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MiRNA-93 functions as an oncogene in glioma by directly targeting RBL2

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Abstract. – OBJECTIVE: Glioma is a tumor of the brain. Although the clinical regimens and surgical techniques for glioma have improved, therapies of advanced glioma remain challenging, carrying dismal overall survival and therapeutic success rates. Evidence has shown that miRNAs played important roles in glioma development. The current study aimed at investigating the function of a novel cancerogenic miRNA, miR-93, in glioma progression by investigating the expression and mechanism of it.

PATIENTS AND METHODS: qRT-PCR was conducted to assess the miR-93 expression and the mRNA expression of target gene in glioma tissues and cells. The invasion and migration abilities of the glioma cells were determined by transwell assays. Luciferase reporter assay was performed to confirm the target of miR-93.

RESULTS: The results indicated that miR-93 expression in glioma tissues and cells was increased significantly than that in normal brain tissues and cells. Furthermore, miR-93 promoted glioma cell migration and invasion. RBL2 was recognized as a direct target of miR-93 in glioma cells, and overexpression of RBL2 could reverse the stimulative effect of miR-93 in glioma cell.

CONCLUSIONS: The above findings suggested that miR-93 together with RBL2 could be diagnostic targets and novel prognostic markers for glioma.

Key Words:
miRNA-93, Oncogene, Glioma, RBL2.

Introduction

Glioma, one of the most prevalent lethal brain cancers, is associated with high morbidity and lethality[1]. Although multiple treatments, including surgery, radiotherapy, and chemotherapy, are constantly improved, the overall survival of glioma patients is still 12-14 months[2]. Mostly because of the highly invasive growth pattern, prominent tumor angiogenesis, and frequent resistance to therapies. The prognosis for glioma patients is dismal, and the treatment efficacy is poor[3,4]. Therefore, exploring novel markers for early diagnosis and treatment of glioma is critical. At the same time, the illustration of the molecular mechanisms underlying glioma also offers new orientation toward the therapies. Several miRNAs were related to glioma development, such as miR-342-3p[5], miR-148[6], and miR-320a[7]. In addition, regulation of miRNAs was found to have important effects on the progress of glioma.

miRNAs are a type of small non-coding RNAs playing crucial roles in regulating multiple biological processes by interacting with the target genes[8]. miRNA has shown great potential to be a biomarker for detection and prognosis of various tumors lately. For example, Zhang et al[9] found that miR-30a-5p inhibited proliferation, invasion, and tumor growth of hepatocellular carcinoma cells by targeting FOXA1. Xiao et al[10] demonstrated that miR-138 acted as a tumor suppressor in non small cell lung carcinoma *via* targeting YAP1. Besides, Zhang et al[11] found that downregulation of miR-449 could promote invasion and migration of breast carcinoma cells by targeting tumor protein D52 (TPD52). miR-93 was also widely described as an ideal biomarker for diagnosis of multiple human tumors. However, the functions of miR-93 in modulating glioma cell migration and invasion needs to be fully elucidated.

Retinoblastoma (Rb) family has an important function in the regulation of cell cycle and it contains three members which are retinoblastoma protein (pRb), retinoblastoma like protein-1 (RBL1), and retinoblastoma-like protein-2 (RBL2)[12]. Among the three members, RBL2 has been reported to show suppressive function intumor[13]. However, the specific functions of RBL2

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in glioma still remain unclear. The present study aimed to investigate the correlation between miR-93 and RBL2 in glioma cell migration and invasion.

Patients and Methods

Human Tissues and Cell Culture

A total of 52 pairs of glioma tissue samples and non-neoplastic brain tissues were acquired from the Beijing Tsinghua Changgung Hospital between 2015 and 2017 and then snap-frozen in liquid nitrogen and reserved at -80°C for further use. All patients involved in this study were histologically diagnosed in line with the 2016 WHO classification of the central nervous system tumors. All the tissue samples were collected. The current study obtained approval from the Ethics Committee of Beijing Tsinghua Changgung Hospital. Signed written informed consents were obtained from the patients and/or guardians.

