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Effects of miR-211-3p/RHBDD1 axis on cell proliferation, cell cycle progression, and epithelial-mesenchymal transition in glioma

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Abstract

This study was designed to elucidate the relationship of miR-211-3p and rhomboid domain containing 1 (RHBDD1) in glioma. Here, we first observed that miR-211-3p directly targets the 3′-UTR of RHBDD1 in glioma cells using dual-luciferase reporter assay, RNA immunoprecipitation (RIP) assay, reverse transcription-quantitative polymerase chain reaction (RT-qPCR), and Western blot analysis. Pearson's correlation analysis showed that miR-211-3p expression was negatively correlated with RHBDD1 expression in glioma tissues. CCK-8 assay, flow cytometry, and transwell assay were applied to assess cell proliferation, cell cycle distribution, migration, and invasion. The results showed that RHBDD1 knockdown inhibited cell proliferation, cell cycle G1/S transition, migration, and invasion in two glioma cell lines (U87 and LN-229). Knockdown of miR-211-3p obtained opposite results. Moreover, overexpression of RHBDD1 counteracted suppressive effects of miR-211-3p on glioma cells. Furthermore, decreased expression of CDK4, cyclin D1, N-cadherin, and vimentin as well as increased E-cadherin expression induced by miR-211-3p was reversed by RHBDD1 overexpression. Our results suggested that targeting miR-211-3p/RHBDD1 axis might be a novel effective therapeutic target for glioma treatment.

Key words: *glioma, miR-211-3p, RHBDD1, proliferation, EMT.*

Introduction

Glioma, accounting for nearly 30% of all brain tumors, is considered as a frequently occurred primary malignant neoplasm in the central nervous system [35]. Despite recent advances in extensive tumor resection and adjuvant chemotherapy/radiotherapy, the prognosis of glioma patients is still unfavorable, with a median survival time of 12-14 months [1,11]. This undesirable result is due to frequently recurrence after treatment, which is associated with highly aggressive infiltration and quick glioma cell prolifer-

ation [19,27]. Therefore, it is vital to find potential biological markers for helping explore novel therapeutic treatment for glioma patients.

MicroRNAs (miRNAs/miRs), a class of non-coding, endogenous, and single-stranded RNAs with a length of approximately 19-24 nucleotides [13], could induce mRNA degradation or translational repression *via* binding to partially complementary sequences in 3′-untranslated regions (3′-UTRs) of their target genes [29]. Functional experiments have suggested the involvement of miRNAs in series of biological

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cellular processes, including cell proliferation, cell cycle, apoptosis, and metastasis [4,9,22]. In recent years, miR-211-3p has been reported to participate in the pathogenesis of many types of cancers, including non-small cell lung cancer [21], breast cancer [12], bladder cancer [5], colorectal cancer [36], and oral squamous cell carcinoma [37]. Although these studies have consistently demonstrated tumor suppressive role of miR-211-3p in cancer progression, the expression levels and biological function of miR-211-3p have not been yet reported in glioma.

Rhomboid protein family mainly consists of active protease and inactive members lacking catalytic residues, which physiologically function as an intra-membrane serine protease [7,20]. Of note, rhomboid domain containing 1 (RHBDD1), a novel member of rhomboid family [32], has been reported to have a strong relationship with carcinogenesis and serve as prognostic indicators. For instance, RHBDD1 was highly up-regulated in breast cancer tissue and promoted breast cancer progression by regulating p-Akt and CDK2 levels [40]. Song *et al.* [30] provided evidence of a growth-promoting role for RHBDD1 in colorectal cancer and RHBDD1 closely associated with survival in patients. In addition, knockdown of RHBDD1 could inhibit cell migration, invasion, and epithelial-mesenchymal transition (EMT) in breast cancer [10], renal cell carcinoma [14], and colorectal cancer [39]. Our previous work showed that RHBDD1 was the target gene of miR-211-3p based on TargetScan prediction, which made us hypothesize that miR-211-3p modulated glioma cell functions by targeting RHBDD1, and the present study was designed to explore this hypothesis.

Here, we first examined the relationship between miR-211-3p and RHBDD1 in glioma cell lines and tissue samples. Subsequently, we investigated the biological function of RHBDD1 and miR-211-3p alone in glioma cells. Moreover, we further evaluated whether RHBDD1 takes part in miR-211-3p-mediated effects in glioma cells. Our work may provide novel insights into the important role of the miR-211-3p/RHBDD1 axis in glioma.

Material and methods

Tissue specimens and cell lines

In total, forty-five pairs of tumor tissues and matched adjacent tissues were collected from glioma patients (age range, 18-67 years) after surgeries performed in the Inner Mongolia Baogang Hospital (Inner Mongolia, China), and were quickly frozen in liquid nitrogen until further use. Prior to operation, none of the glioma patients had undergone radiotherapy or chemotherapy, and each patient signed an informed consent. This study obtained the approval from the Research Ethics Committee of the Inner Mongolia Baogang Hospital (Approval No.: IMB-87B, Inner Mongolia, China).

U87 and LN-229 glioma cell lines were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified eagle medium (DMEM) basic media (Thermo Fisher Scientific, Waltham, Massachusetts, USA), with 10% fetal bovine serum (FBS), 100 mg/ml streptomycin, and 100 U/ml penicillin (Thermo Fisher Scientific) at 37°C in a humid atmosphere containing 5% CO_2.

Cell transfection

GenePharma Co. Ltd. (Shanghai, China) provided miR-211-3p mimics, mimics negative control (NC), miR-211-3p inhibitor, and inhibitor NC. The eukaryotic expression vector containing complete sequence of RHBDD1 cDNA was labelled as pcDNA3.1-RHBDD1, and corresponding empty vector pcDNA3.1 was prepared by Sigma-Aldrich Co., LLC (St. Louis, MO, USA). U87 and LN-229 cells were seeded in six-well plates. After grown until 60-70% confluence, cell transfection was performed in glioma cells with lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) for 48 hours.

Dual-luciferase reporter assay

The relationship between miR-211-3p and RHBDD1 was predicted by TargetScan release 7.0, and then validated by dual-luciferase reporter assay in glioma cells. Briefly, chemical synthesis of 3'-UTR of RHBDD1 mRNA containing wild-type sequence (WT) or containing mutations in putative miR-211-3p binding site (MUT) was performed, which was accordingly inserted into luciferase vector psi-CHECK-2 (Promega Corp., Madison, WI, USA) to generate recombinant vectors. Subsequently, U87 and LN-229 cells were co-transfected with 200 ng of WT or MUT RHBDD1 recombinant plasmid and 100 ng of miR-211-3p mimics or inhibitor as well as the corresponding NC using lipofectamine 2000 (Invitrogen). After incubation for 48 hours, we collected the transfected cells and quantified the luciferase activities

using dual luciferase reporter assay system (Promega Corp.).

