

CircRNA CDR1as Promotes Glioma Progression by Regulating miR-514a-3p/STAT3 Signaling Pathway

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Abstract

Background The pathogenesis of glioma is very complicated and the molecular mechanisms have not been clearly demonstrated so far. CircRNA CDR1as as non-coding RNA was highly expressed in multiple human cancers and promoted tumorigenesis and cancer progression. However, there were no reports to address the roles of CDR1as in glioma.

Methods In this study, we used glioma cell lines to investigate the function of CDR1as. Cell viability, colony formation, migration, invasion, gene expression, luciferase activity, miRNA-circRNA interaction, protein-circRNA interaction, and tumor formation *in vivo* were assessed.

Results We found that CDR1as is up-regulated in human glioma tissues and cell lines, and its expression is significantly associated with the prognosis of patients with glioma. Loss-of-function studies revealed that CDR1as increases glioma cell proliferation, migration, and invasion *in vitro* and tumor growth *in vivo*. We showed that CDR1as can bind miR-514a-3p and act as a sponge of miR-514a-3p. Furthermore, we identified that the expression of STAT3 is regulated by miR-514a-3p and CDR1as as the direct target of miR-514a-3p. Additionally, the decreased glioma cell malignancy by CDR1as knockdown can be reversed by miR-514a-3p inhibitor and overexpression of STAT3. CDR1as knockdown can result in the down-regulated expression of PCNA, Ki67, N-cadherin, and MMP9 and up-regulated expression of E-cadherin. Moreover, the aberrant expression of these genes by CDR1as knockdown also can be reversed by miR-514a-3p inhibitor and overexpression of STAT3.

Conclusions Taken together, our findings uncover the molecular mechanisms of CDR1as-mediated the progression of glioma through the miR-514a-3p/STAT3 signaling pathway. These results will provide a theoretical basis for further understanding of the molecular mechanisms of glioma tumorigenesis and developing new therapeutic targets.

Background

Glioma is the most common primary malignant brain tumor and arises throughout the central nervous system, which is associated with high mortality, high recurrence rate, and poor prognosis. To develop effective treatments for glioma, exploring the mechanisms of tumorigenesis and finding effective therapeutic targets in glioma have attracted wide attention. Many studies showed that the non-coding RNAs (ncRNAs) play important roles in the regulation of cancer-related signaling pathways and some of them had become effective therapeutic targets or biomarkers in clinical studies. CircRNA CDR1as as non-coding RNA was highly expressed in multiple cancers and played important roles in tumorigenesis and cancer therapies, such as nasopharyngeal carcinoma (NPC), hepatocellular carcinoma (HCC), gastric cancer (GC), ovarian cancer (OC), and so on. For example, CDR1as was highly expressed in NPC tissues. CDR1as could up-regulate the expression of E2F3 through negative regulation of miR-7-5p, and promote the growth and glucose metabolism of NPC cells. Moreover, CDR1as could enhance NPC progression in the xenograft mice model (1). Besides, CDR1as was also highly expressed in HCC cell lines and tissues.

CDR1as overexpression could promote HCC cell proliferation and migration. CDR1as could promote the expression of AFP by sponging miR-1270. And exosomes extracted from CDR1as-overexpressed HCC cells increased proliferation and migration of surrounding normal cells (2). Knockdown of CDR1as specifically triggered low-dose Diosbulbin-B (DB) induced GC cell death by regulating miR-7-5p/REGY axis (3). CDR1as was down-regulated in cisplatin-resistant OC patient tissues and cell lines. Overexpression of CDR1as could promote the cisplatin-induced cell apoptosis in OC cells (4). However, there were no reports to address the roles of CDR1as in glioma.

CDR1as is involved in many disease processes as the molecular sponge for multiple miRNAs, including miR-7(1, 5, 6), miR-135a (7), MiR-1270 (4, 8), miR135b-5p (9), miR-641 (10), and so on. For example, CDR1as contains the binding sites for miR-7, which plays an important role in the progress of cancers (11), Alzheimer's disease (12), insulin secretion (13), and osteoblastic differentiation of stem cells (14) by regulating different signaling pathways. It was reported that CDR1as contains more than 70 selectively conserved miRNA target sites (5). To address the molecular mechanisms underlying the functions of CDR1as in glioma, finding the putative miRNAs as the targets of CDR1as is an effective way to explore CDR1as-mediated downstream signaling pathway.

In the present study, we showed that CDR1as is highly expressed in human glioma tissues and cell lines, and knockdown of CDR1as significantly inhibits glioma cell proliferation, migration, and invasion *in vitro* and attenuates tumorigenicity *in vivo*. The bioinformatic analysis and molecular experiments revealed that CDR1as is a sponge for miR-514a-3p and STAT3 expression is regulated by miR-514a-3p as the direct target of miR-514a-3p. Knockdown of CDR1as strongly promotes miR-514a-3p expression, resulting in decreased levels of STAT3. Additionally, the decreased glioma cell malignancy or deregulated expression of the proliferation and epithelial-mesenchymal transition (EMT) markers by CDR1as knockdown can be reversed by miR-514a-3p inhibitor and overexpression of STAT3. All the data suggested that CDR1as promotes human glioma progression through the miR-514a-3p/STAT3 signaling pathway.

Materials And Methods

Human tissue samples

Human tissue samples of glioma and adjacent normal brain were obtained from the Fudan University Shanghai Cancer Center (Shanghai, China). These tissue samples were immediately frozen in liquid nitrogen after surgery. The study was approved by the Clinical Research Ethics Committee of Fudan University Shanghai Cancer Center with informed written consent.

Cell culture, transfection, and virus

The normal human astrocyte (NHA) cell line and glioma cell lines A172, U251, U87, and SHG44 (from ATCC) were cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 10% FBS, 2 mM glutamine, 100 µg/mL streptomycin, and 100 U/mL penicillin at 37°C in a 5% CO₂/95% air incubator.

Lentiviruses encoding a corresponding control (sh-NC) or CDR1as short hairpin RNAs (sh-CDR1as) were obtained from Hanbio (Shanghai, China). The miR-514a-3p mimics and nonspecific miRNA negative control (miR-NC) were purchased from RiboBio (Guangzhou, China). Cells were transfected using Lipofectamine 2000 (Invitrogen). All cell lines were tested to be mycoplasma-free.