Normal human astrocytes (NHAs) and two human glioma cell lines (U251 and U87) were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and maintained in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA, USA) which contained 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) and 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) in an atmosphere with 5% CO_2 at 37°C.

Cell Transfection

Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was applied to transiently transfect the miR-93 mimics or inhibitor as well as the overexpression vector of RBL2 into glioma cells according to the manufacture's instruction. The glioma cells with different transfections were used for subsequent assays.

Quantitative Real time-PCR (qRT-PCR)

TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) was applied to extract the total RNA from glioma tissues and cells as well as the normal tissues according to the manufacture's recommendation. Reverse-transcribed complementary DNAs (cDNAs) were prepared according to the PrimeScript RT reagent Kit (TaKaRa, Dalian, China), and qRT-PCR was conducted with SYBR Premix ExTaq (TaKaRa, Dalian, China). The miR-93 expression was normalized to U6 while the RBL2 was normalized to GAPDH. The relative expression ratios of genes were calculated by the $2^{-\Delta\Delta CT}$ method. The primers involved in this assay were shown in Table I.

Western Blots

Cells were lysed in Protein Lysis Buffer (Beyotime, Shanghai, China) in line with the manufacturer's proposal and the obtained cell lysates were prepared for Western blotting. Total protein was extracted from whole cells and the concentrations of proteins were determined using the BCA Protein Assay Kit (Wuhan Boster Biological Technology, Ltd., Wuhan, China) following the manufacturer's directions. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins, and then, the separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane which was incubated at 4°C overnight with mouse anti-RBL2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or mouse anti-GAPDH antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in 5% milk. Then, a secondary incubation step was performed with appropriate secondary antibody at room temperature for one hour. The proteins were detected by Chemoluminescence method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Abcam, Cambridge, MA, USA) was used to be an internal loading control.

Table I. Primer sequences for qRT-PCR.

Primer	Sequence
miR-93 forward	5'-AGTCTCTGGGCTGACTACATCACAG-3'
miR-93 reverse	5'-CTACTCACAAAACAGGAGTGGAATC-3'
U6 forward	5'-CTCGCTTCGGCAGCACA-3'
U6 reverse	5'-AACGCTTCACGAATTTGCGT-3'
RBL2 forward	5'-GGAGGAAATTGGGACTCTCTCA-3'
RBL2 reverse	5'-AGACGACTCAAGCTATGCGTA-3'
GAPDH forward	5'-GATATTGTTGACATCAATGAC-3'
GAPDH reverse	5'-TTGATTTTGGAGGGATCTCG-3'

U6: small nuclear RNA, snRNA; RBL2: retinoblastoma like protein-2; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

