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MicroRNA-378 acts as a prognosis marker and inhibits cell migration, invasion and epithelial-mesenchymal transition in human glioma by targeting IRG1

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Abstract. – OBJECTIVE: Glioma is one common intracranial malignancy. Recently, there has been a large volume of published studies describing the functions of microRNAs as potential diagnostic markers for glioma. Data from several sources revealed that miR-378 played crucial roles in multiple tumors. However, much uncertainty still exists about the functions and underlying mechanism of miR-378. The purpose of the present work was to evaluate the potential effect of miR-378 and verify its influence on the function of IRG1 in glioma.

PATIENTS AND METHODS: The miR-378 expression was examined in 52 pairs of glioma tissues using quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). Transwell assays were conducted to detect the capability of glioma cell migration and invasion with different transfections. Luciferase reporter was used to confirm whether miR-378 could regulate immune responsive gene 1 (IRG1). Western blot was used to measure the expressions of EMT-related markers.

RESULTS: miR 378 expressions were notably reduced in glioma cells and tissues in comparison with controls. The declined miR-378 expressions were correlated with the poor OS and worse clinicopathological parameters of glioma patients. Overexpression of miR-378 repressed glioma cell epithelial-mesenchymal transition (EMT) and metastasis as well as the tumor growth rate and tumor size of glioma mice. Additionally, IRG1 was markedly up-regulated in glioma and was confirmed as a direct target for miR 378 in glioma.

CONCLUSIONS: We showed that the suppressive role of miR-378 in glioma, which was regulated by IRG1, suggested that the miR-378/IRG1 axis may be an effective target for glioma treatment.

Key Words:
MiR-378, Glioma, Migration, Invasion, Epithelial-mesenchymal transition, IRG1.

Introduction

Glioma, one common malignancy of the central nervous system, presented series of aberrantly aggressive phenotypes[1]. Nowadays, targeted therapy, chemotherapy, radiotherapy, and surgery are the standard therapies for glioma patients[2]. Although evident progress has been made in glioma therapies, one question that needs to be taken into account is that the prognosis for these patients is still poor[3]. This is the result of the difficulties in surgical excision, high tumor aggressiveness, and unlimited proliferation[4,5]. Additionally, the mechanism of the glioma occurrence and progression still need to be further elaborated. Therefore, understanding the mechanism about glioma progression will be beneficial to develop novel biomarkers for its diagnosis and therapies.

In the progression of epithelial-mesenchymal transition (EMT), epithelial cells adopt the phenotypes of mesenchymal, migrating to other sites, and proceeding to colonize[6]. During the EMT process, tumor cells will be endowed with invasive and migratory properties, allowing them to migrate through the extracellular matrix to distant organs[7]. Prior studies[8,9] have noted that EMT has important functions in glioma pathogenesis and progression. Hence, identifications of key

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inducers of EMT are of critical significance to investigate the mechanisms of glioma metastasis and to develop novel therapies.

It has been reported that microRNA (miRNA) is implicated in 90% of translation processes and gene transcriptions, affecting the cellular progress and protein expressions[10]. A considerable amount of literature[11,12] has shown that miRNA is an important regulator of various tumors, functioning as an oncogene or a tumor suppressor. Ectopic expression of miRNA may aid in diagnosis, therapy, and prognosis of tumors[13]. Zhang et al[14] showed that miR-491-5p served as a potential biomarker in gastric cancer and inhibited gastric cancer progression *via* regulating JMJD2B. Baltruskeviciene et al[15] pointed out that downregulation of miR-625-3p and miR-148a in colorectal carcinoma was related to tumor budding. Zhang et al[16] maintained that miR-185 suppressed colon cancer cell invasion and migration through Wnt1. Therefore, the investigation on expressions, functions, and mechanism of miRNAs in glioma will be helpful to identify efficient therapies. Nevertheless, the functions of miR-378 in glioma progression are still unclear and need further elucidation.

Cellular and molecular biological properties indicate that aberrant expressions of genes regulate apoptosis, differentiation, proliferation, metastasis of tumors, including gliomas. As an LPS-inducible gene, immune responsive gene 1 (IRG1) was found to play a key role in neurodegeneration and embryonic implantation[17]. Reactive oxygen species (ROS) production is the one crucial biological function of IRG1, which can promote the expression of some transcription factors including A20[18,19]. The investigations of gene expression profiles on microglial cells and murine macrophages have demonstrated that IRG1 is upregulated under proinflammatory conditions[20], providing an important link between metabolism and immunology[21]. A large and growing body of literature[22-24] revealed that chronic inflammation is implicated in tumorigenesis and tumor development, being identified as one independent risk factor. Therefore, it is essential to further identify the biological functions of IRG1 on glioma progression.