Rt-qpcr

Total RNA was isolated from the tissues or cultured cells using TRizol (Tiangen Biotech, China), treated with DNase I (Thermo Fisher Scientific, USA), and then subject to cDNA synthesis with high-capacity cDNA reverse transcription kits (Applied Biosystems Life Technologies, USA). The RT-qPCR was performed with the SYBR Green RT-qPCR kit (Soochow GenePharma, China) and normalized to the internal control GAPDH RNA.

Western Blotting

Protein samples were separated by SDS-PAGE, transferred to PVDF membrane, and visualized by enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA). The following antibodies were used: anti-PCNA, anti-Ki67, anti-N-cadherin, anti-E-cadherin, anti-MMP9, anti-STAT3, and anti-GAPDH.

Cell viability assay

Cell viability was measured using the Cell Counting Kit-8 (CCK-8 Kit, Bossed, China) according to the provider's manual. Cells were seeded in 96-well plates at a density of 3×10^3 cells per well in triplicate and incubated for 0 h, 24 h, 48 h, 72 h, and 96 h. 10 μ L of CCK-8 solution was subsequently applied into each well and allowed for the reaction at 37 °C for 1 h. The absorption at 450 nm was recorded by a microplate reader (Xmark, Bio-Rad, CA, USA).

Colony formation

About 200 cells were suspended with complete medium and seeded in 6-well plates. After incubation for 2 weeks, cell colonies were stained with 0.005% crystal violet and visualized using a microscope. And the colony number in each well was calculated.

Boyden chamber migration and invasion assays

Cell migration and invasion assays were performed using the transwell cell culture chambers (8 μ m pore size, Millipore, USA). 5×10^4 cells were suspended in serum-free media and seeded onto the noncoated (migration) or ECM (Sigma-Aldrich, E1270)-coated (invasion) upper chamber. 10% FBS in the bottom chamber was used as a chemoattractant. Cells were incubated for 24 hours, fixed with methanol, and stained with 0.1% crystal violet. Colonies were imaged using a microscope (Olympus, Japan) and counted in five randomly selected fields.

Animal studies

Four- to six-week-old female BALB/c nude mice ($n = 6$ per group) were used for animal studies, which have been approved and conducted under the oversight of the Animal Care and Use Committee of Fudan University. A total of 1×10^6 U251 cells infected with the lentivirus expressing either sh-NC or sh-CDR1as were injected subcutaneously into the flank of BALB/c nude mice. Tumor growth was monitored with a caliper every 7 days starting 7 days after implantation and calculated with the following formula: $V(\text{mm}^3) = \text{width}^2 \times \text{length} \times 0.5$. After euthanasia, the tumors were harvested, and the wet weight of each tumor was measured.

Dual-luciferase assay

Wild-type (WT) or mutant (MUT) 3' UTR fragments (CDR1as or STAT3) were cloned into downstream of the *Renilla* luciferase gene in the psiCHECK-2 vector (Promega, Madison, WI, USA). The 3' UTR regions of CDR1as or STAT3 were mutated in the complementary site for the seed region of miR-514a-3p. All plasmids were verified by DNA sanger sequencing. U87 or U251 cells were cotransfected with the reporter vector and miR-514a-3p mimics or miR-NC mimics by using Lipofectamine 2000. Luciferase activities were detected via a dual-luciferase assay system (Promega, USA). Each experiment was conducted in triplicate.

Statistical analysis

Statistical analysis was performed by Student's *t* test between two groups, and one-way or two-way ANOVA between multiple groups. Data were expressed as mean \pm SD. $P < 0.05$ is considered statistically significant.

Results

CDR1as is up-regulated in human glioma tissues and cell lines

To study the clinical importance of CDR1as, we performed RT-qPCR to analyze its expression in 50 pairs of human glioma and normal/benign brain tissues. CDR1as RNA levels were significantly higher in human glioma tumors as compared to control brain tissues (Fig. 1A). We also performed RT-qPCR in Normal Human Astrocyte (NHA) cells and four glioma cell lines, including A172, U251, U87, and SHG44. CDR1as expression was evidently up-regulated in glioma cell lines compared with control cells (Fig. 1B). Next, we analyzed the prognostic role of CDR1as in patients with glioma. The OS was analyzed according to the expression of CDR1as. The data showed that the patients with high expression of CDR1as had shorter OS than those with low expression of CDR1as (Log-rank test, $P = 0.024$) (Fig. 1C).

Knockdown of CDR1as represses glioma cell proliferation, migration, and invasion in vitro and also inhibits tumor growth in vivo

To explore the oncogenic role of CDR1as in glioma cells, we generated CDR1as knockdown in U251 and U87 cell lines via lentiviral infection, which expressed higher levels of CDR1as. The RT-qPCR data showed that CDR1as knockdown was successful in the knockdown cell lines (Fig. 2A). Knockdown of CDR1as markedly inhibited proliferation of U87 and U251 cells as CCK-8 assays indicated (Fig. 2B). Similarly, the colony numbers of U87 and U251 cells were also reduced by CDR1as knockdown (Fig. 2C). The Boyden transwell assays showed that knockdown of CDR1as significantly decreased migration and invasion of U87 and U251 cells as compared with the sh-NC group (Fig. 2D and 2E). PCNA and Ki67 are the markers of proliferation, which are commonly used to assess the growth fraction of the cells. Immunoblot analysis showed that the protein levels of PCNA and Ki67 were impaired by CDR1as knockdown in U87 and U251 cells, which were consistent with the cell proliferation assays. E-cadherin, N-cadherin, and matrix metalloproteinase-9 (MMP9) are key EMT markers and involved in cell migration and invasion. The protein levels of N-cadherin and MMP9 were down-regulated and the E-cadherin protein level was up-regulated by CDR1as knockdown in U87 and U251 cells. The signal intensity for each protein was quantified and normalized to GAPDH. Then the ratios were further normalized to sh-NC and shown next to the immunoblot images (Fig. 2F). To investigate the effect of CDR1as on glioma cell motility *in vivo*, sh-NC or sh-CDR1as U251 cells were subcutaneously implanted into nude mice. The data showed that CDR1as knockdown dramatically inhibited primary tumor volume and weight in mice (Fig. 2G). Taken together, all the results suggested that CDR1as knockdown represses glioma cell proliferation, migration, and invasion *in vitro* and tumor growth *in vivo*.

