR-29a ameliorates CD133-mediated chemoresistance in glioblastoma stem cells, suggesting it as a potential therapeutic target for treating glioblastoma.

Keywords Glioblastoma · CD133 · miRNA-29a · Cisplatin · Chemoresistance

Introduction

Glioblastoma (GBM) is the most common malignancy in the central nervous system and the most aggressive glioma with poor prognosis and high recurrence rates. In the recent years, the cancer stem cell hypothesis has been proposed, providing a new avenue for understanding tumorigenesis as well as developing therapeutic strategies against solid tumors including GBM (Lathia et al. 2015; Reya et al. 2001; Stopschinski et al. 2013). Cancer stem cells are able to self-renew and contribute to tumor recurrence and therapeutic resistance (Lathia et al. 2015). CD133 is a cell surface marker of cancer stem cells, and CD133 positive (CD133$^+$) cells are regarded as tumor initiation cells in GBM (Pollard et al. 2009). Injection of CD133$^+$ cells in mice generates tumors that possess the properties of human patient tumor (Singh et al. 2004). Additionally, higher expression of CD133 is associated with more severe disease status and poorer prognosis (Han et al. 2016).

Previous studies have established an association between CD133 and radio-resistance of GBM. CD133$^+$ cells can survive and accumulate after high dose of radiation therapy, leading to tumor recurrence in patients (Tamura et al. 2010). Similar results are also observed in cell culture and mouse brains where CD133$^+$ tumor cells are enriched as a result of ionizing radiation (Bao et al. 2006). The role of CD133 in chemotherapy is not clearly documented. Previously, it has been shown that CD133 expression was increased in cells cultured under hypoxic conditions, and these GBM cells also showed increased cisplatin resistance (Ahmed et al. 2018). Knockdown of CD133 improved sensitivity to cisplatin treatment, suggesting that CD133 may play a role in cisplatin resistance in GMB cells under hypoxia conditions. Although it is not clear how CD133 is associated with cisplatin resistance, there seems to be a general consensus that high CD133 expression confers resistance to cancer therapy. In fact, we have previously

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shown that $CD133^+$ GBM stem cells exhibited increased proliferation and migration (Yang et al. 2016), consistent with the fact that $CD133^+$ cells are the source of tumor recurrence. Targeting pathways that suppress CD133 expression may thus represent an effective therapeutic strategy. In the current study, we further explored the functions of CD133 in cisplatin resistance by comparing $CD133^+$ and CD133 negative ($CD133^-$) GBM cells. Aiming to find a molecular mechanism that regulates CD133 expression in response to cisplatin treatment, we further searched potential microRNAs that target CD133.

MicroRNAs are small non-coding RNAs that regulate target gene expression. Differential expression of various microRNAs is closely associated with cancer development, including GBM (Agrawal et al. 2014). For example, our previous study identified miR-154 to be overexpressed in $CD133^+$ GBM cell lines and miR-154 knockdown suppressed $CD133^+$ GBM cell proliferation and migration (Yang et al. 2016). However, a microRNA that directly targets CD133 has not been previously identified and how microRNAs are involved in the cisplatin treatment of $CD133^+$ tumors are not known. In this study, we identified a specific microRNA miR-29a that directly regulates CD133 expression, and its levels are suppressed in $CD133^+$ cells treated with cisplatin. Importantly, we found that its overexpression could significantly extend the survival of $CD133^+$ tumor bearing mice in response to cisplatin treatment.

Materials and Methods

Cell Culture and Drug Treatments

Human GBM T98G and U87MG cells were acquired from ATCC (MD, USA) and maintained according to standard protocols in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Cells were treated with 20-μM cisplatin or vehicle for 72 h before they were harvested for further analysis.

Flow Cytometry Analysis

Both T98G and U87MG cells were divided into CD133 positive ($CD133^+$) and negative ($CD133^-$) portions through fluorescence-activated cell sorting (FACS) following anti-CD133 antibody staining as previously described (Yang et al. 2016). Apoptosis of GBM cells was analyzed using an Annexin V-FITC & PI apoptosis kit (Biouniquer) according to the manufacturer's instruction. Apoptotic cells were then detected by flow cytometry.