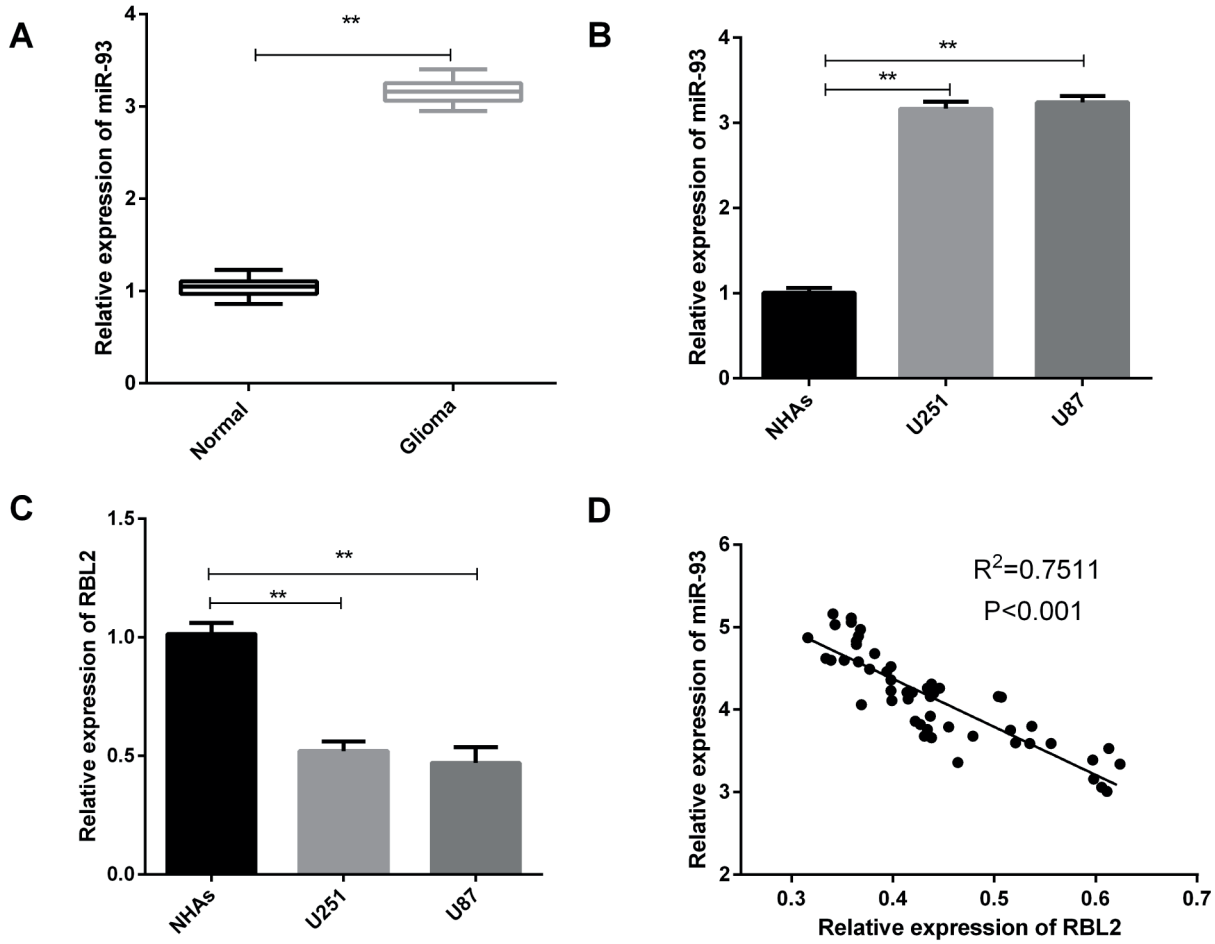

Figure 1. miR-93 expression elevated and RBL2 expression reduced in glioma. ***A,*** qRT-PCR was applied to measure the miR-93 expression in glioma tissues (n=52) and matched para-carcinoma tissues (n=52) (**p<0.01). ***B,*** miR-93 expressions in glioma cells were evaluated by qRT-PCR (**p<0.01). ***C,*** The RBL2 expression was measured using qRT-PCR in glioma cells (**p<0.01). ***D,*** Regression analysis of correlation between miR-93 and RBL2 expressions.

Transwell Assays

The invasion and migration abilities of the glioma cells were determined by transwell assays. The invasion ability was assessed by transwell chambers (Corning, Corning, NY, USA) coated with Matrigel (Clontech, Mountain View, CA, USA). Then, glioma cells were added to the top chamber and cultivated for 48 hours. The bottom chambers contained DMEM including 10% FBS. After cultivated for 48 hours, the glioma cells on the top surface of the chambers were rubbed off by cotton swabs carefully. Subsequently, the invaded glioma cells on the bottom surface were fixed and stained for detecting the results. The migration ability was detected using transwell chambers without being pretreated with Matrigel. Results of invasion and migration were detected under an inverted microscope (Olympus, Tokyo, Japan) in five randomly selected fields. All of the experiments were performed in triplicate.