RNA immuno-precipitation assay

Using Magna RIP kit purchased from Millipore (Bedford, Massachusetts, USA), we performed RNA immuno-precipitation (RIP) experiment to validate the relationship between miR-211-3p and RHBDD1. In brief, U87 and LN-229 cells were harvested after 48 hours transfection of miR-211-3p mimics or mimics NC, and lysed in RIP lysis buffer with RNase inhibitor and protease inhibitor (Roche, Basel, Switzerland). The cell lysates were then incubated with RIP buffer containing magnetic beads conjugated with human anti-Ago2 antibody (Abcam, Cambridge, MA, USA) or IgG as NC. After proteinase K treatment, the extracted immuno-precipitated RNAs were purified and analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

RT-qPCR

TRIzol reagent (Thermo Fisher Scientific) was used to isolate total RNA from tissue samples or cell lines. Total 1 µg RNA of each sample was reversed transcribed to complementary DNA (cDNA) using Prime-Script® RT reagent kit (Takara, Shiga, Japan). PCR amplification was performed using 1 µg cDNA for SYBR® green master mix (Thermo Fisher Scientific) on ABI StepOne plus system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Relative gene expression was measured with the $2^{-\Delta\Delta Ct}$ method [18]. The primers used in this analysis were as follows: miR-211-3p, forward: 5'-GTCGTATCCAGTGCGTGTCGTGG-3' and reverse: 5'-AGTCGGCAATTGCACTGGATACG-3'; U6, forward: 5'-CTCGCTTCGGCAGCACA-3' and reverse: 5'-AACGCTTCACGAATTTGCGT-3'; RHBDD1, forward: 5'-ACGCAGGCGGGTCGTA-3' and reverse: 5'-GGGCCAAAGTTGCTAGGGT-3'; GAPDH, forward: 5'- GGTGAAGGTCGGAGTCAACG-3' and reverse: 5'- GCATCGCCCCACTTGATTTT-3'.

Cell counting kit-8 assay

Cell counting kit-8 (CCK-8) assay was utilized to assess the cell proliferation status in glioma cells. Seeding of transfected cells into 96-well plates was first performed at a density of 5,000 cells per well at 37°C, with 5% CO_2. At 0, 24, 48, and 72 hours, 10 µl of CCK-8 reagent (Dojindo Co., Ltd., Kumamoto, Japan) was added to each well. Following 2 hours

incubation at 37°C, we used a microplate reader (Bio-Rad, San Jose, California, USA) to detect the optical density (OD) value at 450 nm.

Cell cycle analysis

Seeding of transfected cells was first performed in six-well plates at a density of 1×10^5 cells per well for 48 hours. After centrifuged at 360 × g for 5 min, cells were collected and fixed with 70% ethanol overnight at 4°C. Cells were then washed with PBS twice, and stained with 20 µg/ml PI and 200 µg/ml RNaseA both from Becton (Dickinson and Company, Franklin Lakes, NJ, USA) at 37°C for 30 min in dark. Finally, stained cells were immediately analyzed by FACSCalibur flow cytometer (Becton, Dickinson and Company).

Transwell assay

The 24-well transwell chambers (8 µm pore size; Costar, Kennebunk, ME, USA) were used to analyze cell migration (pre-coated without Matrigel) and invasion (pre-coated with diluted 250 µg/ml/well Matrigel, Corning). In brief, transfected cells were cultured in 200 µl of serum-free medium for 12 hours and adjusted approximately, and 1×10^5 cells/ml were seeded into the upper chamber of each test group. The lower chamber was added with 500 µl of FBS-supplemented medium. After 24 hours incubation, the migrated cells in the lower chamber were fixed with methanol and stained with 0.1% crystal violet solution for 20 min. Finally, migrated/invasive cells were photographed under a light microscope (Olympus Corporation; magnification, 200×) and counted in three independent fields.

Western blot analysis

Extraction of total protein sample was performed with radio-immuno-precipitation assay (Beyotime, Nanjing, Jiangsu, China). After protein quantification by a BCA kit (Beyotime Biotechnology), equal amount of protein (30 µg) was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred into polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Afterwards, the membranes were blocked with 5% skimmed milk in tris buffered saline containing 0.1% tween-20 (TBST) for 2 hours at room temperature, and incubated with specific primary antibodies against RHBDD1 (1 : 1000, HPA013972, Sigma),

PCNA (1 : 500, ab18197, Abcam, Cambridge, United Kingdom), E-cadherin (1 : 1000, ab219332, Abcam), N-cadherin (1 : 1000, ab18203, Abcam), vimentin (1 : 1000, ab137321, Abcam), and GAPDH (1 : 5000, 10494-1-AP, Proteintech) at 4°C overnight, followed by incubated with horseradish peroxidase-conjugated secondary antibodies (#7074; 1 : 5000, Cell Signaling Technology) for 2 hours at room temperature. The immuno-reactive proteins were detected through an enhanced chemiluminescent detection system (Thermo Fisher Scientific).

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Data analysis was performed using Graph-Pad Prism 6.0. Pearson's correlation analysis was applied to analyze the associations between miR-211-3p and RHBDD1 levels in glioma tissues. Analysis of statistical difference was performed using Student's t-test for two groups and one-way ANOVA, along with Tukey's post-hoc test for three groups. Statistically significant differences were accepted when a p-value was less than 0.05.

Results

RHBDD1 as a target gene of miR-211-3p in glioma cells

By searching bioinformatics analytical tool Target-Scan, we found a putative binding site of miR-211-3p in the 3'-UTR regions of human RHBDD1 mRNA (Fig. 1A), indicating RHBDD1 may be a direct target of miR-211-3p. Dual-luciferase reporter assay was then applied to validate the association between RHBDD1 and miR-211-3p. As shown in Figures 1B, C, the luciferase activity of the cells co-transfected with WT RHBDD1 and miR-211-3p mimics was remarkably decreased, while no significant difference was observed in miR-211-3p mimics with MUT RHBDD1 in both U87 and LN-229 cells. In contrast, miR-211-3p inhibitor transfection significantly increased the luciferase activity in cells transfected with WT RHBDD1 luciferase plasmid in both U87 (Fig. 1D) and LN-229 (Fig. 1E) cells. Moreover, an anti-Ago2 RIP assay was conducted in U87 and LN-299 cells to pull down the RNA transcripts binding to Ago2, and immunoglobulin G (IgG) was used as a negative control. We found that RHBDD1 was significantly enriched with the Ago2 antibody in U87 (Fig. 1F) and LN-229 (Fig. 1G)

cells. Therefore, our findings indicated that RHBDD1 was a target of miR-211-3p in glioma cells.

Inverse correlation of miR-211-3p and RHBDD1 expression levels in glioma

Using RT-qPCR assay, we analyzed the expression levels of miR-211-3p and RHBDD1 in 45 pairs of glioma tissues and adjacent tissues. The results showed that miR-211-3p expression level (Fig. 2A) was significantly downregulated, while RHBDD1 mRNA levels (Fig. 2B) were upregulated in glioma tissues, compared with matched adjacent tissues. Pearson's analysis revealed a significant negative correlation between miR-211-3p and RHBDD1 mRNA levels in glioma tissues (Fig. 2C, $r = -0.3152$, $p = 0.0350$). In addition, we analyzed the regulatory effects of miR-211-3p on RHBDD1 expression levels using RT-qPCR and Western blot analysis. As expected, overexpression of miR-211-3p by miR-211-3p mimics transfection (Fig. 2D) resulted in a significant reduction of RHBDD1 mRNA (Fig. 2E) and protein (Fig. 2F) levels in U87 and LN-229 cells. Conversely, knockdown of miR-211-3p by miR-211-3p inhibitor transfection (Fig. 2G) upregulated the expression of RHBDD1 at the mRNA (Fig. 2H) and protein (Fig. 2I) levels in U87 and LN-229 cells. The above findings collectively indicated that miR-211-3p negatively regulated RHBDD1 in glioma by targeting its' 3'-UTR.