Patients and Methods

Human Tissue Samples

A total of 52 pairs of glioma tissues were obtained from glioma patients in the Yantai Yuhuangding Hospital from October 2015 to May 2017. Eleven normal brain tissue samples obtained from patients with cerebral trauma were adopted as controls. The collected tissue samples were frozen immediately in liquid nitrogen and stored at -80°C for further use. Written informed consents were received from all the patients. This study was approved by the Ethics Committee of Yantai Yuhuangding Hospital.

Cell Lines

Normal human astrocytes (NHAs) and human glioma cells (SHG44, A172, LN229, LN18 and T98) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (HyClone, South Logan, UT, USA) with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) in 5% CO_2 at 37°C.

Cell Transfections

MiR-378 mimics, inhibitors and negative miRNA control (NC) were obtained from GenePharma (Shanghai, China) and transfected into glioma cells by Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' proposals. Efficiencies of the transfected cells were analyzed by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) 48 h after the transfection.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA from glioma cells or tissues. Subsequently, the extracted total RNA was reversely transcribed into complementary Deoxyribose Nucleic Acid (cDNA) by PrimeScript RT reagent kit (TaKaRa Bio, Inc., Shiga, Otsu, Japan). qRT-PCR reactions were performed with an ABI 7900 Real-Time PCR Detection System (Applied Biosystems, Foster City, CA, USA) by SYBR Green (TaKaRa Bio, Inc., Shiga, Otsu, Japan). Data of PCR were analyzed using the $2^{-\Delta\Delta CT}$ method. Expressions of U6 were used as an endogenous control for miR-378 while glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was an internal control for IRG1 and EMT related genes. The sequences of the primers were described in Table I.

Transwell Assay

Transwell chambers (8.0 µm pore size, Corning, Corning, NY, USA) with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) were used to

Table I. Primer sequences for qRT-PCR.

Primer	Sequence
miR-378 forward	5'- GGGACTGGACTTGGAGTCA-3'
miR-378 reverse	5'- GTGCGTGTCGTGGAGTCG-3'
U6 forward	5'- CTCGCTTCGGCAGCACA-3'
U6 reverse	5'- AACGCTTCACGAATTTGCGT-3'
IRG1 forward	5'- GTTAACTACACTTCTCAAAGGACCC-3'
IRG1 reverse	5'- TACAAACCAAATCATTGAATCTCCC-3'
GAPDH forward	5'- TGTGGGCATCAATGGATTTGG-3'
GAPDH reverse	5'- ACACCATGTATTCCGGGTCAAT-3'
E-cadherin forward	5'- CACCTGGAGAGAGGCCATGT-3'
E-cadherin reverse	5'- TGGGAAACATGAGCAGCTCT-3'
Vimentin forward	5'- CTTGAACGGAAAGTGGAATCCT-3'
Vimentin reverse	5'- GTCAGGCTTGGAAACGTCC-3'

U6: small nuclear RNA, snRNA; IRG1: Immune responsive gene 1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

perform the invasion or migration assays. Glioma cells cultured with serum-free medium were placed into the top chambers. In the meantime, medium containing 10% FBS, as a chemoattractant, was placed to the bottom chambers. After incubation at 37°C with 5% CO_2 for 48 h, cells left on the upper surface of the chambers were gently removed with a cotton swab, while the cells attached to the lower surface were fixed with methanol and stained with crystal violet. Then, the cells were photographed and counted under a microscope (Olympus, Tokyo, Japan) from five randomly selected visual fields.

Western Blot

Cells were lysed on ice in radioimmunoprecipitation assay (RIPA) buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing protease inhibitors. Subsequently, a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) was utilized to quantify the concentration of the extracted protein. Then, proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred into polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), which was blocked in Tris-buffered saline and Tween (TBS-T) with 5% skim milk at room temperature for 2h. Soon afterwards, the proteins were incubated with specific primary antibodies overnight at 4°C. The following primary antibodies were used: rabbit antibody against IRG1 (1:1000, ab222417, Abcam, Cambridge, MA, USA), rabbit antibody against E-cadherin (1:1000, ab133597, Abcam, Cambridge, MA, USA), rabbit antibody against Vimentin (1:1000, ab137321, Abcam, Cambridge, MA, USA) and rabbit antibody against GAPDH (1:2000, ab128915, Abcam, Cambridge, MA, USA). Followed by being washed with TBS-T for 3 times, the membranes were incubated with goat anti-rabbit secondary antibody (1:5,000, ab6721; Abcam) at room temperature for 2 h. GAPDH was used as an internal control. Protein bands were detected using the Enhanced Chemifluorescence Western blotting kit (Pierce, Rockford, IL, USA).