Luciferase Reporter Assay

The amplified RBL2-3'-UTR-WT and corresponding RBL2-3'-UTR-MUT were respectively cloned into pGL3 luciferase vector (Promega, Madison, WI, USA). Glioma cells were co-transfected with miR-93 mimics and luciferase reporter vectors of the wide type or mutant type 3'-UTR of RBL2 gene using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). Subsequently, the Dual-Luciferase Reporter Assay kit (Promega, Madison, WI, USA) was used to detect the relative luciferase activities 48 hours after the transfections.

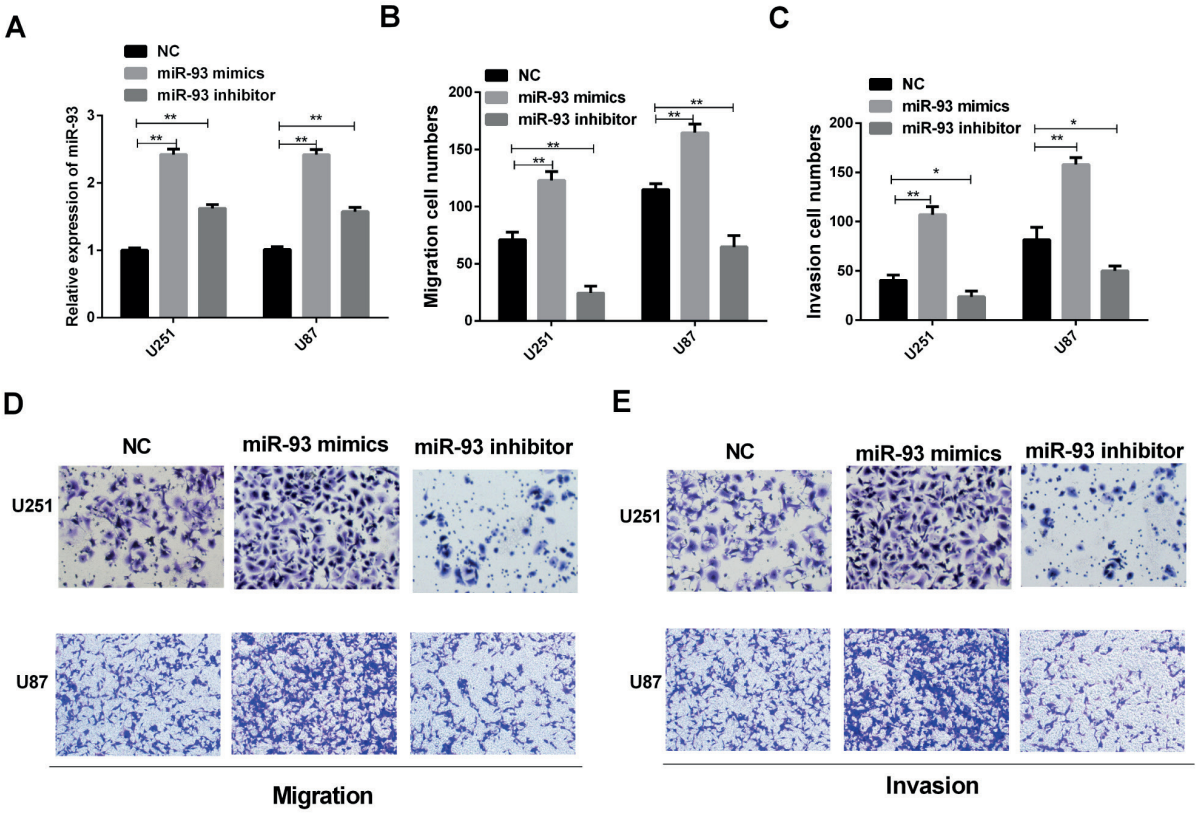

Figure 2. miR-93 accelerated glioma cell migration and invasion. *A,* The miR-93 expressions in transfected U251 and U87 cells were detected using qRT-PCR (**p<0.01). ***B,*** The migration cell numbers of glioma cells were counted (**p<0.01). ***C,*** The invasion cell numbers of glioma cells were counted (*p<0.05,**p<0.01). ***D,*** Cell migration was observed by the transwell assay in transfected glioma cells. ***E,*** The transwell assay was conducted to detect cell invasion in transfected glioma cells.