Quantitative Real Time-PCR (qRT-PCR)

CD133 mRNA levels were measured by qRT-PCR and normalized to the housekeeping gene *HPRT* as previously described (Ahmed et al. 2018). Primers for CD133 are 5′-CAATCTCCCTGTTGGTGATTTG-3′ and 5′-ATCACCAGGTAAGAACCCGGA-3′. Primers for *HPRT* are 5′-ATTATGCTGAGGATTTGGAAAGGG-3′ and 5′-GCCTCCCATCTCCTTCATCAC-3′. Briefly, total RNA was extracted using TRIzol® RNA Isolation Reagents (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using an equivalent amount of RNA through the iScript™ cDNA Synthesis Kit. qRT-PCR was performed using SsoAdvanced™ Universal SYBR® Green Supermix according to the standard protocol.

Lentivirus-Mediated Overexpression of miR-29a

Lentiviral overexpression of miR-29a was performed according to a previously described protocol (Zollner et al. 2014). Briefly, small hairpin-miR-29a was cloned into pLKO.1 puro vector (addgene, no. 8453) and packaged into lentivirus in HEK 293T cells. Subsequently, $CD133^+$ T98G or U87MG GBM stem cells were infected with lentiviruses expressing miR-29a as indicated.

Xenograft Mouse Model

BALB/C nude mice were housed in the Second Hospital of Hebei Medical University animal facility under standard environment with free access to water and food. Tumor model of BALB/C mice was established by subaxillary inoculation of 1×10^6 $CD133^+$ T98G cells at the logarithmic growth phase. Eighty mice were randomly assigned to four groups with 20 mice per group: control, cisplatin treatment, miR-29a overexpression, and cisplatin treatment + miR-29a overexpression. Cisplatin was injected intraperioneally (ip) at a dose of 10 mg/kg once a day for 10 consecutive days. miR-29a was administered by ip injection once a day for 10 consecutive days. All mouse experiments were approved by the Second Hospital of Hebei Medical University's Institutional Animal Care and Use Committee and conducted in accordance with USA National Institutes of Health guidelines.

Statistical Analysis

Data were analyzed by SPSS 21.0 (SPSS Inc., Chicago, IL, USA) and presented as mean ± standard deviation (S.D.). Independent sample t test was used to determine the statistical difference between two groups. The difference was regarded statistically significant when p value < 0.05.

Results

CD133 Suppresses Cisplatin-Induced Apoptosis in Glioblastoma Stem Cells

We first investigated the role of CD133 in cisplatin resistance in GBM stem cells. T98G and U87MG cells were divided into $CD133^+$ and $CD133^-$ portions by FACS sorting as previously described (Yang et al. 2016). We assessed apoptosis of each cell type after cisplatin treatment (Fig. 1a). We found that cisplatin treatment induced dramatic apoptosis of $CD133^-$ T89G and U87MG cells (Fig. 1b, c). Importantly, apoptosis was greatly reduced in $CD133^+$ T89G and U87MG cells upon cisplatin treatment. These results suggest that the presence of CD133 in GBM cells may cause resistance to cisplatin treatment.

Cisplatin Treatment Increases CD133 Expression in $CD133^+$ Glioblastoma Stem Cells

We then investigated whether cisplatin treatment altered CD133 expression in GBM stem cells. We found that mRNA levels of CD133 were significantly increased in