Inhibitory effects of RHBDD1 knockdown on glioma cell proliferation, migration, and invasion

Considering the increased RHBDD1 expression in glioma, we then investigated the biological function of RHBDD1 in glioma cells by performing loss-of-function assays. First, two different siRNAs targeting RHBDD1 (si-RHBDD1#1 or si-RHBDD1#2) were transfected into U87 and LN-299 cells. As shown in Figure 3A, the protein expression of RHBDD1 was downregulated after transfection with si-RHBDD1#1 or si-RHBDD1#2, compared with si-NC transfection in U87 and LN-229 cells. By performing CCK-8 assay, we found that siRNA-mediated downregulation of RHBDD1 significantly inhibited the proliferation rate of U87 and LN-229 cells (Fig. 3B). Compared with si-RHBDD1#2, si-RHBDD1#1 transfection presented stronger suppressive effects on RHBDD1 expression and proliferation ability in glioma cells. We thus selected si-RHBDD1#1 transfection for subsequent
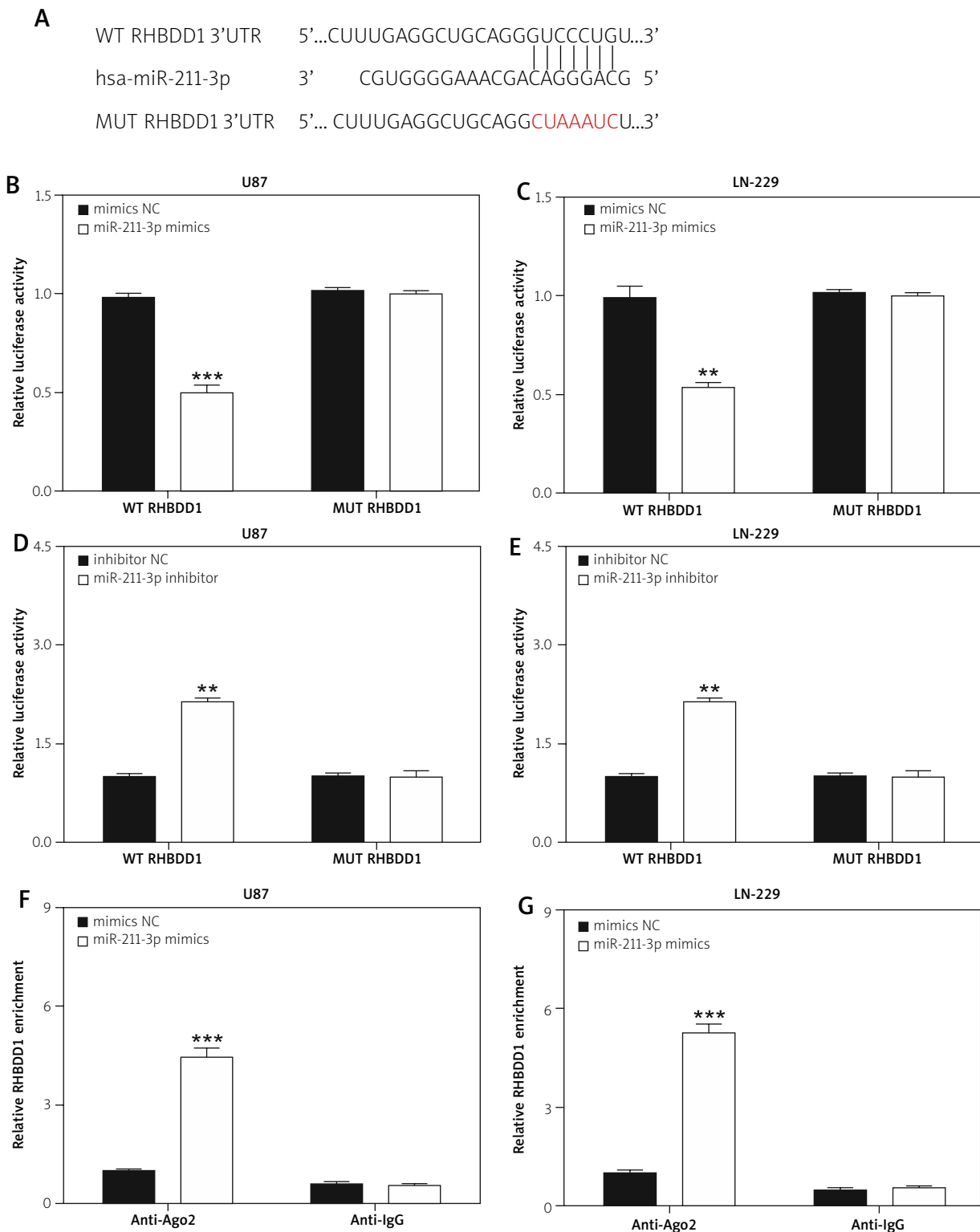
A

WT RHBDD1 3'UTR   5'...CUUUGAGGCUGCAGGGUCCCUGU...3'

                             | | | | | | |

hsa-miR-211-3p   3'   CGUGGGGAAACGACAGGGACG 5'

MUT RHBDD1 3'UTR   5'... CUUUGAGGCUGCAGGCUAAAUCU...3'

Fig. 1. RHBDD1 as the target gene of miR-211-3p in glioma cells. **A**) Putative complementary sites between miR-211-3p and RHBDD1 3'-UTR, and mutant sites in RHBDD1 3'-UTR-MUT reporter. **B, C**) U87 and LN-229 cells were co-transfected with RHBDD1 3'UTR-WT, RHBDD1 3'UTR-MUT reporter and miR'211-3p mimics or mimics NC for 48 h. Luciferase reporter assay was then performed in glioma cells. **D, E**) U87 and LN-229 cells were co-transfected with RHBDD1 3'UTR-WT, RHBDD1 3'UTR-MUT reporter and miR'211-3p inhibitor or inhibitor NC for 48 hours. Luciferase reporter assay was then performed in glioma cells. **F, G**) RNA RIP experiments were performed in U87 and LN-229 cells, and co-precipitated RNA was analyzed by RT-qPCR. **$p < 0.01$, ***$p < 0.001$ vs. mimics NC or inhibitor NC.
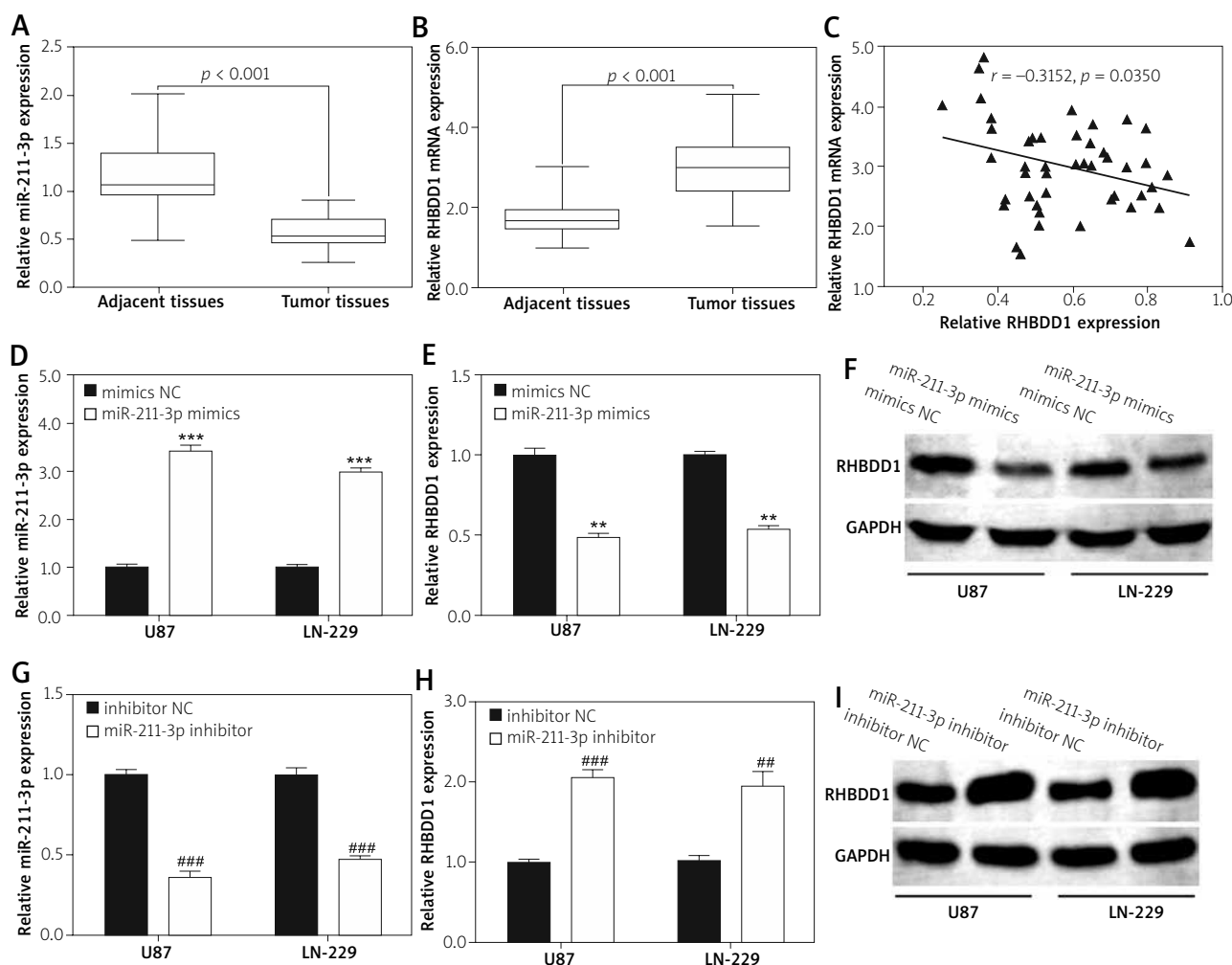
Fig. 2. Inverse correlation of miR-211-3p and RHBDD1 expression levels in glioma. **A**) MiR-211-3p expression in 45 pairs of samples was detected by RT-qPCR. **B**) Relative mRNA expression of RHBDD1 in 45 pairs of samples was determined by RT-qPCR. **C**) Spearman's correlation analysis was performed to assess the correlation of miR-211-3p and RHBDD1 in glioma tissues. Expression levels of: **D**) miR-211-3p, **E**) RHBDD1 mRNA, and **F**) RHBDD1 protein were determined in U87 and LN-229 cells after transfection with miR-211-3p mimics or mimics NC. Expression levels of: **G**) miR-211-3p, **H**) RHBDD1 mRNA, and **I**) RHBDD1 protein were determined in U87 and LN-229 cells after transfection with miR-211-3p inhibitor or inhibitor NC. $**p < 0.01$, $***p < 0.001$ vs. mimics NC; $^{##}p < 0.01$, $^{###}p < 0.001$ vs. inhibitor NC.