miR-514a-3p is directly regulated by CDR1as

Multiple lines of evidence suggest that CDR1as serves as a sponge for multiple miRNAs. Thus, we hypothesized that CDR1as could also target the specific miRNA in glioma and regulate its downstream pathways. Using the Starbase database, we identified that CDR1as contains the putative target sites for miR-514a-3p in its 3' UTR, suggesting CDR1as may have direct interaction with miR-514a-3p as a target of CDR1as. Then we performed the luciferase reporter assays in U87 and U251 cells to determine whether miR-514a-3p can directly bind the 3' UTR region of CDR1as. The results demonstrated that miR-514a-3p mimics overexpression significantly repressed the luciferase activity of the reporter plasmid containing WT 3'UTR but had no effect for the vector containing MUT 3'UTR (Fig. 3A). RNA pull-down assay revealed that CDR1as could be pulled down by biotin-labeled miR-514a-3p rather than miR-NC in both U87 and U251 cells (Fig. 3B). Then we performed RIP-RT-qPCR to address whether CDR1as and miR-514a-3p can specifically bind to Ago2 protein in U87 and U251 cells. The data showed that CDR1as and miR-514a-3p were accumulated in anti-Ago2 groups other than anti-IgG groups (Fig. 3C). Taken together, these results suggested that CDR1as has direct interaction with miR-514a-3p. Next, we carried out RT-qPCR assays in CDR1as knockdown U87 and U251 cells to examine the correlation of CDR1as and miR-514a-3p expression. The result revealed that the miR-514a-3p RNA level was elevated by CDR1as knockdown and negatively associated with CDR1as RNA level (Fig. 3D). In conclusion, our findings identified that CDR1as acts as a sponge for miR-514a-3p and negatively regulates its expression.

CDR1as positively regulates STAT3 via sponging miR-514a-3p

To further investigate the downstream signaling pathways mediated by miR-514a-3p, we also used the Starbase database to predict the target gene of miR-514a-3p. The result showed that the signal transducer and activator of transcription 3 (STAT3) was one of the miR-514a-3p targets and contained miR-514a-3p target sites in its 3'UTR. Mutations were generated in the complementary sites for the seed region of miR-514a-3p (Fig. 4A). Then We carried out luciferase reporter assays to determine whether miR-514a-3p can directly target the 3'UTR region of STAT3. As expected, overexpression of miR-514a-3p mimics dramatically inhibited the luciferase activity of the reporter with WT 3'UTR of STAT3 as compared with miR-NC mimics in both U87 and U251 cells, but failed to inhibit the luciferase activity of the reporter with MUT 3'UTR (Fig. 4B).

To detect whether miR-514a-3p regulates STAT3 expression, we generated miR-514a-3p overexpression and knockdown cell lines in U87 and U251 cells via transfection of miR-514a-3p mimics and miR-514a-3p inhibitor. miR-NC and NC inhibitor were used as negative controls. RT-qPCR assays verified successful overexpression and knockdown of miR-514a-3p in both U87 and U251 cells as compared with negative controls (Fig. 4C). Immunoblot analysis showed that miR-514a-3p overexpression significantly reduced CDR1as protein levels in U87 and U251 cells. The signal intensity for each protein was quantified and normalized to GAPDH. Then the ratios were further normalized to miR-NC. The RT-qPCR analysis showed that overexpression of miR-514a-3p also significantly reduced STAT3 mRNA levels, suggesting that miR-514a-3p regulates STAT3 at the transcriptional level (Fig. 4D).

Because miR-514a-3p is negatively regulated by CDR1as. Then we want to test whether CDR1as can regulate STAT3 expression. The western blot data showed that STAT3 protein levels were reduced by CDR1 knockdown in both U87 and U251 cell lines. Then we further studied whether CDR1as can regulate STAT3 expression via sponging miR-514a-3p. As shown in Fig. 3D, the RNA levels of miR-514a-3p were elevated in CDR1as knockdown cells. We transfected miR-514a-3p inhibitors to CDR1as knockdown cells and addressed whether the reduction of STAT3 protein levels by CDR1 knockdown can be rescued by transfection of miR-514a-3p inhibitors. As expected, transfection of miR-514a-3p inhibitors could partially rescue the reduced protein levels of STAT3 in CDR1as knockdown cells. The RT-qPCR analysis showed that transfection of miR-514a-3p inhibitors also partially rescued the reduced RNA levels of STAT3 in CDR1as knockdown cells. This suggested that CDR1as positively regulates STAT3 via sponging miR-514a-3p (Fig. 4E).

CDR1as promotes glioma progression by regulating miR-514a-3p/STAT3 signaling pathway

We have approved that CDR1as knockdown could cause decreased glioma cell malignancy and it positively regulates STAT3 via sponging miR-514a-3p. Then we want to demonstrate whether CDR1as promotes glioma progression through miR-514a-3p/STAT3 signaling. To answer this question, we further

transfected miR-514a-3p inhibitors or STAT3-overexpressed vector into CDR1as knockdown cells. The immunoblot and RT-qPCR analysis revealed that STAT3 was successfully overexpressed in both U87 and U251 cells (Fig. 5A). Knockdown of CDR1as could inhibit the cell proliferation rate of U87 and U251 cells (Fig. 2B and 5B). And miR-514a-3p RNA level was increased in CDR1as knockdown cells (Fig. 3D). Our results showed that the decreased cell proliferation rate by CDR1as knockdown can be reversed partially by the transfection of miR-514a-3p inhibitor in U87 and U251 cells (Fig. 5B). The RNA and protein levels of STAT3 were reduced in CDR1as knockdown cells (Fig. 4E). Furthermore, the decreased cell proliferation rate by CDR1as knockdown can also be reversed partially by overexpression of STAT3 (Fig. 5B). These data suggested that CDR1as promotes glioma cell proliferation through miR-514a-3p/STAT3 signaling.