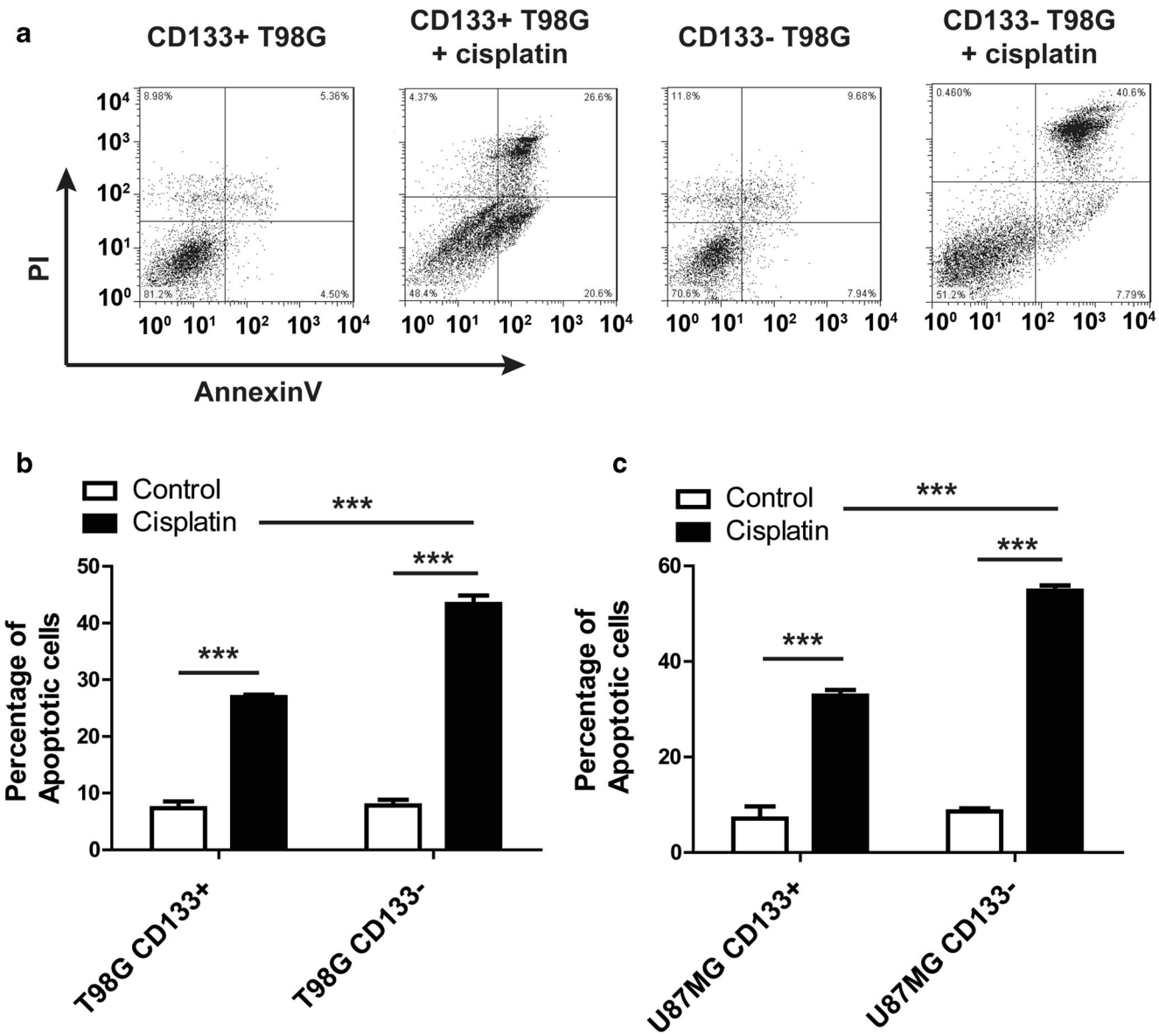

Fig. 1 CD133 expression contributed to cisplatin resistance in glioblastoma stem cells. **a** Apoptosis of $CD133^+$ or $CD133^-$ T98G cells with indicated treatments was determined by the annexin-V-FITC & PI apoptosis kit and assessed by flow cytometry analysis. $n = 3$ independent experiments and this panel presented one of these repeats. Cisplatin concentration was 20 μM. **b** Percentage of apoptotic $CD133^+$ and $CD133^-$ T98G cells. **c** Percentage of apoptotic $CD133^+$ or $CD133^-$ U87MG cells. **b** and **c** Data showed the percentage of ANNEXIN-V and PI double positive cells over total cells. Data were represented as mean ± S.D.; $n = 3$ independent experiments. *** indicated $p < 0.001$

$CD133^+$ T98G and U87MG cells as a result of cisplatin treatment (Fig. 2a). We also examined CD133 expression by flow cytometry (Fig. 2b) and found that consistently, cisplatin treatment significantly increased CD133 expression in $CD133^+$ T98G and U87MG cells (Fig. 2c).

Cisplatin Treatment Down-Regulates miR-29a in $CD133^+$ Glioblastoma Stem Cells

We investigated the molecular mechanism underlying cisplatin resistance in $CD133^+$ GBM stem cells. Since microRNAs