experiments. Flow cytometry experiment results (Fig. 3C) showed that the percentage of cells at G0/G1 phase was significantly increased, while cells at S phase was decreased in si-RHBDD1#1 group, in comparison with si-NC group in both U87 (G0/G1 phase: si-RHBDD1#1 vs. si-NC: 57.27 ±0.35 vs. 46.84 ±0.98; S phase: si-RHBDD1#1 vs. si-NC: 27.74 ±1.22 vs. 38.76 ±1.03), and LN-229 (G0/G1 phase: si-RHBDD1#1 vs. si-NC: 65.05 ±0.17 vs. 57.24 ±0.97; S phase: si-RHBDD1#1 vs. si-NC: 16.82 ±1.02 vs. 24.74 ±0.29) cells, which indicated that knockdown

of RHBDD1 induced glioma cell in G0/G1 phase arrest. In addition, transwell assay was used to test the migration and invasion ability of U87 and LN-229 cells harboring RHBDD1 knockdown. As shown in Figure 3D, the number of migratory cells was obviously decreased in U87 and LN-229 cells after si-RHBDD1#1 transfection, compared with si-NC transfection. Similar to migration, knockdown of RHBDD1 significantly suppressed cell invasive ability compared with NC group in both U87 and LN-229 cells (Fig. 3E).
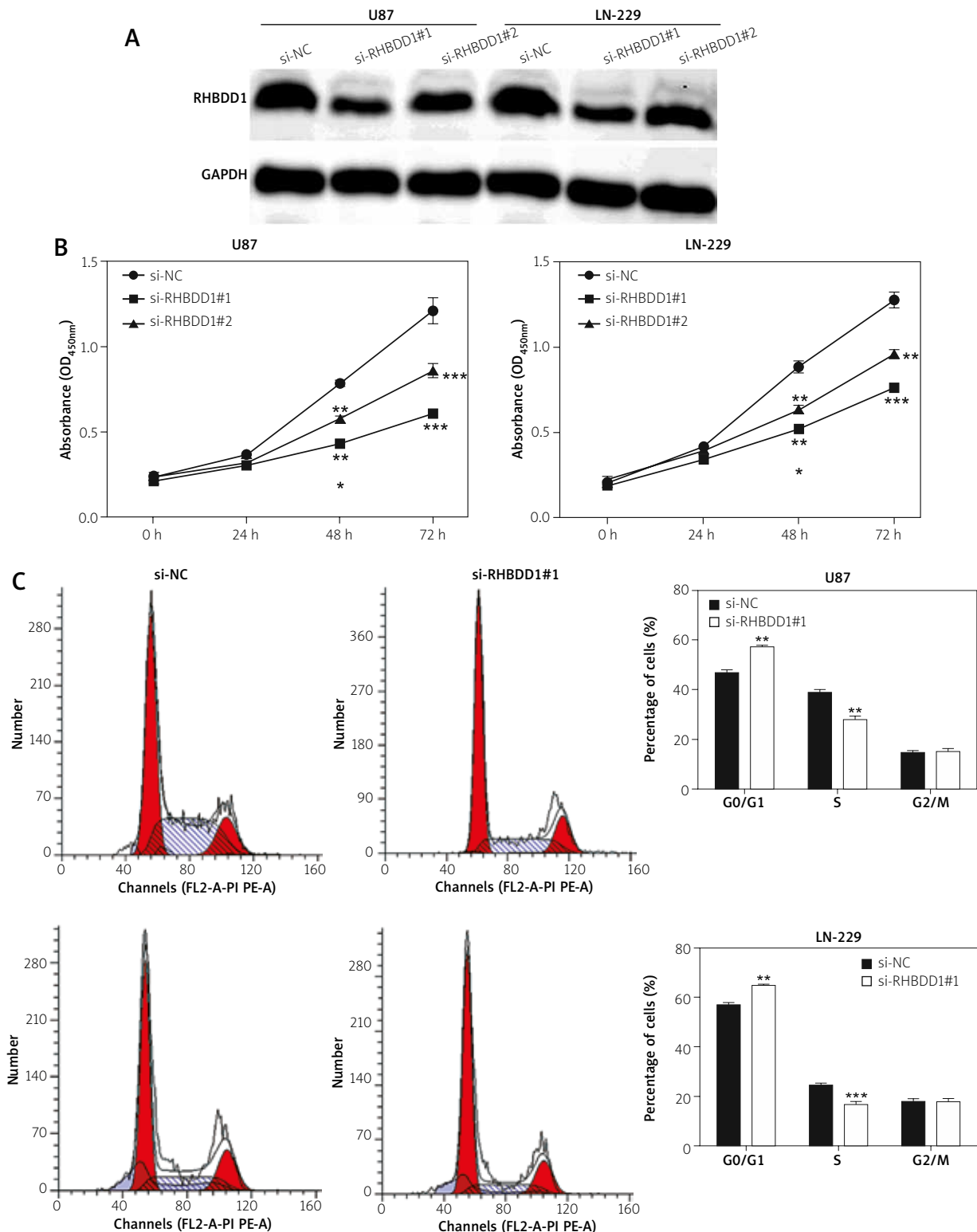
Fig. 3. Inhibitory effects of RHBDD1 knockdown on glioma cell proliferation, migration, and invasion. **A**) Transfection efficiency of si-RHBDD1#1 or si-RHBDD1#2 in U87 and LN-229 cells was detected by Western blot analysis. **B**) CCK-8 assay was used to determine the proliferation ability of U87 and LN-229 cells after transfection with si-RHBDD1#1, si-RHBDD1#2, or si-NC. **C**) Flow cytometry analysis was performed to analyze the cell cycle distribution in U87 and LN-229 cells after transfection with si-RHBDD1#1 or si-NC.
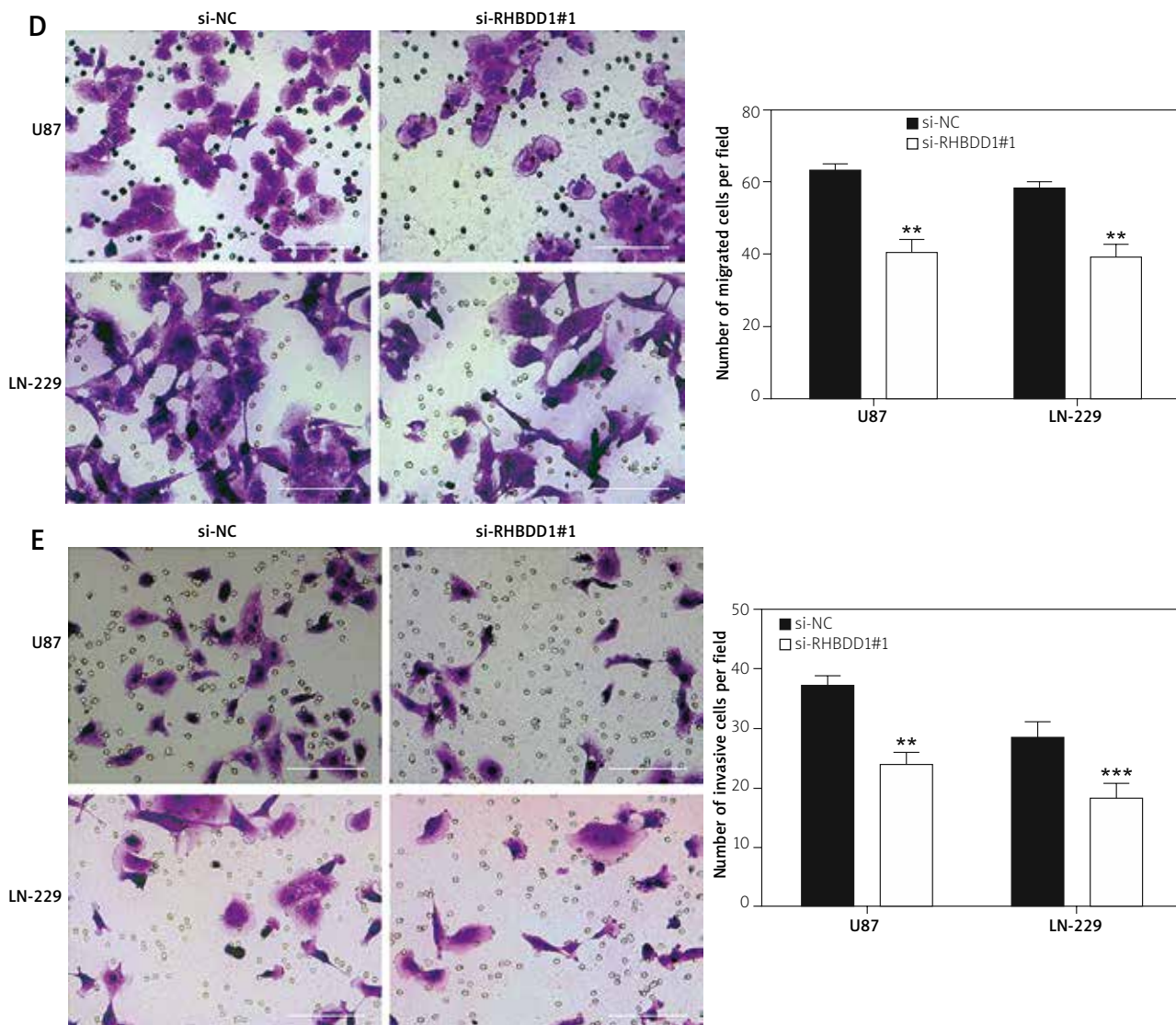