Then we performed cell colony formation, migration, and invasion assays by using the same cell lines. The results revealed that the decreased colony numbers of glioma cells by CDR1as knockdown could be rescued partially by transfection of miR-514a-3p inhibitor or overexpression of STAT3 (Fig. 5C). Meanwhile, the decreased cell numbers of migrated or invaded glioma cells by CDR1as knockdown could also be rescued partially by transfection of miR-514a-3p inhibitor or overexpression of STAT3 (Fig. 5D and 5E).

The protein levels of PCNA, Ki67, N-cadherin, and MMP9 were impaired by CDR1as knockdown and the E-cadherin protein level was up-regulated by CDR1as knockdown (Fig. 2F). And the protein levels of these proteins could be reversed partially by transfection of miR-514a-3p inhibitor or overexpression of STAT3 in both U87 and U251 cells (Fig. 5F), which were consistent with the cell proliferation, migration, and invasion assays. In conclusion, CDR1as promotes glioma cell proliferation, colony formation, migration, and invasion through the miR-514a-3p/STAT3 signaling pathway.

Discussion

In this study, we investigated the function of CDR1as in the pathogenesis of glioma and dissected the molecular mechanism of CDR1as-induced tumorigenesis and cancer progression. We found that CDR1as is up-regulated in human glioma tissues and cell lines. Our results proved that CDR1as acts as a sponge of miR-514a-3p and negatively regulates miR-514a-3p. We also provide evidence that STAT3 is regulated by miR-514a-3p and CDR1as as the direct target of miR-514a-3p. Furthermore, we identified that CDR1as promotes glioma cell proliferation, colony formation, migration, and invasion through miR-514a-3p/STAT3 signaling.

CircRNA CDR1as is known to serve as a sponge of multiple miRNAs (10). In our study, we proved that CDR1as acts as a sponge of miR-514a-3p, which is a newly discovered target. Thus, we further expanded the family of CDR1as target miRNAs. miR-514a-3p has been shown as a tumor suppressor in human renal cell carcinoma, which was significantly down-regulated in renal cell carcinoma (RCC) tissues. Additionally, restoration of miR-514a-3p suppressed RCC cell proliferation, viability, migration, and invasion. MiR-514a-3p negatively regulated EGFR gene expression by directly targeting its 3'UTR (15, 16). In melanoma, the tumor suppressor NF1 was a direct target of miR-514a, and overexpression of miR-

514a inhibited NF1 expression, suggesting that miR-514a negatively regulated NF1 expression as an oncogene (17). Our data indicated that miR-514a-3p was negatively regulated by the oncogene CDR1as, suggesting that miR-514a-3p functions as a tumor suppressor in glioma (17). Thus, miR-514a-3p may function as both oncogene and tumor suppressor in a tissue-specific manner.

We found that STAT3 was the direct target of miR-514a-3p. STAT3 as the transcription factor can regulate cell proliferation, apoptosis, inflammation, and angiogenesis. The aberrant expression and activation of STAT3 play important roles in tumorigenesis (18, 19).

STAT3 is emerging as a potential therapeutic target and multiple STAT3-targeted drugs have been successfully developed. Several STAT3 inhibitors are currently tested in clinical trials in various human cancers (20, 21). Here we characterized a novel factor in regulating STAT3 expression in glioma cells and presented its role in glioma cell malignancy via miR-514a-3p/STAT3 signaling pathway. These findings suggested that CDR1as may be a new target for the treatment of glioma.

Based on the Starbase database, we identified more than 20 miRNAs which may be the targets of CDR1as. And miR-514a-3p is one of the predicted targets. In the future, we will explore whether other miRNAs are involved in CDR1as-mediated glioma cell malignancy. On the other hand, we will use animal models to prove that CDR1as regulates tumor growth or metastasis via the miR-514a-3p/STAT3 signaling pathway. Meanwhile, we will establish the patient-derived tumor xenograft (PDX) mouse model and perform small interfering RNA-mediated knockdown of CDR1as in patient tumors to address whether CDR1as knockdown affects the growth of patient tumors. This will provide evidence to answer whether CDR1as can be an effective therapeutic target.

In summary, we identified that CDR1as promotes glioma progression through the miR-514a-3p/STAT3 pathway. These findings will deepen our understanding of the molecular mechanisms underlying tumorigenesis and progression in glioma, and provide valuable clues for the targeted therapy of glioma.

Conclusions

Taken together, CDR1as acts as a sponge of miR-514a-3p and promotes glioma cell proliferation, colony formation, migration, and invasion through miR-514a-3p/STAT3 signaling. These results will provide a theoretical basis for further understanding of the molecular mechanisms of glioma tumorigenesis and developing new therapeutic targets.

Abbreviations

NPC

nasopharyngeal carcinoma; HCC:hepatocellular carcinoma; GC:gastric cancer; OC:ovarian cancer; RCC:renal cell carcinoma; PDX:patient-derived tumor xenograft; MMP9:matrix metalloproteinase-9; EMT:epithelial-mesenchymal transition; DB:low-dose Diosbulbin-B; ncRNAs:non-coding RNAs

Declarations

Ethics approval and consent to participate: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Consent for publication: Not applicable.

Data availability statement: The authors confirm that the data or material supporting the findings of this study are available from the corresponding author on request.

Conflict of interest: None reported.