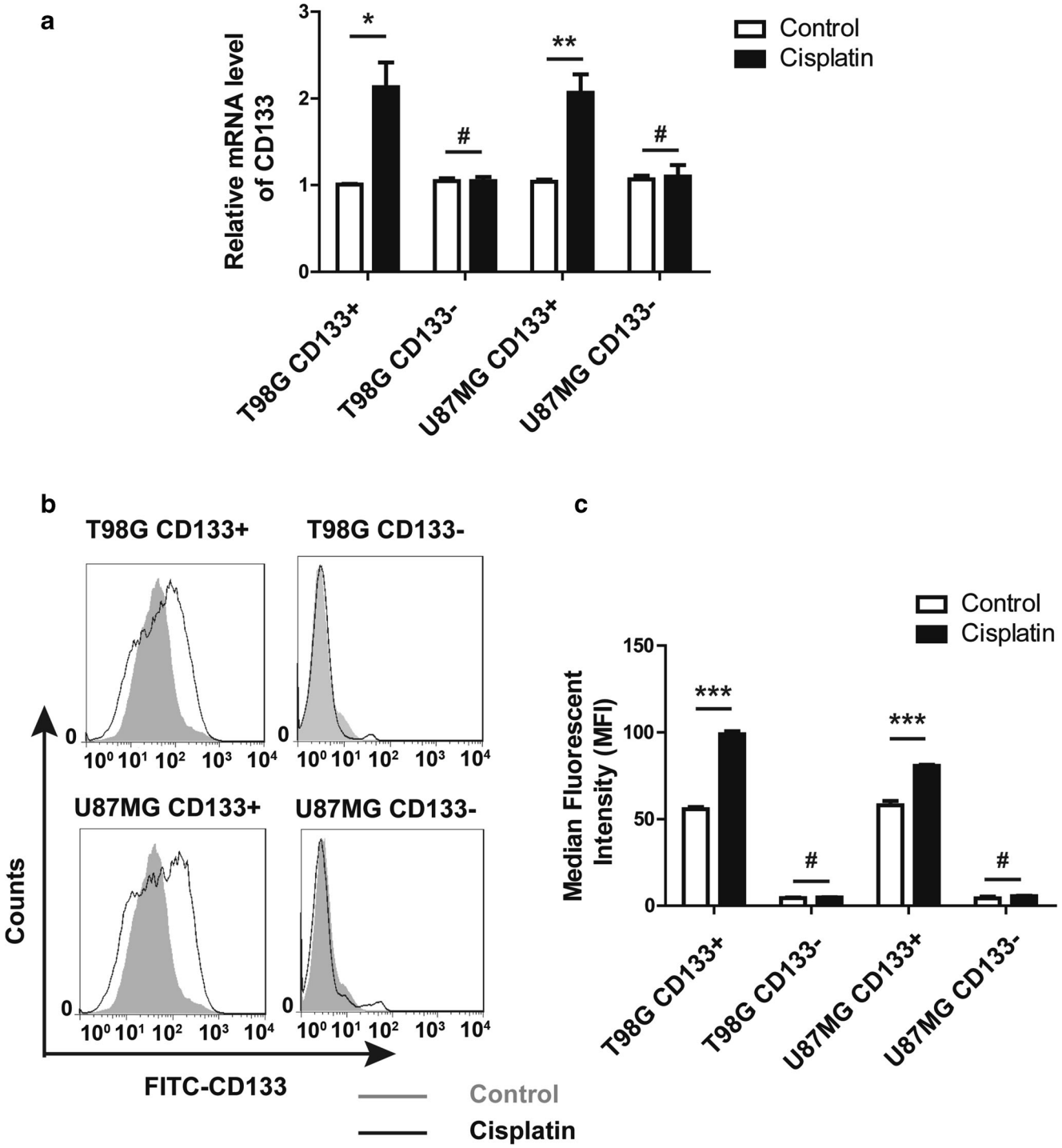

Fig. 2 CD133 expression was upregulated in $CD133^+$ cells treated with Cisplatin. **a** Relative mRNA levels of CD133 was evaluated by qRT-PCR in control or cisplatin (20 μM) treated cells. **b** Histograms showing the fluorescence intensity of CD133 in the response to control or cisplatin treatment. **c** Quantification of median fluorescent intensity (MFI) in panel **b**. Data were represented as mean ± S.D.; $n = 3$ independent experiments, *** indicated $p < 0.001$ and # indicated $p > 0.05$

are involved in tumorigenesis and regulate gene expression, we searched for CD133 targeting microRNAs that are regulated by cisplatin through an online tool (http://www.microrna.org). We examined the expression levels of all 21 candidate microRNAs and found that miR-29a (has-miR-29a) was significantly down-regulated in cisplatin treated CD133$^+$ GBM stem cells compared to that of control treatment (Fig. 3a). We also identified binding sites within miR-29a to the promoter region of CD133 (Fig. 3b), suggesting that cisplatin may regulate CD133 through suppressing miR-29a expression.