Dual-Luciferase Reporter Assay

The predicted IRG1 3'-UTR sequences, which contained the wild-type or mutant binding sites of miR-378, were constructed and cloned into the pGL3 luciferase reporter vectors (Invitrogen, Cambridge, MA, USA), and were named as IRG1-3'UTR-Wt or IRG1-3'UTR-Mut, respectively. Glioma cells were cotransfected with miR-378 mimics and IRG1-3'UTR-Wt or IRG1-3'UTR-Mut. The relative luciferase activities were measured by Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) 48 h after incubation.

Tumor Xenograft Model

Mice were randomly divided into two groups and the mouse xenograft model was developed by subcutaneously injecting the stable expressing cells which was transfected either with lentiviral miR-378 (lenti-miR-378) or the negative lentiviral miR-control (lenti-control) into the right oxter of each mouse. Once tumors became visible, the sizes were measured with an external Vernier caliper. Tumor volumes were calculated as: volume

= 1/2 × length × width^2. Mice were euthanized 30 days after injection, and the tumors were dissected and trimmed.

Statistical Analysis

All experiments were repeated at least 3 times. Statistical Product and Service Solutions (SPSS) software version 17.0 (SPSS Inc., Chicago, IL, USA) was applied to perform the statistical analysis. Student's *t*-test, ANOVA and Scheffe's post-hoc test were applied, where appropriate. Kaplan-Meier method and log-rank test were applied to estimate the survival rates and compare the survival curves respectively. $p < 0.05$ was regarded as statistically significant difference.

Results

MiR-378 Was Downregulated in Glioma Tissue Samples

To examine the functional effects of miR-378 in glioma, firstly, we evaluated the miR-378 expressions in 50 glioma tissues as well as 11 normal brain tissue samples using qRT-PCR. The results indicated that, compared to the normal brain tissues, miR-378 expressions in glioma tissues were markedly decreased (Figure 1A). In addition, we further investigated the clinical significance of miR-378 in glioma. Firstly, the glioma patients were divided into miR-378 high expressing group and low expressing group with the mean miR-378 expression level being regarded as the cutoff. Clinical association analysis demonstrated that the low miR-378 expressions were notably related to advanced WHO grade ($p = 0.0095$) and low KPS ($p = 0.0107$) (Table II). Furthermore, Kaplan-Meier analysis demonstrated that the low miR-378 expressions were prominently associated with shorter OS in glioma patients (Figure 1B).

MiR-378 Repressed Glioma Cell Invasion and Migration Abilities

Subsequently, we also analyzed miR-378 expressions in a series of glioma cell lines and NHAs. The data demonstrated significantly lower miR-378 expressions in glioma cells than that in NHAs (Figure 2A). To examine the functional roles of miR-378 in glioma, we performed miR-378 inhibition or overexpression assays in SHG44 or LN229 cells via transfecting the inhibitor or mimics of miR-378 into SHG44 or LN229. qRT-PCR was performed to examine the efficiencies and results presented successful overexpression or inhibition of miR-378 in LN229 or SHG44 (Figure 2B and 2C). Subsequently, transwell assays were carried out to investigate the functions of miR-378 in glioma cell invasion and migration. As expected, miR-378 overexpression significantly suppressed LN229 cell invasion and migration (Figure 2D). On the other hand, miR-378 inhibition remarkably facilitated SHG44 cell invasion and migration (Figure 2E).