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Authors' contributions: Deheng Li, Liangdong Li, and Xin Chen had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis;

Conception/Design: Deheng Li, Liangdong Li, and Xin Chen;

Provision of study material or patients: All authors;

Collection and/or assembly of data: All authors;

Data analysis and interpretation: Deheng Li, Liangdong Li, and Xin Chen;

Manuscript writing: Deheng Li;

Final approval of manuscript: All authors;

Study supervision: Wentao Yang and Yiqun Cao

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Figures

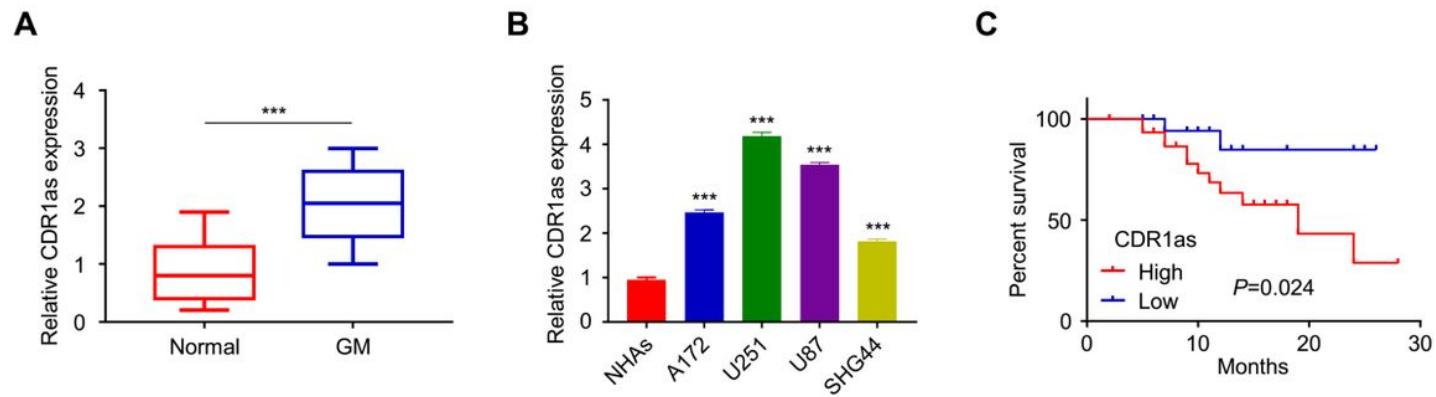


Figure 1

CDR1as is up-regulated in human glioma tissues and cell lines. (A) RT-qPCR analysis of CDR1as RNA in 50 pairs of human glioma and normal/benign brain tissues (mean \pm SD). ***p < 0.001. (B) RT-qPCR analysis of CDR1as RNA levels in several human glioma cell lines (mean \pm SD, n = 3), including A172, U251, U87, and SHG44. Normal human astrocytes (NHAs) were used as control. ***p < 0.001. (C) The PFS distribution for patients with high or low CDR1as expression levels was estimated with the Kaplan-Meier method. Statistical analysis was performed by the Log-rank test between two groups.

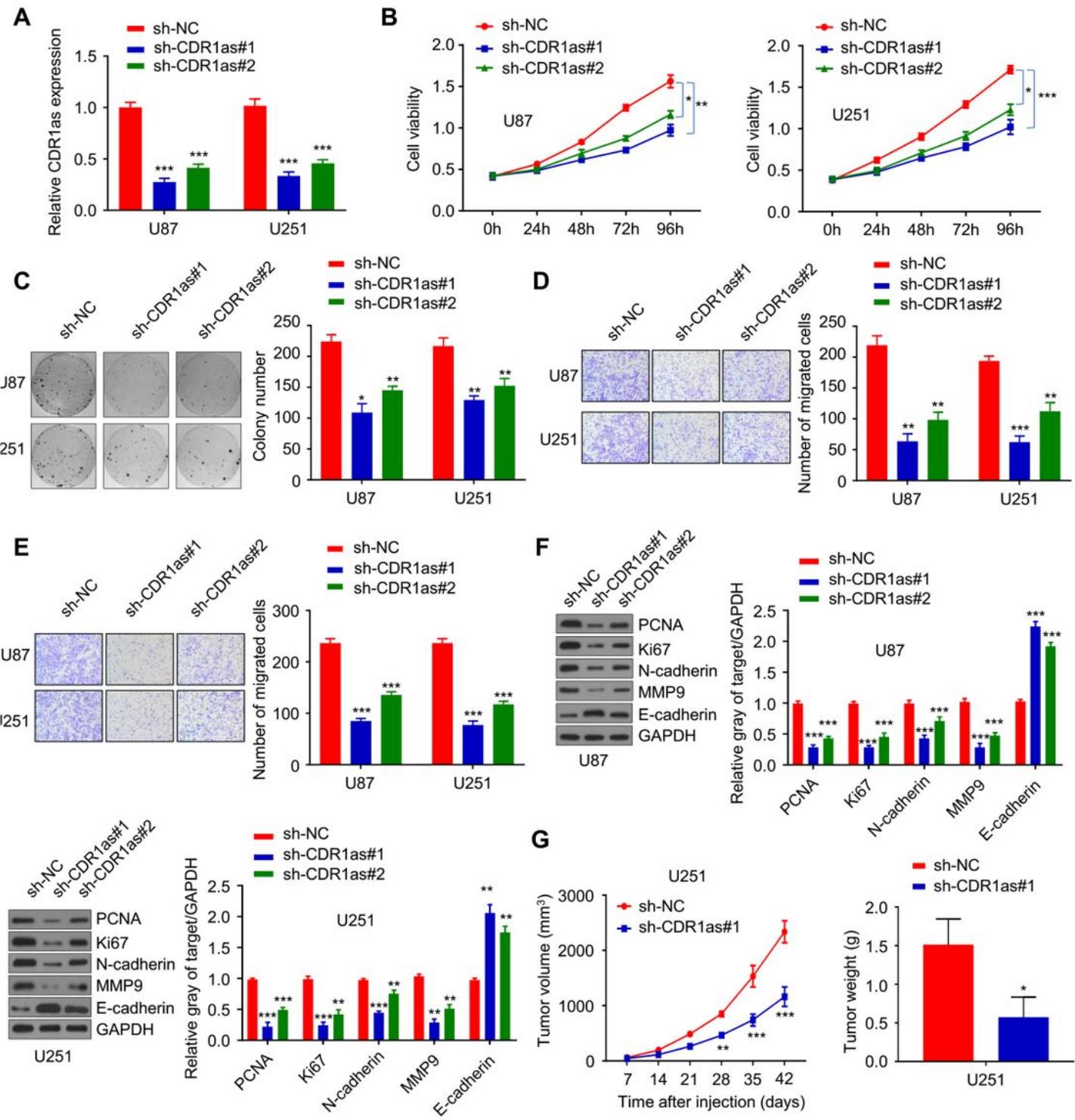


Figure 2

Knockdown of CDR1as represses glioma cell malignancy in vitro and tumor growth in vivo. (A) RT-qPCR analysis of CDR1as RNA levels in two independent sh-CDR1as or sh-NC (NC, negative control) lentivirus infected U87 and U251 stable cells. *** $p < 0.001$ versus sh-NC group. (B) Cell viability of NC or CDR1as knockdown U87 and U251 cells was measured using CCK-8 assay. Cells were collected after incubation at 0 h, 24 h, 48 h, 72 h, and 96 h. The absorption at 450 nm was recorded by a microplate reader. * $p <$