MiR-29a Promotes Cisplatin-Induced Apoptosis by Suppressing CD133 Expression

To determine if cisplatin resistance is mediated through down-regulating miR-29a, we overexpressed miR-29a in CD133$^+$ GBM stem cells and examined apoptosis by cisplatin treatment. We found that CD133 expression was reduced as a result of miR-29a (Fig. 4a, b). Importantly, miR-29a significantly increased apoptosis of CD133$^+$ (Fig. 4c, d) GBM stem cells after cisplatin treatment. These results suggest that miR-29a overexpression is sufficient to reverse cisplatin resistance in CD133$^+$ GBM stem cells.

MiR-29a Expression Significantly Suppresses Tumor Growth in CD133$^+$ Tumor Bearing Mice

Finally, we investigated the activity of miR-29a against CD133 induced cisplatin resistance in vivo. We generated a tumor xenograft mouse model by subaxillary inoculating. We found that cisplatin treatment significantly reduced tumor growth including both volume and weight (Fig. 5a, b). Remarkably, when combined with miRNA-29a expression, cisplatin treatment substantially suppressed tumor growth than cisplatin treatment alone.

Discussion

In the current study, we first assessed the association between cisplatin-induced apoptosis and expression levels of CD133 in GBM cells. We found that not only CD133$^+$ cells showed reduced sensitivity than CD133$^-$ cells in response to cisplatin, but on the other hand, cisplatin further increased CD133 expression compared with control treatment. We examined a series of microRNAs that potentially target CD133 and found that the expression level of miR-29a was significantly reduced by cisplatin treatment in CD133$^+$ cells.

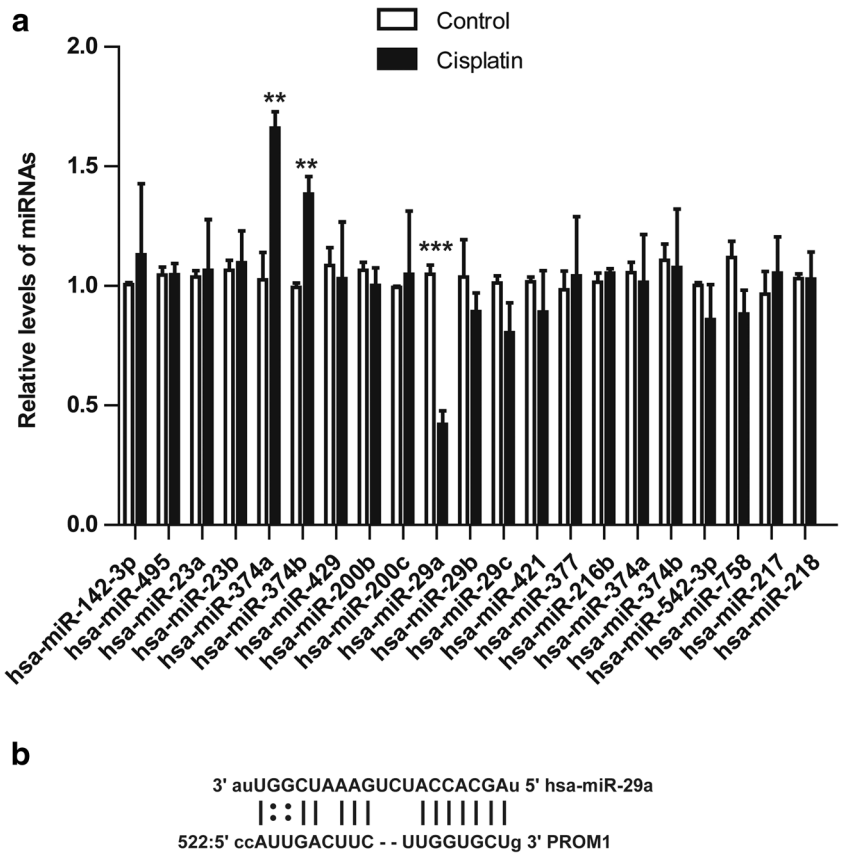

Fig. 3 MiR-29a was down-regulated in cisplatin treated CD133$^+$ T98G cells. MicroRNAs that might target mRNA of CD133 were predicted by online tools available at http://www.microma.org/. The expression levels of all predicted candidates were assessed by qRT-PCR. Note that only hsa-mir-29a was significantly down-regulated by the treatment of cisplatin in CD133$^+$ T98G cells. Data were represented as mean ± S.D., $n = 3$ independent experiments. ** indicated $p < 0.01$, *** indicated $p < 0.001$. **b** The hsa-mir-29a targeting sites of CD133 promoter