Statistical Analysis

All above experiments were performed 3 times. The statistical analysis was evaluated by the GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) together with statistical product and service solutions (SPSS) 18.0 version (SPSS Inc. Chicago, IL, USA). The Student's *t*-test was applied to evaluate the differences between the two groups. The data was indicated as means ± SD. The differences were identified as statistically significant when p<0.05.

Results

miR-93 Expression is Up-Regulated and RBL2 Expression is Down-Regulated in Glioma

We collected glioma tissues and the corresponding normal tissues from 52 glioma patients to detect the expressions of miR-93 and RBL2 using qRT-PCR. At the same time, the miR-93 and RBL2 expressions in two kinds of glioma cell lines were measured. The results of qRT-PCR revealed that the miR-93 expressions in glioma tissues were significantly up-regulated in contrast with that in the matched para-carcinoma tissues (Figure 1A). Similarly, compared to the normal human astrocytes (NHAs), the miR-93 expression in glioma cells was significantly increased (Figure 1B). On the contrary, we found that the mRNA expression of RBL2 in glioma cell lines was decreased significantly in contrast with that in the normal human astrocytes (NHAs) (Figure 1C). Moreover, to better understand the relationship between miR-93 and RBL2 in glioma, we investigated the correlation of miR-93 and RBL2 expression and found a negative correlation between them (Figure 1D).

miR-93 Accelerates Glioma Cell Invasion and Migration

We researched cell invasion and migration abilities of glioma cells with different transfections. First of all, we measured the miR-93 expressions in different treated glioma cells. The qRT-PCR re-

sults showed that the expression of miR-93 mimics was high in U251 and U87 cells (Figure 2A). Subsequently, the Transwell assays were carried out to research the effects of miR-93 on regulating invasion and migration of U251 and U87. The results revealed that overexpression of miR-93 accelerated migration abilities of glioma cells; the results also inversely demonstrated that miR-93 inhibitor could suppress the migration of glioma cells (Figure 2B and 2D). Additionally, according to the results of transwell assays, we found that miR-93 could promote the invasion ability of glioma cells (Figure 2C and 2E).

miR-93 Suppresses RBL2 Gene Transcription in Glioma by Targeting its 3'-UTR

We ulteriorly explored the correlation between miR-93 and RBL2 to better understand the mechanism of miR-93 in regulating glioma. Firstly, we used Target Scan to predict the target sites of miR-93 in the sequence of RBL2 (Figure 3A). Then, the luciferase reporter vectors contained the RBL2 3'-UTR-WT or RBL2 3'-UTR-MUT were constructed and cotransfected into glioma cells with miR-93 mimics. We measured the RBL2 3'UTR luciferase activities by performing luciferase reporter gene assays. The results demonstrated a significant decrease of fluorescence activity in both U251 and U87 cells co-transfected with the RBL2 3'-UTR-WT and miR-93 mimics. Nevertheless, there was no significant difference in the cells co-transfected with the miR-93 and RBL2 3'-UTR-MUT vector in contrast with the control group (Figure 3B and 3C). Furthermore, we also detected the RBL2 expressions at mRNA level as well as at protein level in glioma cell li-