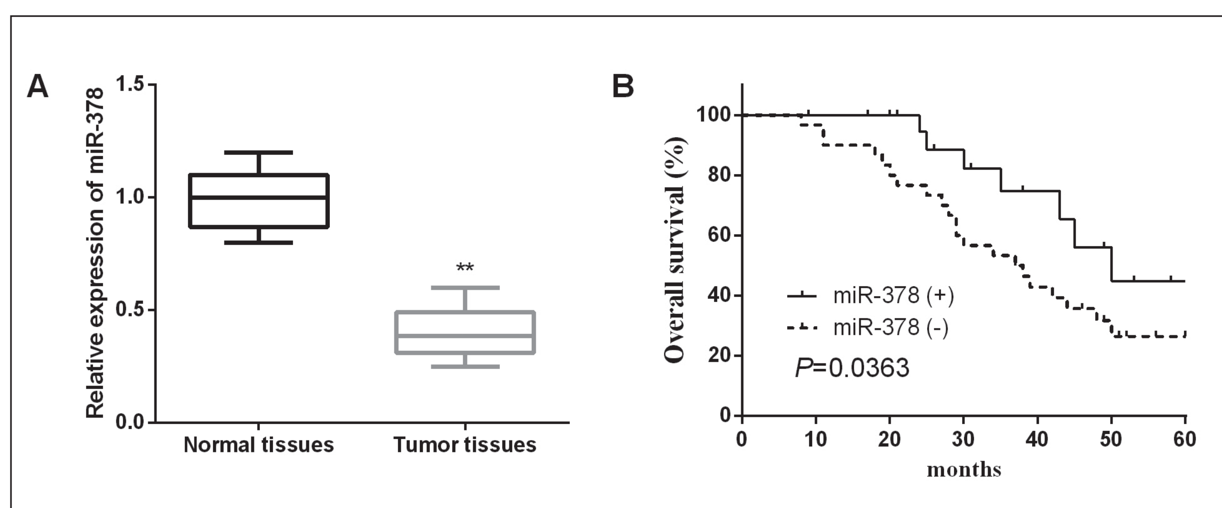

Figure 1. MiR-378 was downregulated in glioma. ***A,*** MiR-378 expressions in glioma tissues and normal brain tissue samples were detected using qRT-PCR. ***B,*** Kaplan-Meier analysis demonstrated a shorter overall survival (OS) of glioma patients with low miR-378 expressions. $^{**}p < 0.01$.

Table II. Correlation of miR-378 expression with the clinicopathological characteristics of the glioma patients.

Clinicopathological features	Cases (n = 52)	miR-378# expression		*p*-value
		High (n = 22)	Low (n = 30)	
Age (years)				0.1936
> 60	29	13	16	
≤ 60	23	9	14	
Gender				0.2124
Male	27	10	17	
Female	25	12	13	
Tumor size (cm)				0.0574
≥ 5.0	25	7	18	
< 5.0	27	15	12	
WHO grade				0.0095*
I-II	23	18	5	
III-IV	29	4	25	
KPS				0.0107*
< 80	29	3	26	
≥ 80	23	19	4	

WHO: World Health Organization; KPS: Karnofsky performance scale. #The mean expression level of miR-378 was used as the cutoff; *Statistically significant.