0.05; **p < 0.01; ***p < 0.001. (C) Colony formation of sh-NC and sh-CDR1as in U87 and U251 cells. Representative images from three experiments are shown on the left. Quantification of colony numbers is shown on the right (mean \pm SD, n = 3). *p < 0.05; **p < 0.01. (D) Transwell migration of NC or CDR1as knockdown U87 and U251 cells. Representative images from three independent experiments are shown. The number of migrated cells was counted. Data are presented as the mean \pm SD (n = 3). **p < 0.01; ***p < 0.001. (E) Transwell invasion of NC or CDR1as knockdown U87 and U251 cells. Representative images from three independent experiments are shown. The number of invaded cells was counted and presented as the mean \pm SD (n=3). ***p < 0.001. (F) Immunoblot analysis of PCNA, Ki67, N-cadherin, MMP9, E-cadherin, and GAPDH in NC or CDR1as knockdown U87 and U251 cells. Representative blots from at least three independent experiments are shown. The relative levels for each protein were also analyzed. ***p < 0.001. (G) NC or CDR1as knockdown U87 and U251 cells were implanted into the flank of BALB/c nude mice. The tumor growth curve is shown on the left (mean \pm SD, n = 6). The tumor weight is shown in on the right (mean \pm SD, n = 6).

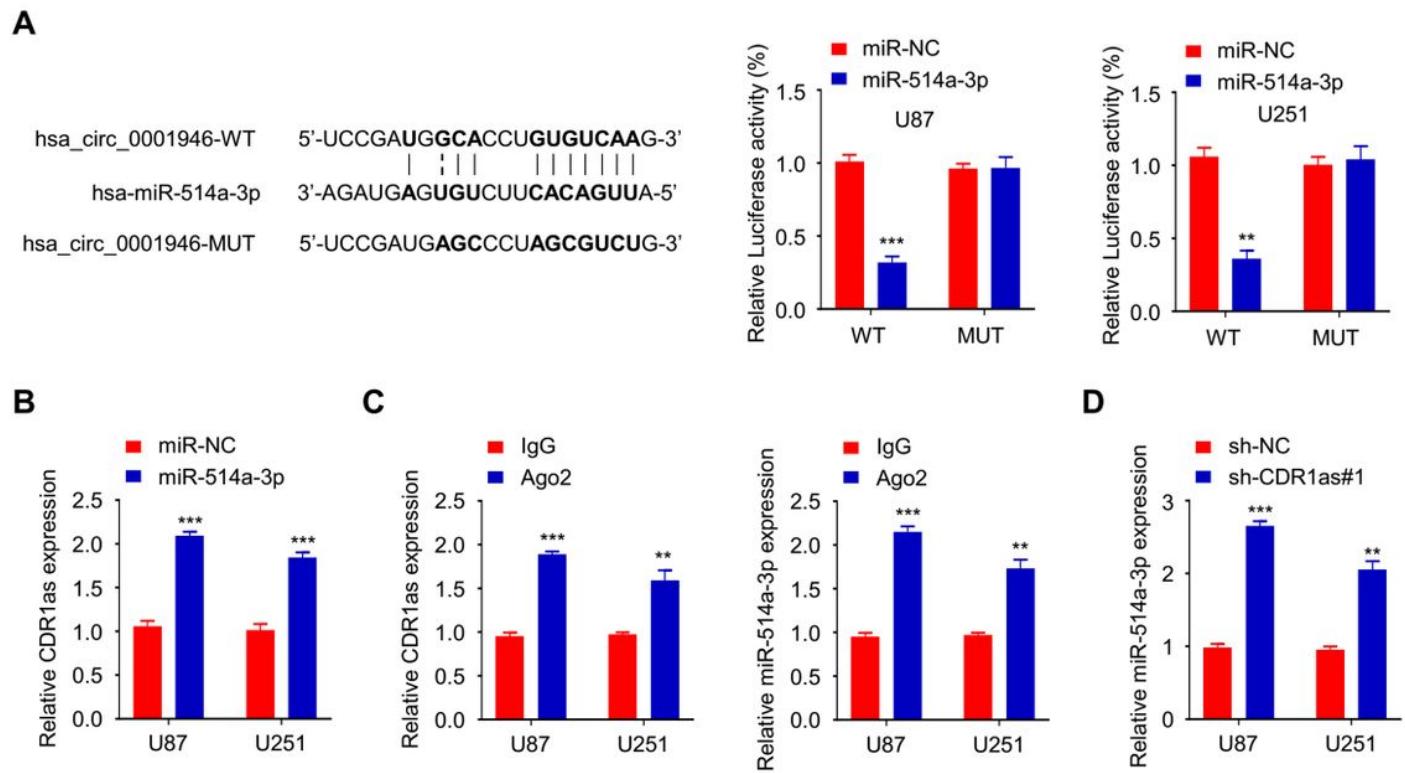


Figure 3

miR-514a-3p is directly regulated by CDR1as. (A) Predicted miR-514a-3p binding sites in the 3' UTR of CDR1as. Mutations were generated in the complementary sites for the seed region of miR-377-3p. U87 or U251 cells were co-transfected with CDR1as wild-type (WT) 3' UTR or mutant (MUT) 3' UTR reporter and miR-514a-3p mimics or miR-NC mimics. The luciferase activities were detected and normalized to the co-transfected group of WT 3' UTR and miR-NC mimics (mean \pm SD, n = 3). **p < 0.01; ***p < 0.001. (B) Biotin-labeled miR-NC or miR-514a-3p was incubated in the presence of streptavidin magnetic beads with lysates from U87 or U251 cells, followed by RT-qPCR analysis with CDR1as. Data are presented as the

mean \pm SD ($n = 3$). *** $p < 0.001$ versus miR-NC group. (C) Anti-Ago2 RNA immunoprecipitation was performed to detect interactions between Ago2 and CDR1as or miR-514a-3p. IgG or anti-AGO2 antibody was incubated with lysates from U87 or U251 cells. RNA was extracted after wash and RT-qPCR analysis was performed for the detection of CDR1as or miR-514a-3p. (D) RT-qPCR analysis of miR-514a-3p in sh-CDR1as or sh-NC transfected U87 or U251 cells.