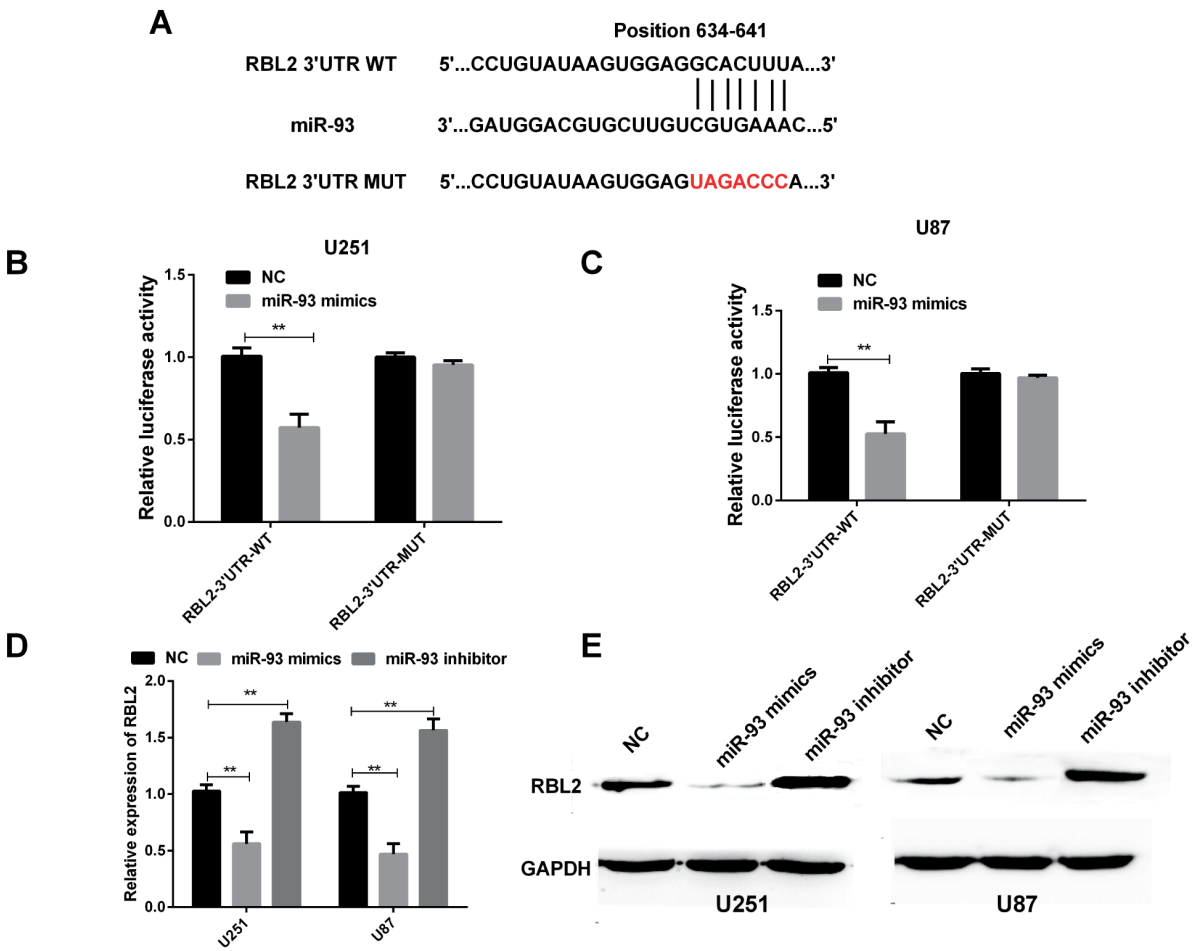

Figure 3. miR-93 de-regulated RBL2 expression *via* binding to the 3'-UTR of RBL2 directly. ***A,*** The miR-93 binding sequence in the 3'-UTR of RBL2. ***B, C,*** The luciferase reporter gene assays were carried out to detect the fluorescence activities of the RBL2 3'UTR in U251 cells (***B,***) and U87 cells (***C,***) which were cotransfected with wild-type RBL2 3'UTR or mutational type RBL2 3'UTR and miR-93 mimics, respectively (**$p<0.01$). ***D,*** qRT-PCR results of the RBL2 mRNA level in U251 cells and U87 cells with different transfections (**$p<0.01$). ***E,*** Western blot results of the RBL2 expression in glioma cells with different transfections.

nes transfected with miR-93 mimics or inhibitor. The results of qRT-PCR and Western blot both demonstrated that miR-93 could inhibit RBL2 expression in glioma cells (Figure 3D and 3E).