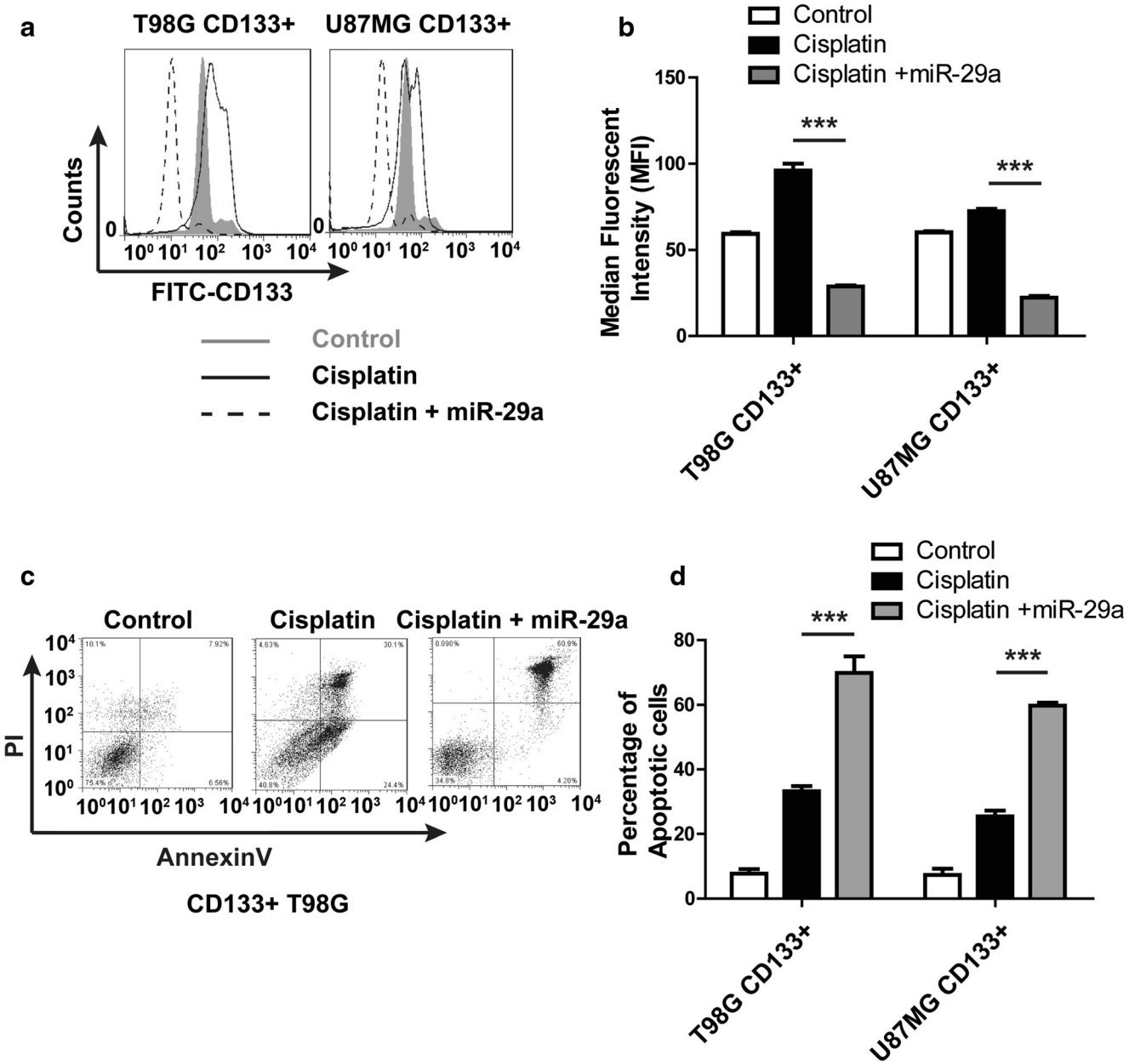

Fig. 4 Overexpression of miR-29a reduced CD133 level and promoted cisplatin induced apoptosis. **a** Histogram showing the fluorescence intensity of CD133 in the $CD133^+$ glioblastoma stem cells overexpressing miR-29a in the response to cisplatin treatment. **b** Quantification of Median Fluorescent Intensity (MFI) as shown in panel **a**. **c** Apoptosis of $CD133^+$ T98G cells with indicated treatments assessed by flow cytometry following annexin-V-FITC and PI labelling. $n = 3$ independent experiments and this panel shows one of these repeats. **d** Percentage of apoptotic $CD133^+$ T98G and U87MG cells. Data were represented as mean ± S.D.; $n = 3$ independent experiments. *** indicated $p < 0.001$

Overexpression of miR-29a suppressed CD133 expression and restored cisplatin sensitivity in GBM cells. Furthermore, we found that combination of miR-29a overexpression and cisplatin treatment in $CD133^+$ GBM tumor bearing mice substantially suppressed tumor growth in those mice. Our study thus uncovered a novel pathway that connects cisplatin resistance to CD133 expression in GBM stem cells.

Being a cancer stem cell marker, CD133 is responsible for resistance to various therapeutic strategies in various cancer types and $CD133^+$ cells are frequently associated with tumor recurrence (Brescia et al. 2013; Kozovska et al. 2014; Nadal et al. 2013). Although a direct link between CD133 and cisplatin resistance has not been established before in GBM, cisplatin treatment has been shown to induce accumulation