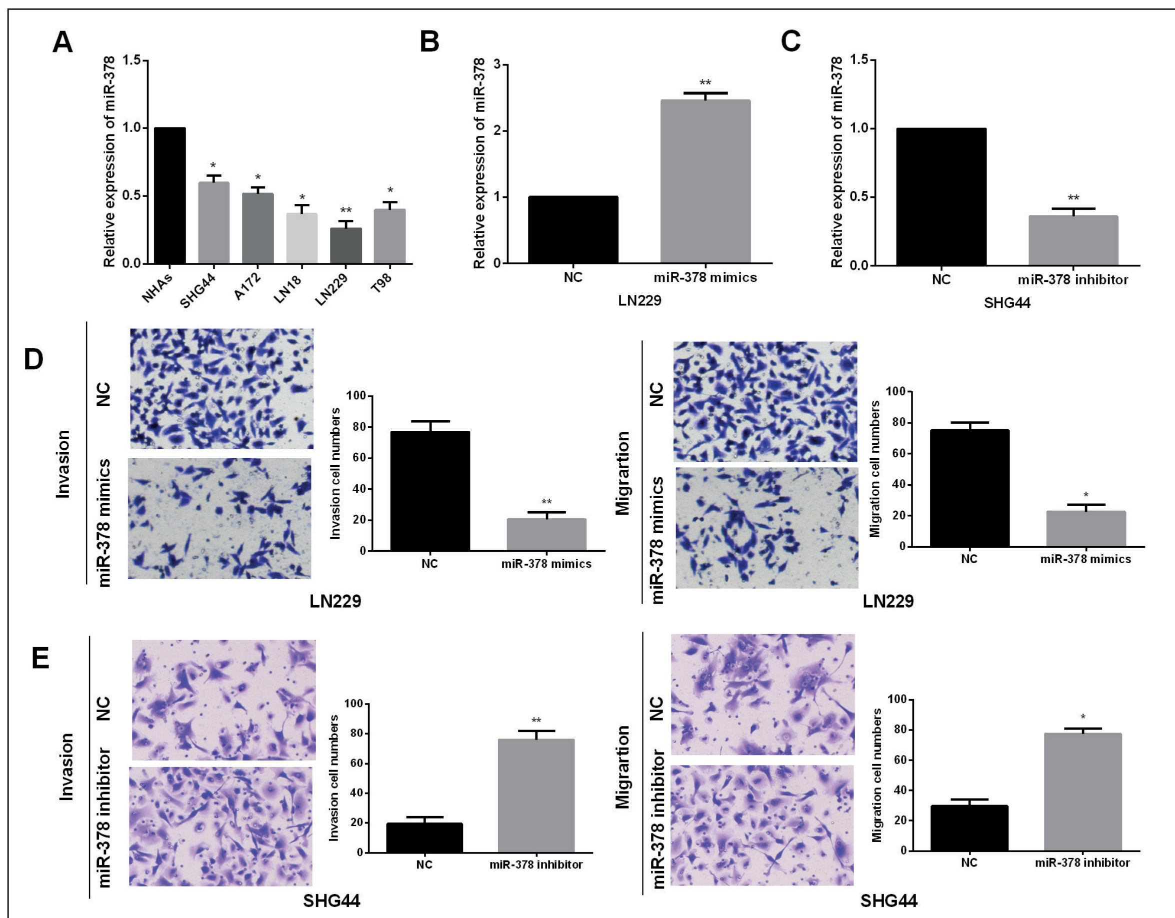

Figure 2. MiR-378 overexpression inhibited glioma cell invasion and migration. ***A,*** qRT-PCR analysis of miR-378 expression levels in glioma cell lines compared with the NHAs. ***B,*** MiR-378 expressions in LN229 with transfections of miR-378 mimics. ***C,*** MiR-378 expressions in SHG44 with transfection of miR-378 inhibitor. ***D,*** Cell invasion and migration were observed by transwell assays in LN229 treated with miR-378 mimics and ***(E)*** SHG44 treated with miR-378 inhibitor. $**p < 0.01$, $*p < 0.05$.

IRG1 Was a Direct Target of MiR-378 in Glioma Cells

Candidate target genes of miR-378 were explored to elucidate the mechanism about the suppression effects on glioma cell invasion and migration mediated by miR-378. Targetscan was utilized to search for potential target genes of miR-378. Data suggested that IRG1 might be a direct target of miR378 in glioma (Figure 3A). To confirm the relationship between IRG1 and miR-378, dual-luciferase reporter assays were carried out and findings showed that luciferase activity was markedly declined by co-transfection with IRG1-3'UTR-Wt and miR-378 mimics in glioma cells, whereas co-transfection with IRG1-3'UTR-Mut and miR-378 mimics had no prominent effect on the luciferase activity of glioma cells (Figure 3B). Then, the regulatory function of miR-378 in endogenous expressions of IRG1 was further determined. Results revealed that the IRG1 expressions in miR-378 mimics-transfected LN229 cells were significantly reduced in comparison with the negative controls (Figure 3C). In the meantime, miR-378 inhibition significantly decreased the IRG1 expressions in SHG44 cells (Figure 3D). These data confirmed that IRG1 was a direct target of miR-378 in glioma cells.

MiR-378 Suppressed Glioma EMT by Targeting IRG1

The biological functions of IRG1 in glioma progression were next investigated. Firstly, the IRG1 expressions in glioma tissues and normal brain tissues were measured by qRT-PCR. The data demonstrated that, compared to the normal tissues, the expression levels of IRG1 in glioma tissues were remarkably increased (Figure 4A). In the meantime, the