Fig. 3. Cont. **D, E**) Migration and invasion ability were evaluated by transwell assay in U87 and LN-229 cells after transfection with si-RHBDD1#1 or si-NC. Magnification 200×; scale bar 100 μm; **$p < 0.01$, ***$p < 0.001$ vs. si-NC.

Knockdown of miR-211-3p promoted glioma cell proliferation, migration, and invasion

We established stable cells to examine the functions of miR-211-3p in glioma cells, and miR-211-3p inhibitor was transfected into U87 and LN-229. CCK-8 assay indicated that miR-211-3p knockdown remarkably promoted the proliferative abilities of U87 and LN-229 cells (Fig. 4A). The results from flow cytometry assay indicated that knockdown of miR-211-3p promoted cell cycle of G1/S transition, as reflected by a decreased proportions of cells at G0/G1 phase ($p < 0.01$), and an increased proportions of cells at S phase ($p < 0.01$) in U87 and LN-229 cells (Fig. 4B). The results from transwell assays showed that low expression of miR-211-3p significantly promoted cell migration (Fig. 4C) and invasion (Fig. 4D) abilities in both U87 and LN-229 cells.

Overexpression of RHBDD1 ablated inhibitory effects of miR-211-3p in glioma cells

Given evidence has indicated that RHBDD1 was the direct target of miR-211-3p in U87 and LN-229 cells, RHBDD1 might take part in miR-211-3p-mediat-

ed inhibitory effects in glioma cells. Firstly, overexpression of RHBDD1 was confirmed by Western blot analysis in U87 and LN-229 cells after pcDNA3.1-RHBDD1 transfection, compared with pcDNA3.1 transfection (Fig. 5A). Then, we performed rescue experiments to detect whether overexpression of RHBDD1 would sim-

