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Effects of miR-18a on proliferation and apoptosis of gastric cancer cells by regulating RUNX1

B. QI[1], Y. DONG[2], X.-L. QIAO[2]

[1]Department of Abdominal Emergency Surgery, The First Affiliated Hospital of Dalian Medical University, Dalian, China
[2]Department of Gastroenterology, The Second Affiliated Hospital of Dalian Medical University, Dalian, China

Abstract. – OBJECTIVE: The aim of this study was to explore the effects of micro ribonucleic acid (miR)-18a on the proliferation and apoptosis of gastric cancer (GC) cells, and to elucidate the possible underlying mechanism.

PATIENTS AND METHODS: In this study, the expression of miR-18a in GC tissues and para-cancer tissues was verified by in situ hybridization (ISH) of GC tissue microarray (TMA). Meanwhile, the effect of miR-18a expression on the prognosis of GC patients was evaluated. GC AGS cell line was selected and transfected with miR-18a mimic and mimic control (NC) to up-regulate miR-18a expression *in vitro*. Thereafter, changes in cell proliferation, apoptosis and migration after transfection were detected by biological functional assays. Luciferase reporter gene assay was carried out to verify the target gene Runt-related transcription factor 1 (RUNX1) modulated by miR-18a. Finally, the Spearman's grade correlation coefficient was calculated to explore the correlation between the expressions of miR-18a and RUNX1.

RESULTS: ISH results of TMA showed that overexpression of miR-18a in GC tissues was significantly associated with low survival rate of patients ($p<0.001$). High expression of miR-18a remarkably enhanced the proliferation, migration and invasion of GC cells ($p<0.05$). Besides, it has been predicted in biology that RUNX1 is one of the target genes of miR-18a. Luciferase reporter gene assay showed that Luciferase activity in cells transfected with wild-type (WT) RUNX1 3' untranslated region (3'UTR) was significantly reduced ($p<0.05$). Moreover, the protein expression of RUNX1 decreased remarkably in GC cells with over-expression of miR-18a ($p<0.05$). All these findings indicated that the expression of miR-18a was negatively correlated with RUNX1 in GC cells ($p<0.001$, r=0.86).

CONCLUSIONS: MiR-18a exerts a high predictive value for the prognosis of GC patients by directly targeting the transcription factor RUNX1. All our findings may provide therapeutic candidates for GC identification.

Key Words:
MiR-18a, Transcription factor RUNX1, Proliferation, Apoptosis.

Introduction

Gastric cancer (GC) is the fourth most common cancer in the world. Despite its decreasing morbidity and mortality rates in recent years, it remains the second leading cause of tumor-related death worldwide[1,2]. Currently, the ratio of morbidity rate to mortality rate of GC is close to 1 in more than 70% of countries, with approximately three quarters of new cases in Asia[1]. Molecular biomarkers for predicting GC outcomes will enable appropriate treatment regimens to be applied at the early stage of GC. Therefore, there is an urgent need to search for novel diagnostic and prognostic indicators for GC treatment.

Micro ribonucleic acids (miRNAs), a kind of regulatory factors, exert a vital role in the pathogenesis of GC development. In terms of base composition, they are homologous to the 3' untranslated region (3'UTR) of target messenger RNAs (mRNAs) and can suppress gene expression at the transcription level. MiR-18a encoded by miR-17-92 cluster is remarkably up-regulated in GC tissues when compared with normal gastric tissues. It is also highly expressed in several malignancies, including nasopharyngeal carcinoma, glioblastoma and other digestive tract cancers[3,4]. Hence, miR-18a is speculated to affect the occurrence and development of tumors. However, the potential biological functions of miR-18a in the occurrence and development of have not been fully elucidated.

Mammal Runt-related transcription factor 1 (RUNX1) belongs to the runt domain family of tran-

Corresponding Author: Xiuli Qiao, MM; e-mail: dongying0721@dmu.edu.cn

scription factors. In this family, RUNX1, RUNX2 and RUNX3 are lineage-specific regulators of gene expression in main developmental pathways[5]. These three RUNX proteins recognize the same deoxyribonucleic acid (DNA) motif, but there is little overlap in function. Meanwhile, their biological functions differ. No functional redundancy is caused by the strict regulation on the spatio-temporal expression of RUNX through transcriptional and post-transcriptional control mechanisms[6-10]. As a core regulator of hematopoiesis, the expression of Runx1 is specifically regulated by miRNA lineage[11]. Fontana et al[12] have reported that during monocyte production, RUNX1 expression is attenuated by miR-7-5p-20a-106a cluster. In this study, the potential miRNA binding sites in the longest 3'UTR (3.8 kb) of RUNX1 was identified. Moreover, RUNX1 was manifested to bind to miR-27a, miR-9, miR-18a, miR-30c and miR-199a*, thus weakening RUNX1 expression.

In this study, the expression of miR-18a in GC tissues and para-cancer tissues was first determined. The predictive value of miR-18a for the prognosis of GC patients was explored through the *in situ* hybridization (ISH) of tissue microarray (TMA). Moreover, the biological function of miR-18a and its potential signaling pathway were investigated. All our findings might help to provide therapeutic candidates for GC identification.