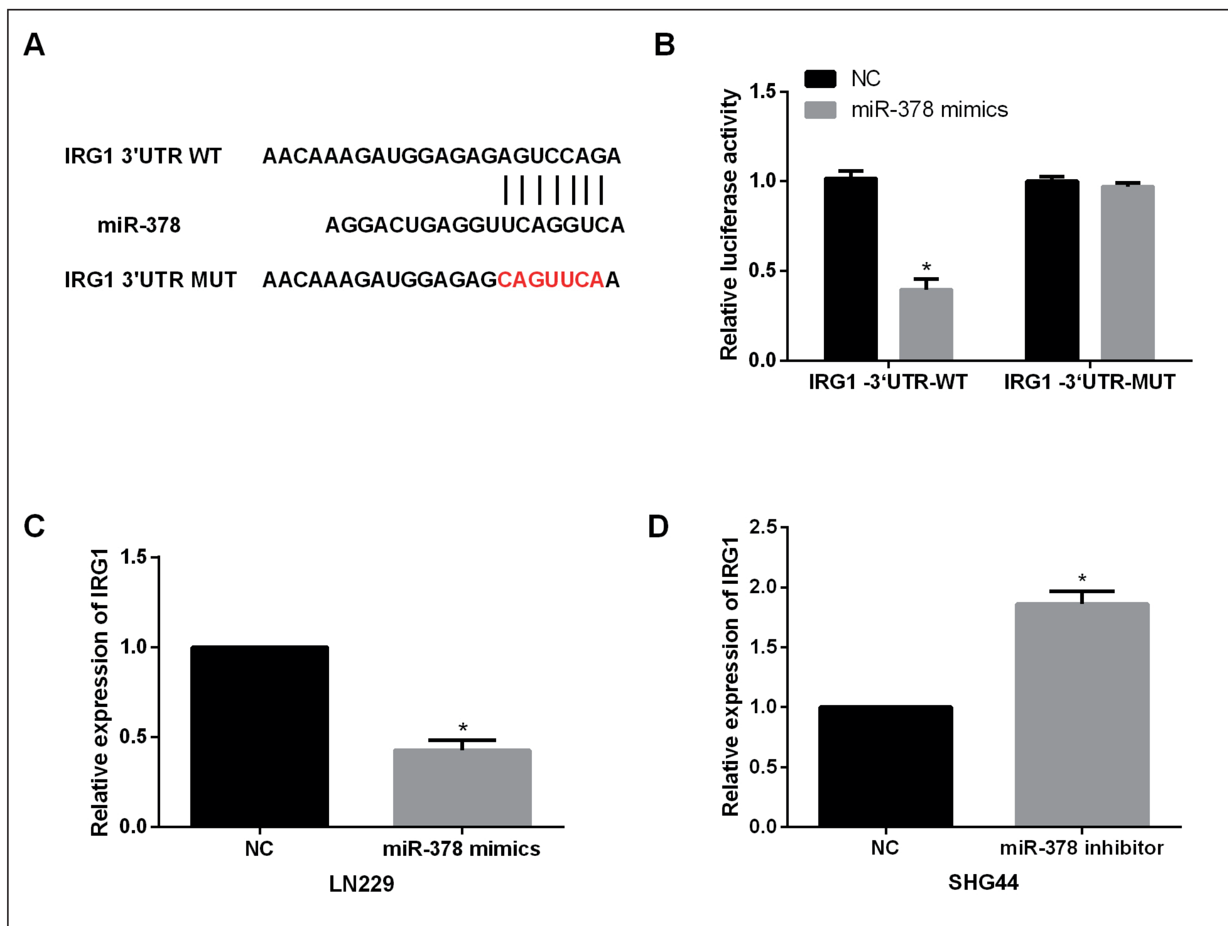

Figure 3. IRG1 was a direct target of miR-378 in glioma. ***A,*** The putative binding sites of miR-378 in the IRG1 3'-UTR. ***B,*** Luciferase activities in glioma cells cotransfected with luciferase reporters containing IRG1-WT or IRG1-MUT and miR-378 mimics. ***C, D,*** IRG1 expressions in LN229 or SHG44 treated with miR-378 mimics or inhibitor respectively. $^*p < 0.05$.