Overexpression of RBL2 Markedly Reversed miR-93-Medicated Promotion of Cell Migration and Invasion in Glioma Cells

We explored the functions of RBL2 in regulating the glioma cell invasion and migration mediated by miR-93. Firstly, overexpression vector of RBL2 and miR-93 mimics were transfected into glioma cells and the results were detected by qRT-PCR as well as Western blot. From the results, we found that the RBL2 expression in glioma cells transfected with RBL2 was significantly up-regulated in contrast to the control group (Figure 4A and 4B). Furthermore, we measured the cell invasion and migration in glioma cell lines co-transfected with RBL2 and miR-93 mimics by Transwell assays. The results revealed that the restoration of RBL2 markedly reversed miR-93-medicated promotion of cell invasion and migration in glioma cells (Figure 4C and 4D).

Discussion

Despite the improvement in treatment options, there has been little or no improvement in the survival of glioma patients. The traditional clinicopathologic features, such as clinical symptoms and tumor size, are still regarded as the gold standard diagnosis for the prognosis of patients with glioma[14]. However, the significant variations and heterogeneities in glioma patients at the same tumor stage result in an intensive evaluation of other histologic, molecular, and genetic characteristics[15,16]. More and more evidence has indicated

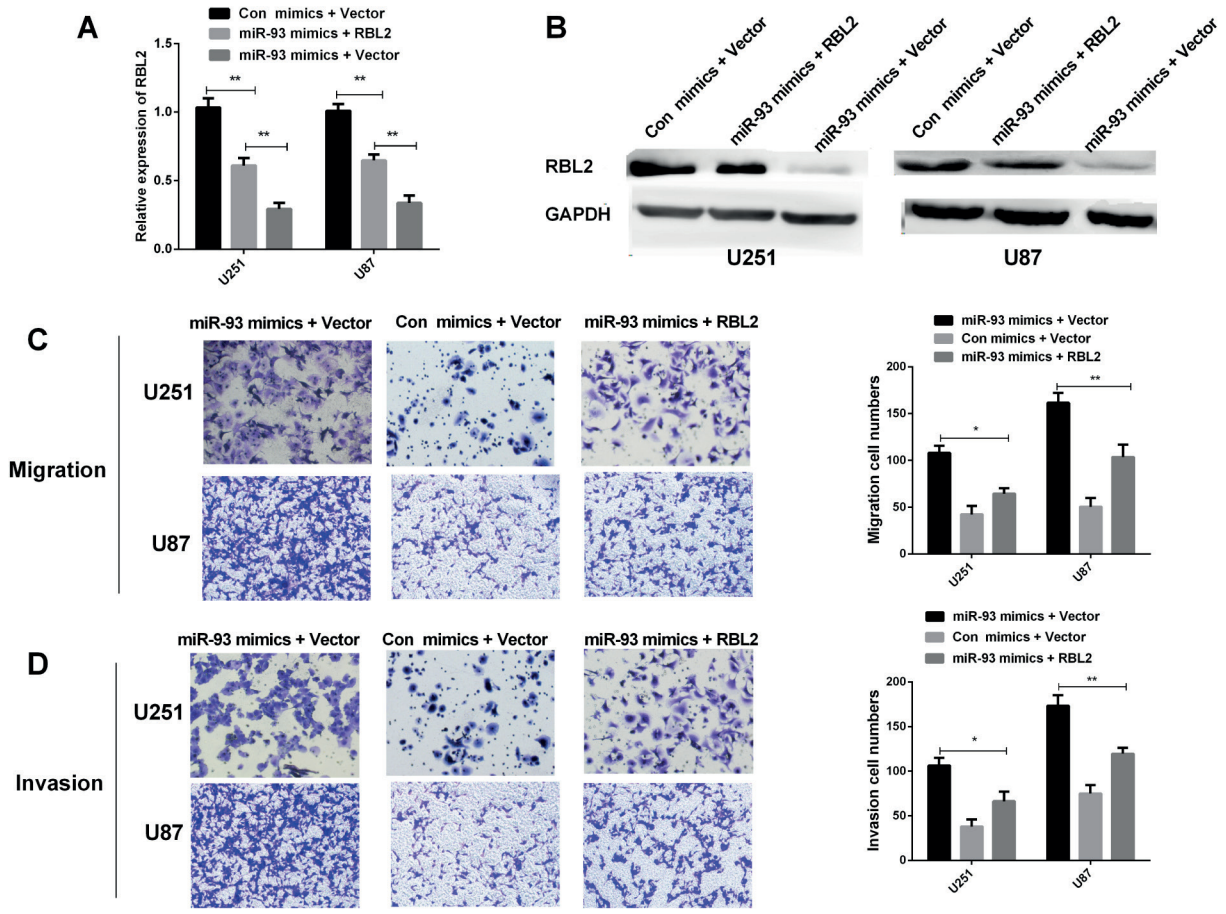

Figure 4. Restoration of RBL2 expression abrogated miR-93-mediated cell migration and invasion. ***A, B,*** The RBL2 mRNA or protein expression level was detected using Western blot or qRT-PCR in glioma cells co-transfected with RBL2 overexpression vector and miR-93 mimics (***p*<0.01). ***C, D,*** Transwell assays were conducted to detect migration and invasion ability in glioma cells co-transfected with RBL2 overexpression vector and miR-93 mimics (**p*<0.05, ***p*<0.01).

that it is emergent to clarify the molecular mechanisms involved in glioma initiates, progresses, invades, migrates, and recurs. At the same time, to explore novel and effective therapeutic strategies for glioma is also important.