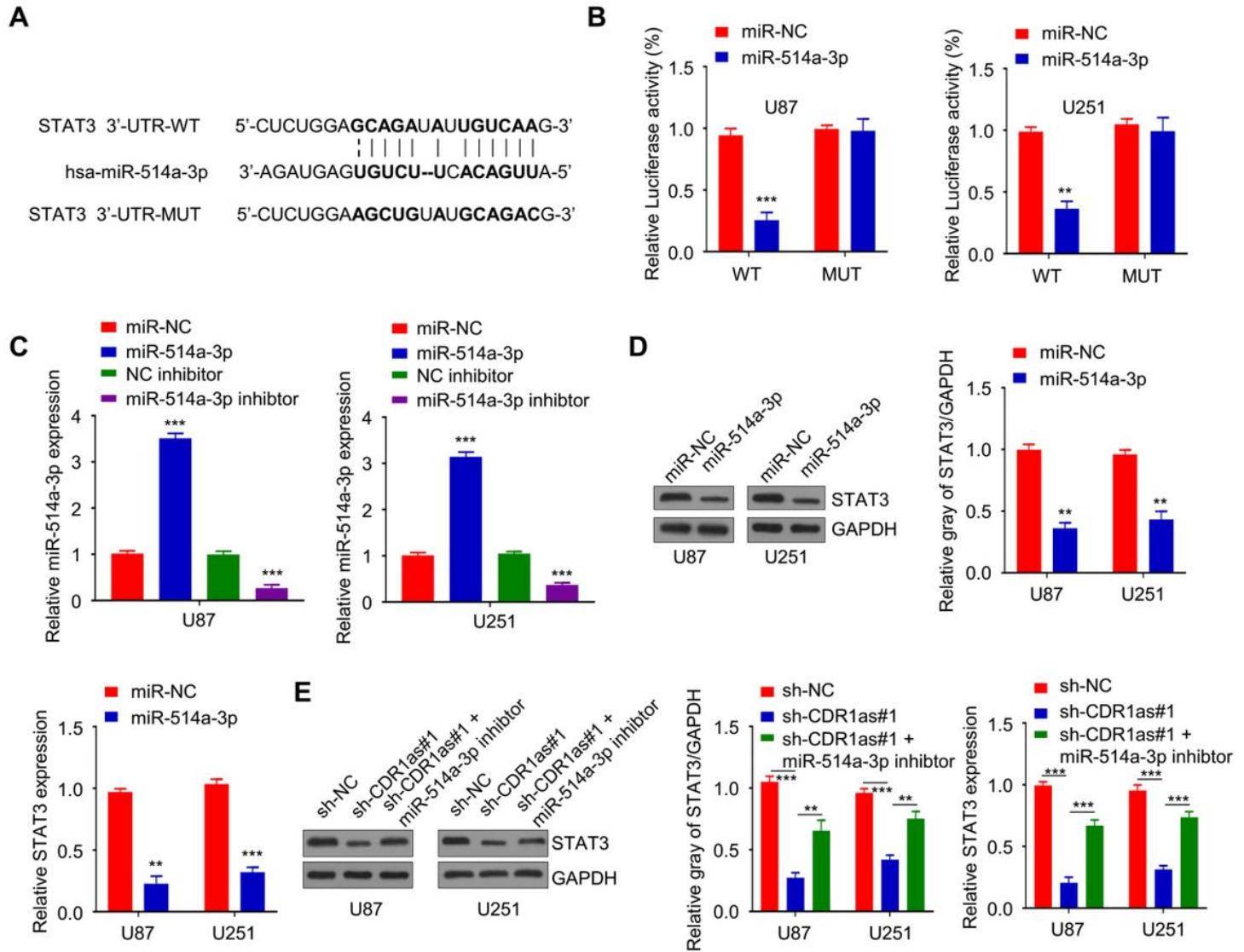


Figure 4

CDR1as positively regulates STAT3 via sponging miR-514a-3p. (A) The predicted miR-514a-3p binding sites in the 3' UTR of STAT3 were shown. The mutation sites were generated in the 3' UTR of STAT3 in a complementary site for the seed region of miR-377-3p. (B) The wild-type (WT) 3' UTR or mutant (MUT) 3' UTR fragments for STAT3 were cloned into the luciferase reporter vector. Then U87 or U251 cells were co-transfected with the reporter vectors and miR-514a-3p mimics or miR-NC mimics. (C) RT-qPCR analysis of miR-514a-3p RNA levels in miR-NC mimics, miR-514a-3p mimics, miR-NC inhibitor, or miR-514a-3p inhibitor transfected U87 and U251 cells. (D) Western blot analysis of STAT3 and GAPDH in miR-NC or

miR-514a-3p mimics transfected U87 and U251 cells. The relative protein levels of STAT3 were shown. The protein levels were normalized to GAPDH first and the ratios were determined by further normalization to the miR-NC group. **p < 0.01 versus miR-NC cells. The mRNA levels of STAT3 was also analyzed by RT-qPCR in miR-NC or miR-514a-3p mimics transfected U87 and U251 cells. (E) Western blot analysis of STAT3 and GAPDH in NC, CDR1as knockdown, CDR1as knockdown plus miR-514a-3p inhibitor in U87 and U251 cells. The relative protein levels of STAT3 were shown. RT-qPCR analysis of STAT3 mRNA levels was also performed in these cell lines. **p < 0.01 versus sh-NC; ***p < 0.001 versus sh-NC.