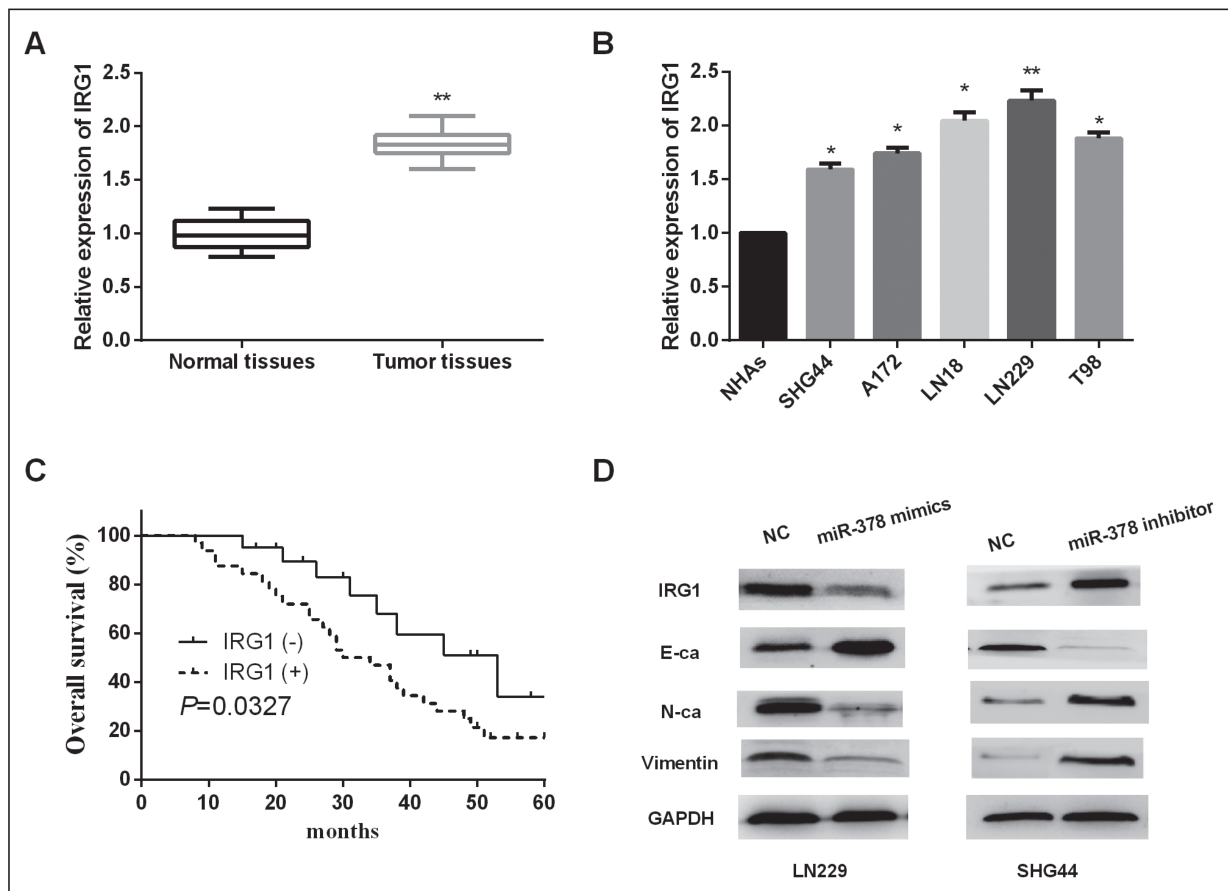

Figure 4. MiR-378 repressed glioma cell EMT by targeting IRG1. ***A,*** IRG1 expressions in glioma tissues and normal brain tissue samples were detected using qRT-PCR. ***B,*** qRT-PCR analysis of IRG1 expression levels in glioma cell lines compared with the NHAs. ***C,*** OS of IRG1highly-expressing and lowly-expressing glioma patients. ***D,*** The protein expression levels of EMT-related markers were measured in LN229 or SHG44 treated with miR-378 mimics or inhibitor respectively. **$p < 0.01$, *$p < 0.05$.

IRG1 expressions in glioma cells were also evaluated and, as expected, there was an increase of IRG1 expressions in glioma cells compared to the NHAs (Figure 4B). Additionally, the glioma patients were divided into two groups according to the Kaplan-Meier analysis results (Figure 4C). EMT is an important mechanism underlying the metastasis of tumor cells. Therefore, we investigated the functions of miR-378 in suppressing the glioma cell metastasis *via* regulation of EMT phenotypes. Western blot analysis indicated that miR-378 overexpression in LN229 cells significantly enhanced the E-cadherin expressions whereas reduced the N-cadherin and vimentin expressions. Additionally, the expressions of IRG1 were markedly declined by miR-378 overexpression. However, the E-cadherin expressions were significantly decreased while the N-cadherin and vimentin expressions were remarkably potentiated by inhibition of miR-378 in SHG44 cells, where the IRG1 expressions were also prominently increased (Figure 4D).

MiR-378 Suppressed The Tumor Growth of Glioma In Vivo

Furthermore, glioma cells were stably transfected with lentiviral miR-378 (lenti-miR-378) or the negative lentiviral miR-control (lenti-control), and then injected into mice to establish glioma xenograft models. The results showed that lenti-miR-378 overexpressing had a significantly decreased tumor growth rate and tumor volume in comparison to the control group (Figure 5A and 5B).