ulate miR-211-3p-mediated effects by co-transfection with miR-211-3p mimics and RHBDD1 overexpression plasmid. As expected, the inhibitory effects of miR-211-3p mimics transfection on cell proliferation (Fig. 5B) and G1/S transition (Fig. 5C) were restored after co-transfected RHBDD1 and miR-211-3p
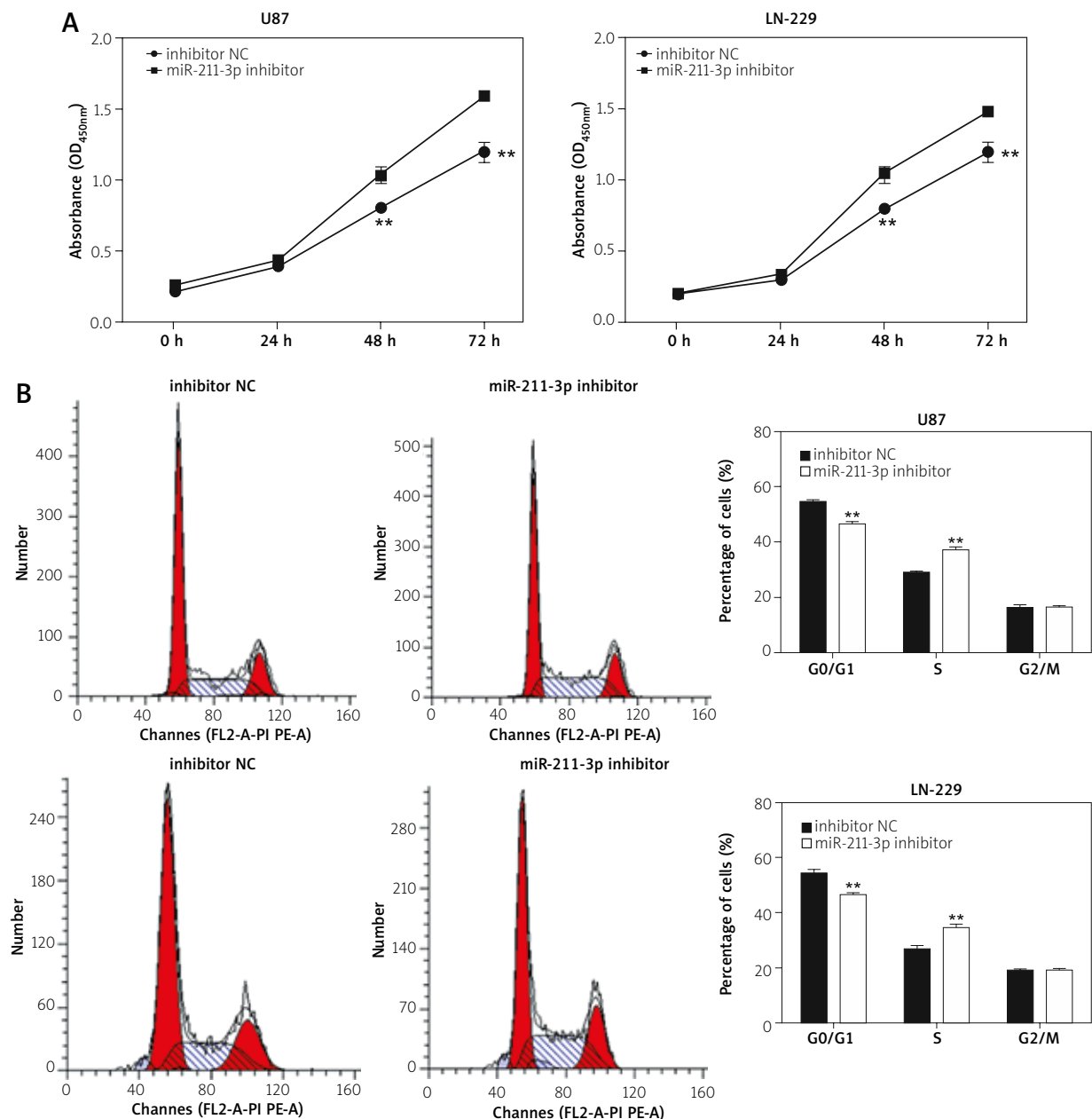

Fig. 4. Knockdown of miR-211-3p promoted the glioma cell proliferation, migration, and invasion. U87 and LN-229 cells were transfected with miR-211-3p inhibitor or inhibitor NC, respectively. **A**) CCK-8 assay was used to determine the proliferation ability of transfected U87 and LN-229 cells. **B**) Flow cytometry analysis was performed to analyze the cell cycle distribution in transfected U87 and LN-229 cells.
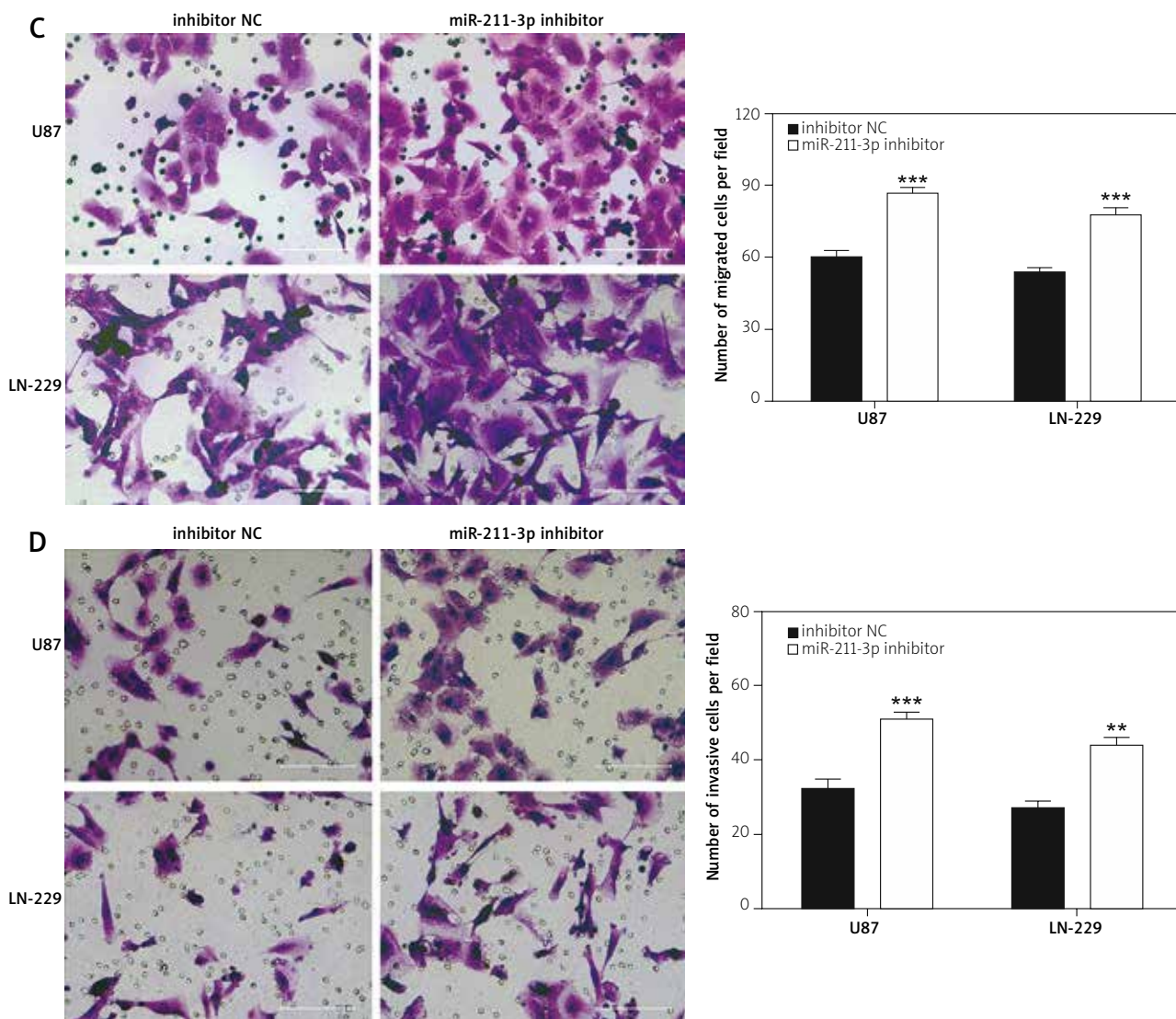
C

D

Fig. 4. Cont. **C, D**) Migration and invasion ability were assessed by transwell assay in transfected U87 and LN-229 cells. Magnification 200×; scale bar 100 μm; Data shown as mean ±SD of the three independent experiments. **$p < 0.01$, ***$p < 0.001$ vs. inhibitor NC.

mimics in both U87 and LN-229 cells. Additionally, overexpression of RHBDD1 significantly reversed the miR-211-3p-inhibiting effects on cell migration (Fig. 5D) and invasion (Fig. 5E) abilities in both U87 and LN-229 cells. Furthermore, Western blot analysis was applied to measure the protein markers associated with G1/S transition and cell mobility. As shown in Figure 6, miR-211-3p overexpression induced downregulation of CDK4, cyclin D1, N-cadherin, and vimentin as well as upregulation of E-cadherin, which were all reversed after co-transfection of miR-211-3p mimics and RHBDD1 in both glioma cells.