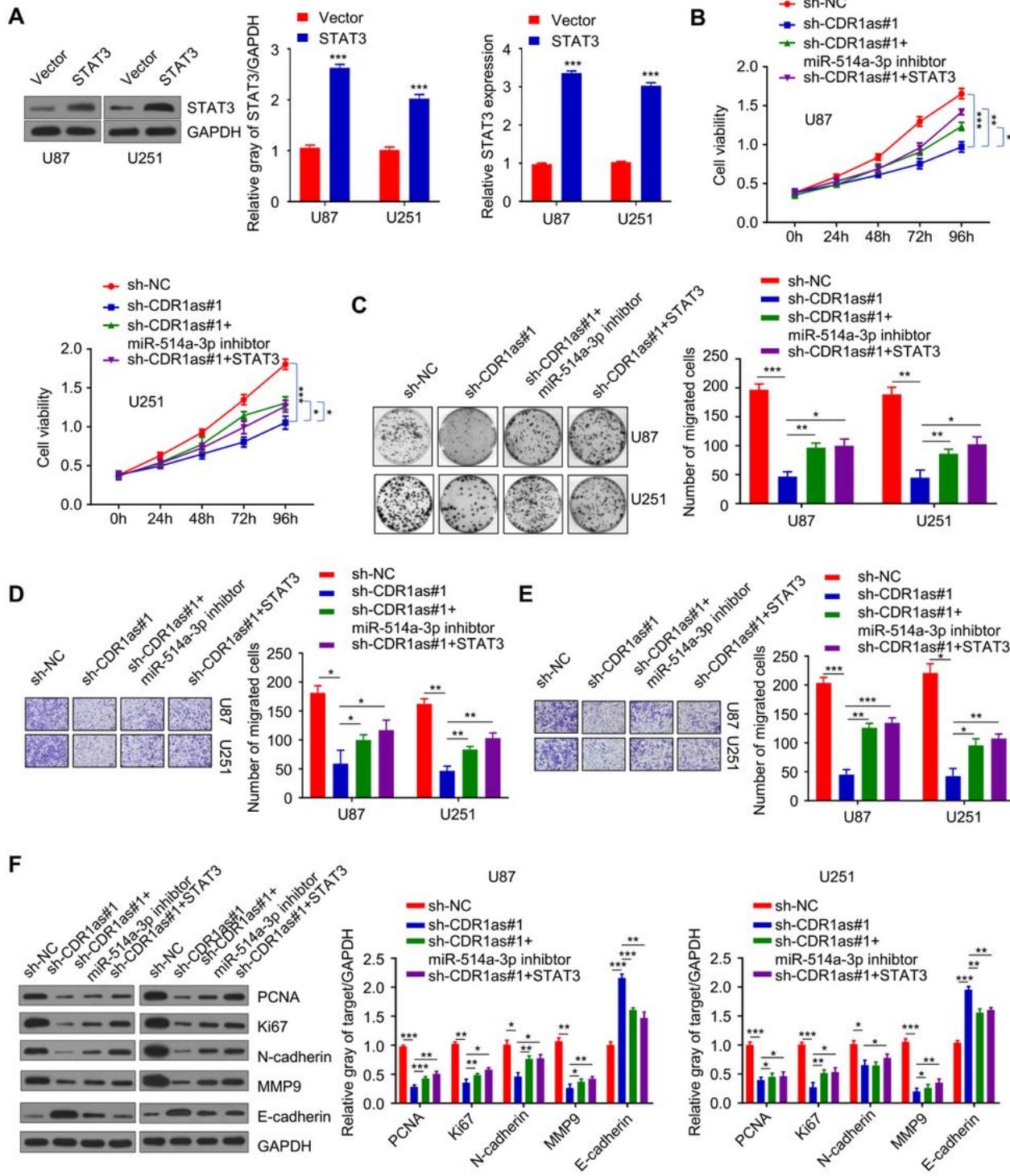


Figure 5

CDR1as promotes glioma progression by regulating miR-514a-3p/STAT3 signaling. (A) The protein levels of STAT3 and GAPDH in empty vector or STAT3-overexpressed U87 and U251 cells. The relative mRNA levels of STAT3 were also analyzed by RT-qPCR in these cells. (B) Cell viability of NC, CDR1as knockdown, CDR1as knockdown plus miR-514a-3p inhibitor, and CDR1as knockdown plus STAT3 overexpression in U87 and U251 cells was measured using CCK-8 assay. Cells were collected after

incubation at 0 h, 24 h, 48 h, 72 h, and 96 h. The absorption at 450 nm was recorded by a microplate reader. *p < 0.05; **p < 0.01; ***p < 0.001. (C) Colony formation of NC, CDR1as knockdown, CDR1as knockdown plus miR-514a-3p inhibitor, CDR1as knockdown plus STAT3 overexpression in U87 and U251 cells. Representative images from three experiments are shown. Quantification of colony numbers is shown on the right (mean ± SD, n = 3). *p < 0.05; **p < 0.01; ***p < 0.001. (D) and (E) Transwell migration (D) and invasion (E) of NC, CDR1as knockdown, CDR1as knockdown plus miR-514a-3p inhibitor, CDR1as knockdown plus STAT3 overexpression in U87 and U251 cells. Representative images from three independent experiments are shown on the left. The number of migrated or invaded cells was counted and presented as mean ± SD (n = 3). *p < 0.05; **p < 0.01; ***p < 0.001. (F) Immunoblot analysis of PCNA, Ki67, N-cadherin, MMP9, E-cadherin, and GAPDH in NC, CDR1as knockdown, CDR1as knockdown plus miR-514a-3p inhibitor, CDR1as knockdown plus STAT3 overexpression in U87 and U251 cells. Representative blots from three independent experiments are shown. The relative levels of each protein were analyzed. ***p < 0.001 versus sh-NC group. The mRNA levels of PCNA, Ki67, N-cadherin, MMP9, and E-cadherin were also analyzed by RT-qPCR in these cell lines. *p < 0.05; **p < 0.01; ***p < 0.001.