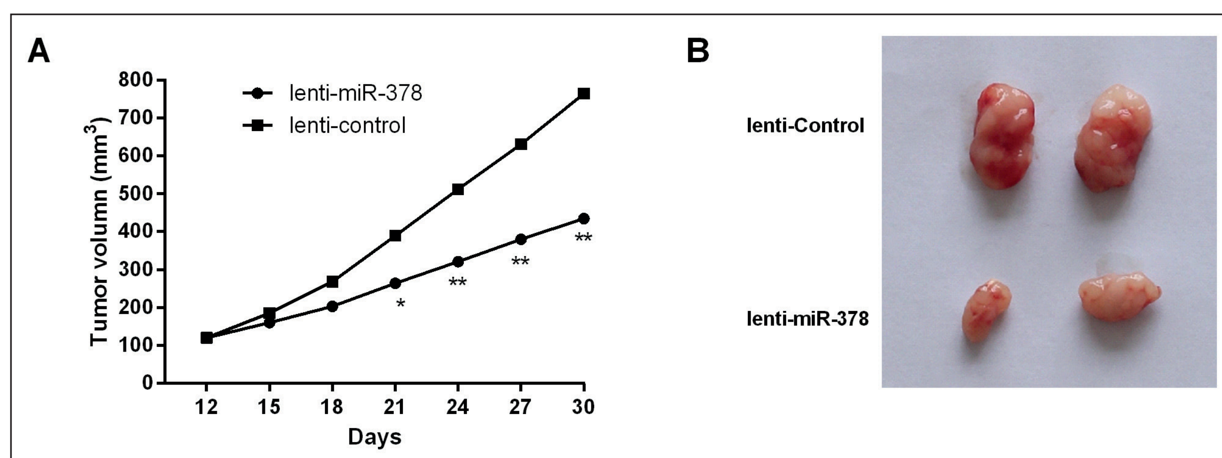

Figure 5. ***A,*** The tumor growth curves of mice which were subcutaneously injected with lenti-miR-378or lenti-control. ***B,*** The tumor volumes were detected in lenti-miR-378 group and lenti-control group every 3 days from day 12 to 30. $**p < 0.01$, $*p < 0.05$.

Discussion

The high morbidities, recurrence rates, and mortalities of glioma patients, which were mainly caused by the angiogenic behaviors, aggressive nature, and heterogeneities of glioma, might result in short survival time and poor prognosis of glioma patients[25,26]. Numerous studies have attempted to explain the functional effects of miRNAs on glioma formation and progression, serving tumorigenesis or anti-cancer functions through negatively regulating their targets. For instance, Li et al[27] claimed that miR-130b overexpression promoted glioma cell proliferation via regulation of ERK/MAPK pathway. Zhou et al[28] reported that miR-141-3p promoted temozolomide resistance and growth of glioma cells *via* regulation of p53. Ji et al[29] pointed out that miR-375 inhibited the migration and proliferation *via* the downregulation of RWDD3 in glioma. The present study was designed to identify miRNAs implicated in the regulation of glioma progression.

There is a large volume of published investigations describing that miR-378 is a key regulator of different human tumors. For example, Ma et al[30] pointed out that miR-378 promoted liver carcinoma migration *via* downregulating Fus. According to Zeng et al[31], miR-378 suppressed colon cancer progression *via* repressing SDAD1. Chen et al[32] demonstrated that miR-378 suppressed cell growth *via* inhibiting MAPK1 prostate cancer. A previous research[33] revealed that miR-378 played a suppressive role in glioma, but the underlying mechanisms are still unclear. In the present work, we firstly found that miR-378 was downregulated in glioma tissues and cells. In addition, the downregulated miR-378 was related to poor OS and worse clinicopathological parameters of glioma patients. Moreover, miR-378 overexpression could suppress glioma cell EMT and metastasis as well as the tumor growth rate and tumor size of glioma mice. The direct target for miR-378 should be determined for a better understanding of its effects on glioma tumorigenesis as well as the progression. Our current study indicated that IRG1 was a candidate target of miR-378. Moreover, the ectopic upregulation of IRG1 in glioma was presented, indicating a poor prognosis of glioma patients. Notably, the suppressive functions of miR-378 in glioma cell metastasis and EMT were confirmed to be regulated by IRG1. These findings about IRG1 in glioma suggested that IRG1 functioned as an oncogene in glioma, being consistent with the study of Pan et al[34], who found that IRG1 could increase glioma growth and tumorigenesis.

Conclusions

We investigated the suppressive role of miR-378 in the metastasis of glioma cells, and demonstrated that miR-378 was a novel anti-tumor miRNA in glioma. MiR-378 has suppres-

sive effects on glioma cell metastasis and EMT through the regulation of IRG1. This newly identified miR-378/IRG1 axis might provide novel insights into the mechanism underlying glioma progression, representing promising therapies for glioma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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