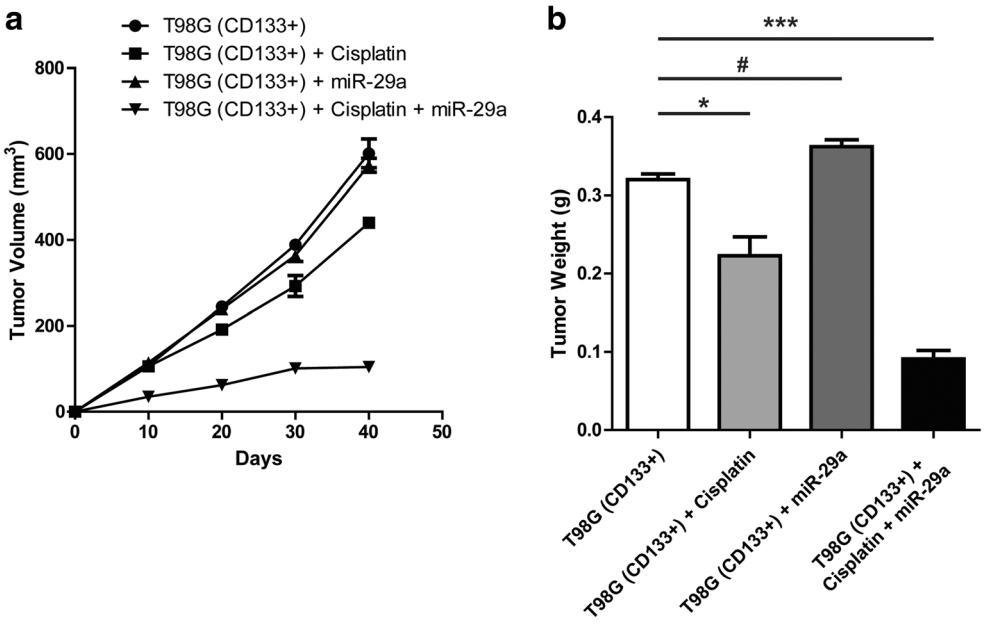

Fig. 5 Combination of miR-29a overexpression and cisplatin treatment improved survival of tumor bearing mice. **a** The growth curves of CD133$^+$ T98G xenograft tumors after treatment as indicated were measured at days 10, 20, 30, and 40. At each time point, three mice were sacrificed and the tumor tissues were taken out then the volume was measured. **b** The weight of ESCC-07 CD133$^+$ T98G xenograft tumors was measured after 40 days of different treatments as indicated. $N = 3$ for each time point. Data were represented as mean ± S.D.; # indicated $p > 0.05$ (not significant) and *** indicated $p < 0.001$

of CD133$^+$ cells in non-small cell lung cancer (Liu et al. 2013). Our study revealed that CD133$^+$ GBM cells were less sensitive to cisplatin treatment. In fact, CD133 expression was also increased as a result of cisplatin treatment. Previously, it has been shown that CD133 knockdown significantly increased cisplatin sensitivity (Ahmed et al. 2018). Based on this study and our observation, we hypothesized that cisplatin regulates CD133 expression in some way which eventually leads to resistance to the treatment. MicroRNAs have received much attention recently because of their function in controlling the expression of a lot of genes involved in tumorigenesis and abnormal expression of microRNAs are frequently reported in cancer. These observations prompted us to search for microRNAs that might directly target CD133 and mediate cisplatin induced CD133 upregulation.