Discussion

Dysregulation of miRNAs has been thoroughly reported to be involved in uncontrolled and progressive human malignancies, including glioma [23,26,33]. Mounting evidences have demonstrated miR-211-3p plays an important role in malignant biological behaviors, such as cell growth, migration, and invasion in different cancers [5,12,21,36,37]. However, the functional role and molecular mechanism of miR-211-3p in glioma have not been investigated until now. Here, we observed that miR-211-3p
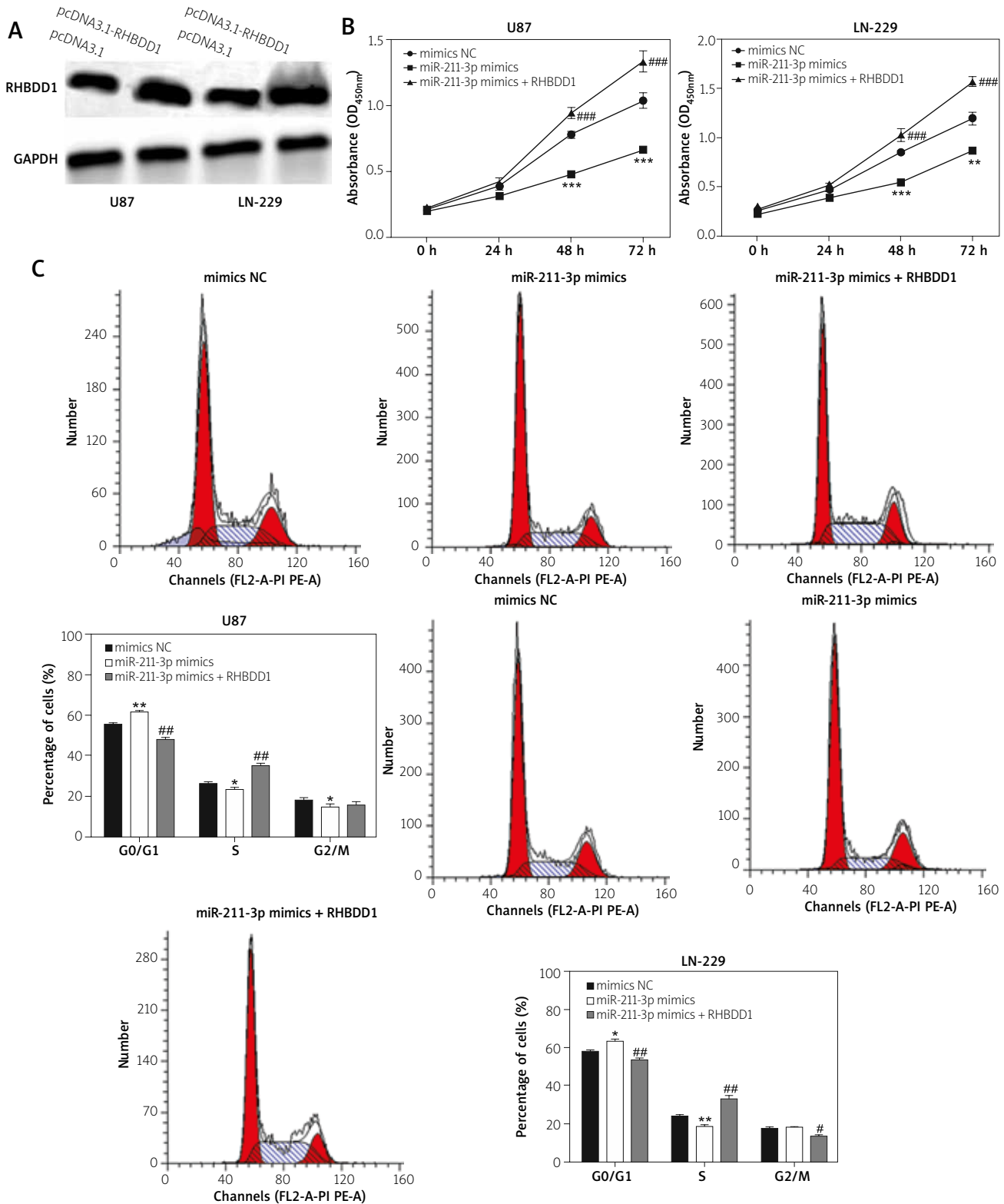
Fig. 5. Overexpression of RHBDD1 ablated the inhibitory effects of miR-211-3p in glioma cells. U87 and LN-229 cells were transfected with pcDNA3.1, pcDNA3.1-RHBDD1, mimics NC, miR-211-3p mimics alone, or co-transfected with miR-211-3p mimics and RHBDD1. **A**) Western blot analysis was applied to determine the protein expression level of RHBDD1. **B**) Proliferation ability of transfected U87 and LN-229 cells was analyzed using CCK-8 assay. **C**) Cell cycle distribution was analyzed by flow cytometry assay in transfected U87 and LN-229 cells.
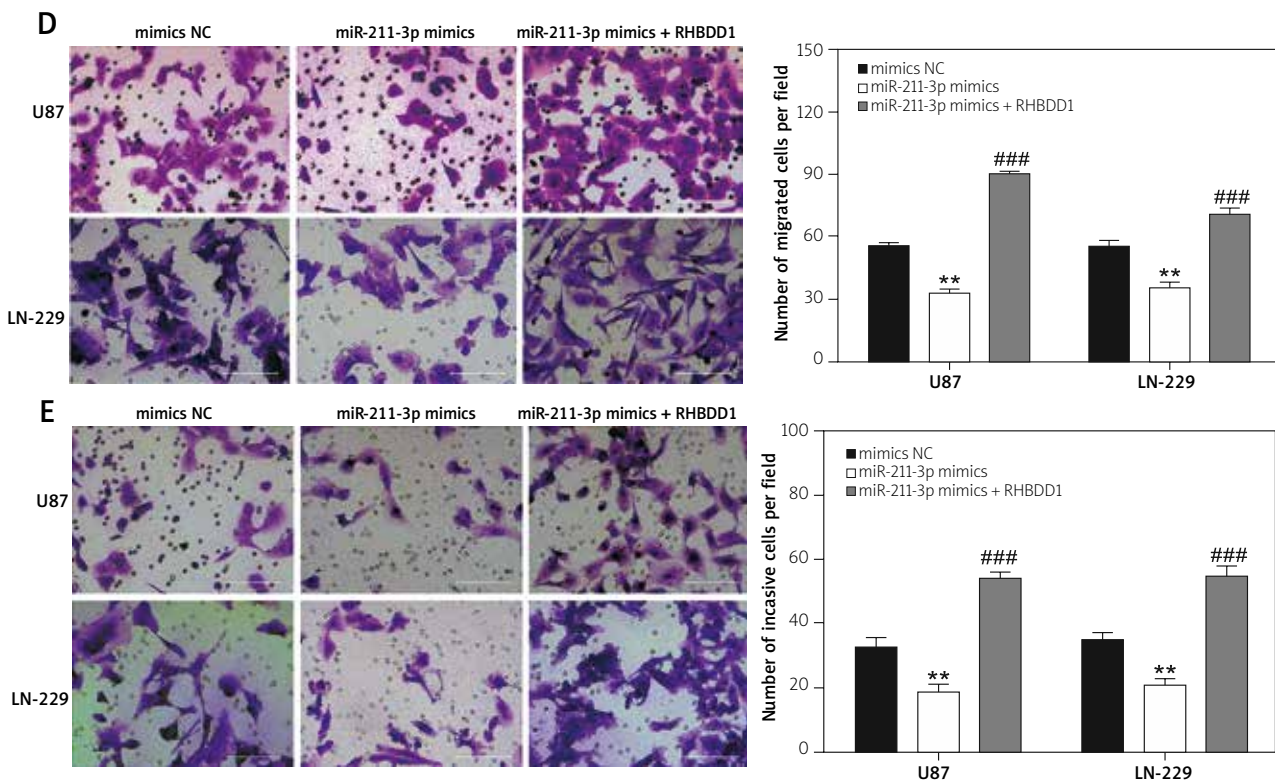
Fig. 5. Cont. **D, E**) Migration and invasion ability were assessed by transwell assay in transfected U87 and LN-229 cells. Magnification 200×; scale bar 100 μm; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. mimics NC; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ vs. miR-211-3p mimics.