miRNAs have been reported to participate in various biological processes by regulating the protein levels of the target genes negatively[17]. In addition, the target genes of miRNAs contain many oncogenes and tumor suppressors, so miRNAs play crucial roles in multiple human tumors, functioning as oncogenes or tumor suppressors[18]. Growing evidence has demonstrated aberrant regulation of miR-93 in multiple tumors. For example, Li et al[19] found that miR-93 could promote tumorigenesis and metastasis via regulating the PI3K/Akt pathway in non-small cell lung cancer. Ji et al[20] reported that miR-93 could promote hepatocellular carcinoma invasion and metastasis by EMT through directly targeting PDCD4. Moreover, Li et al[21] clarified the function of miR-93-5p in accelerating tumor progression by regulating the Hippo signaling pathway in gastric cancer. As we all know, miR-93 has been demonstrated to play important roles in many tumors, but its role in glioma needs to be fully elucidated. This study aimed to explore the function of miR-93 in glioma.

The Rb family has crucial functions in mediating cell proliferation and differentiation[22]. It's reported that deletions or alterations of certain genes, such as CDKN2A, CCND1, and CDK4, involved in regulating Rb proteins, may down-regulate or completely ablate the normal effects of Rb protein family[23,24]. RBL2, a well-known tumor suppressor gene in the Rb family, is inactivated in numerous cancers. For example, miR-17-5p could accelerate the proliferation of pancreatic cancer *via* changing the cell cycle profiles through the disruption of RBL2/E2F4-repressing complexes[25]. This illustrated that interruption of the signaling pathway in which RBL2 participated was a common event in tumorigenesis. Nevertheless, little is known about the possible functions of RBL2 in glioma. In the current study, we investigated the relationship between miR-93 and RBL2 in glioma.

Conclusions

We showed that miR-93 expression was enhanced both in glioma tissues and glioma cells and the miR-93 expression was connected with clinicopathological features of glioma patients. Also, we discovered that the miR-93 expression has a negative correlation with the RBL2 expression in glioma tissues. Moreover, we found that miR-93 exerted tumor carcinogenic functions in glioma by targeting RBL2 directly. In summary, all the results of this study together suggested that miR-93-RBL2 axis may be a novel biomarker for the diagnosis and treatment of glioma.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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