Our search revealed 21 candidate microRNAs that potentially target CD133 and among these microRNAs, we found three were differentially expressed in cisplatin treated CD133$^+$ cells. MiR-29a is the only down-regulated microRNA in response to cisplatin treatment and its binding site was identified in the promoter region of CD133. MiR-29a has been shown to inhibit cell proliferation, migration as well as tumor growth by targeting the oncogenic mucin MUC1 in pancreatic ductal adenocarcinoma (Trehoux et al. 2015). MiR-29a also suppresses cell proliferation by inhibiting β-catenin expression in non-small cell lung cancer (Tan et al. 2013). Combining with our previous study that CD133 promotes GBM cell proliferation, our current study suggests that miR-29a may target CD133 to inhibit cell proliferation.

Since CD133 is a major cause of therapy resistance in GBM, we investigated whether overexpression of miR-29a could reverse CD133 mediated cisplatin resistance. Our finding was very promising. Overexpression of miR-29a significantly suppressed CD133 expression and substantially increased cisplatin-induced apoptosis in CD133$^+$ GBM stem cells. Thus, for the first time, we identified a microRNA that directly targets CD133 and may potentially overcome CD133-mediated resistance to chemotherapy in GBM.

GBM is an aggressive glioma with poor prognosis. The median survival time of patients with GBM is dauntingly short-only about 15 months (Alifieris and Trafalis 2015). It would be a significant progress to suppress tumor growth in GBM patient. We used a mouse model to test whether overexpression of miR-29a could suppress tumor growth in mice bearing GBM tumor. One major obstacle of GBM chemotherapy is blood-brain barrier (Jue and McDonald 2016; van Tellingen et al. 2015). In addition to chemoresistance, penetration of cisplatin in the CNS is very limited (Jacobs et al. 2005). In fact, current standard therapy of GBM is not cisplatin administration, but radiation combined with temozolomide treatment (Jue and McDonald 2016). Therefore, to investigate the efficacy of miR-29a overexpression in cisplatin-mediated tumor suppression without the need to consider blood brain barrier penetration, we implanted CD133$^+$ GBM cells by subaxillary inoculation. Our results suggest that miRNA-29a overexpression alone did not significantly suppress tumor growth. However, it drastically slowed down tumor enlargement and reduced tumor weight when combined with cisplatin treatment.

Although testing the effects of miR-29a overexpression in response to radiotherapy in CD133$^+$ GBM cells is beyond the scope of our current study, our results with cisplatin are very promising. Since radioresistance and chemoresistance are mainly caused by enrichment of CD133$^+$ cancer stem cells that lead to tumor recurrence, suppressing this cell population may represent a highly feasible therapeutic strategy (Liu et al. 2006; Tamura et al. 2010; Yu et al. 2015). One possibility will

be a combination of miR-29a overexpression and cisplatin treatment in addition to radiotherapy. Current development in nanotechnology has already improved cisplatin delivery through the blood brain barrier. One group used magnetic resonance-guided focused ultrasound to deliver cisplatin-gold-nanoparticle conjugates and found significant improvement of blood brain barrier permeability as well as suppression of GBM growth in combination with radiation therapy (Coluccia et al. 2018). With this technique, it is thus possible to utilize miR-29a overexpression and cisplatin treatment to induce $CD133^+$ cell apoptosis, although in the future it is necessary to characterize the functions of miR-29a in patients with GBM first.

Conclusion

In summary, our findings suggest that miR-29a is an appropriate target to suppress CD133-mediated resistance of GBM. Overexpression of miR-29a may be sufficient to overcome therapy resistance by targeting $CD133^+$ cells when combined with radiation and cisplatin treatment. However, further studies are required to explore this possibility.

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Compliance with Ethical Standards

Ethical Approval All applicable international, national, and institutional guidelines for the care and use of animals were followed.

Conflict of Interest The authors have no conflict of interest to disclose.

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