was downregulated in tumor tissues from glioma patients compared with that in adjacent tissues. Functionally, knockdown of miR-211-3p significantly promoted cell proliferation, cell cycle of G1/S transition, migration, and invasion in two glioma cell lines (U87 and LN-229). In agreement with our data, Ma *et al.* [21] not only observed that miR-211-3p expression was significantly lower in non-small cell lung cancer tissues, but also demonstrated that overexpression of miR-211-3p could inhibit the proliferation and migration of A549 and H358 cells. Feng *et al.* [5] found that up-regulated miR-211-3p reduced the tumor volume and weight of nude mice with bladder cancer as well as promoted apoptosis and restrained proliferation of tumor cells. In addition, miR-211-3p was downregulated in breast cancer and inhibited cell proliferation and invasion [12]. In contrast, Liu *et al.* [17] reported that miR-221-3p was markedly upregulated in hepato-cellular carcinoma (HCC) clinical tissue samples, which significantly enhanced proliferation, HBV-DNA replication,

migration, and invasion of HCC cells. Another study by Xu *et al.* [36] demonstrated that miR-211-3p was negatively correlated with lncRNA tumor suppressor candidate 7 (TUSC7) as a potential tumor suppressor in colorectal cancer. From these evidences, we could conclude that whether miR-211-3p function as a tumor suppressor or oncogene depend on different tumor types.

To the best of our knowledge, miRNAs usually exert their functions through inhibiting their target genes' expression [2]. It has been reported that miR-211-3p was linked to the suppression of various target genes, with currently validated targets, including endothelial cell-specific molecule 1 (ESM1) in bladder cancer [5], MAF bZIP transcription factor G (MAFG) in oral squamous cell carcinoma [37], zinc finger protein 217 (ZNF217) in non-small cell lung cancer [21], and CDK6 in colorectal cancer [36]. In this study, we verified that RHBDD1 was a target gene of miR-211-3p and negatively regulated by miR-211-3p in glioma cells. We also observed that
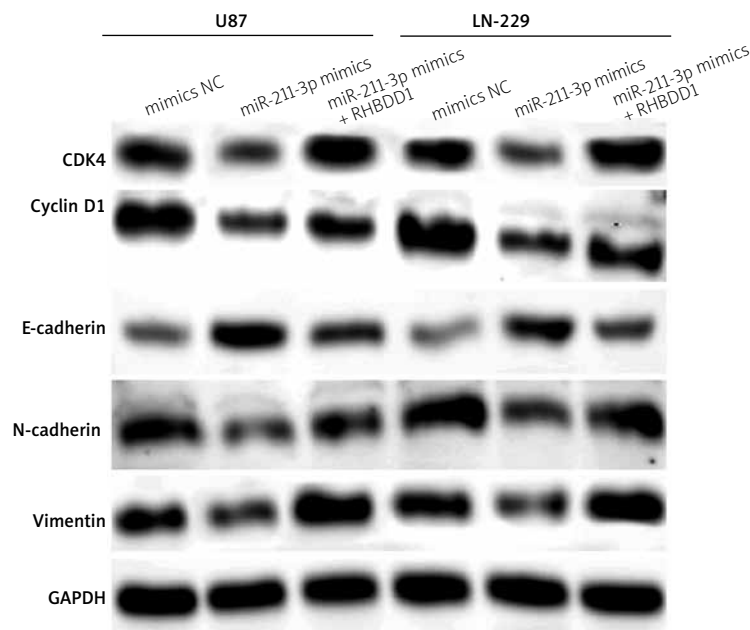
Fig. 6. Overexpression of RHBDD1 reversed miR-211-3p-mediated effects on related protein markers in glioma cells. U87 and LN-229 cells were co-transfected with miR-211-3p mimics and RHBDD1. Western blot analysis was performed to detect protein levels of CDK4, cyclin D1, E-cadherin, N-cadherin, and vimentin in U87 and LN-229 cells.

the expression of RHBDD1 was highly increased in glioma tissues compared with adjacent tissues. If condition permits, we will perform immuno-cyto-chemistry to examine the RHDDB1 protein expression in tissues samples. By loss-of-function assay, we further demonstrated that knockdown of RHBDD1 significantly suppressed cell proliferation, migration, and invasion as well as induced cell cycle of G0/G1 arrest in glioma cells. In fact, RHBDD1 has been widely reported to be an oncogene in tumor cell proliferation and tumor metastasis, including breast cancer [10,40], renal cell carcinoma [14], colorectal cancer [8,30], and HCC [16]. Consistently, Wei *et al*. [34] previously reported that silencing of RHBDD1 caused significant inhibition of cell cycle progression and cell proliferation in glioblastoma cells. Different from a study by Wei *et al*. [34], our data further presented the effects of RHBDD1 in cell migration and invasion *in vitro*. In a similar manner, RHBDD1 has been identified as a target gene of tumor suppressors in different tumor cells, including miR-145-5p in colorectal cancer [24], miR-138-5p in breast cancer [38], and miR-924 in non-small cell lung cancer [31].

Further studies revealed that overexpression of RHBDD1 counteracted the suppressive effects of

miR-211-3p on glioma cell proliferation, cell cycle of G1/S transition, migration, and invasion. At molecular level, the decreased expression of CDK4, cyclin D1, N-cadherin, and vimentin, and increased E-cadherin expression induced by miR-211-3p overexpression, were notably reversed by RHBDD1 overexpression. Recent studies have shown that the expression of CDK4 and cyclin D1 were abnormally overexpressed in a variety of tumors [6,28]. CDK4 can bind with cyclin D1 to form complexes, affecting cell cycle regulation through G1/S transition and cell proliferation [3,6]. Epithelial-mesenchymal transition (EMT), as an effective way for epithelial cells to acquire migration ability, is an important process for more than 90% of epithelial cancer invasion and metastasis in malignant tumors [15]. The EMT process is associated with biochemical changes, in which epithelial cell markers, such as E-cadherin is down-regulated, which leads to loss of cell-cell adhesion, while mesenchymal markers, such as vimentin and N-cadherin are up-regulated, casing cell migration and metastasis [25]. Based on these evidences, we might infer that miR-211-3p suppressed cell proliferation by inducing cell cycle of G0/G1 arrest, and inhibited cell migration and invasion by inhibiting EMT pro-

cess, which were both correlated with suppression of RHBDD1 induced by miR-211-3p in glioma cells.

In summary, the results of this study preliminarily presented the expression pattern and possible biological function of the miR-211-3p/RHBDD1 signaling axis in glioma. Our study laid a solid foundation that miR-211-3p may be considered as a tumor suppressor in the diagnosis and treatment of glioma. However, the further function and molecular mechanism underlying miR-211-3p/RHBDD1 signaling axis still need to be confirmed by *in vivo* experiments and more clinical studies.

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Disclosure

The authors report no conflict of interest.

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