Patients and Methods

Experimental Materials

This investigation was approved by the Ethics Committee of The First Affiliated Hospital of Dalian Medical University. Signed written informed consents were obtained from all participants before the study. The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). Paired tissues were collected from 90 GC patients who underwent radical resection in our hospital. Primary antibody (CST, Danvers, MA, USA), miRcute miRNA isolation kit and complementary DNA (cDNAs) synthesis kit (TIANGEN, Beijing, China), MTS reagent (Sigma-Aldrich, St. Louis, MO, USA), TMA (Shanghai Outdo Biotech Co., Ltd. (Shanghai, China) and pGL4 vector (Promega, Madison, WI, USA).

Research Objects

Samples in TMA assay were divided into GC tissues and para-cancer tissues. Cells used in this study included GC cell lines (AGS, MKN-28, MKN-45 and HGC-27) and gastric epithelial cell line (GES-1). AGS cells transfected with miR-18 mimic and mimic control (NC) were allocated into mimic group and NC group, respectively. 293T cells transfected with wild-type (WT) or mutant (MUT) RUNX1 3'UTR sequences were assigned into WT group and MUT group, respectively.

Immunohistochemistry

Paraffin-embedded tissue sections were first deparaffinized, and antigen retrieval was carried out by boiling in an autoclave with citrate. After cooling at room temperature for 40 min, each site was covered with primary antibody to ensure that all antibody sites were covered. Subsequently, paraffin-embedded tissue sections were incubated in a refrigerator overnight. On the next day, primary antibody was washed away with phosphate-buffered saline-tween (PBST). Next, the sections were incubated with secondary antibody for 30 min, and diaminobenzidine (DAB) staining (Solarbio, Beijing, China) was performed. Finally, the sections were counterstained by hematoxylin after 3 min of staining, followed by mounting.

MiRNA Extraction and Fluorescence Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total miRNAs in cell lines was extracted according to the instructions of the miRcute miRNA isolation kit. 140 ng of total miRNAs were subjected to polyadenylation and reverse transcription into cDNAs using the miRcute miRNA first-strand cDNA synthesis kit. Next, PCR was performed using the miRcute miRNA qPCR detection kit based on the reaction system. U6 was used as an internal reference. Relative miR-18a level was calculated by the $2^{-\Delta \text{cycle threshold (Ct)}}$ method, where ΔCt = Ct (has-miR-18a) - Ct (control). Primer sequences used in this study were as follows: miR-18a-F: 5'-GATAGCAGCACAGAAATATTGGC-3', miR-18a-R: 5'-TGGTGTCGTGGAGTCG-3'. U6-F: 5'-CTCGCTTCGGCAGCACA-3', U6-R: 5'-AACGCTTCACGAATTTGCGT-3'.

Cell Culture and Transfection

GC cell lines and gastric epithelial cell line were cultured with complete Roswell Park Memorial Institute-1640 (RPMI-1640). The sequences of transfection reagents miR-18a mimic and NC were listed as below: miR-18a mimic-F: 5'-UAAGGUGCAUCUAGUGCAGAUAG-3' and miR-18a mimic-R: 5'-CUAUCUGCACUA-

GAUGCACCUUA-3'. NC was celmiR-67-3p mimic, whose sequences are mimic-F: 5'-UCACAACCUCCUAGAAAGAGUAGA-3' and mimic-R: 5'-UCUACUCUUUCUAGGAGGUUGUGA-3'.

Cell transfection was performed at a concentration of 100 nM according to the manufacturer's specifications of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfection efficiency was verified by qRT-PCR.

Cell Proliferation

AGS cells with miR-18a overexpression were inoculated into 96-well plates, with 5 replicates for each well. After 24 h, 10 μL of Cell Counting Kit-8 (CCK-8) reagent (Dojindo Molecular Technologies, Kumamoto, Japan) was added to each well, followed by 2 h of incubation in the dark. Optical density (OD) values at 450 nm were detected by a micro-plate reader, and cell proliferation curve was finally recorded.

Construction of RUNX1 3'UTR Reporter Plasmids and Luciferase Reporter Gene Assay

The fragments of RUNX1 3'UTR were amplified from cDNAs using PCR amplification techniques and inserted into luciferase XbaI and FseI sites. Next, the successfully constructed plasmids were transferred into the cells. Briefly, 1.5×10^4 293T cells were inoculated into 96-well plates and culture for 24 h. Subsequently, RUNX1 3'UTR expression plasmids and miRNA mimic were co-transfected into 293T cells. Dual-Luciferase reporter assay system was then applied to detect Luciferase activity changes at 48 h after transfection. Primer sets utilized to generate specific fragments included 3'UTR-F: ATGACATCTAGAGCTGAGCGCCATCGCCATCG and 3'UTR-R: AGTTCAGGCCGGCCAAGGGTATAAAATCTTTCTTTTATTCACAGCATTG.

Western Blotting

Cell lysate was applied to lyse cell precipitates, and total protein in cells was extracted. 60 μg of proteins were separated by 12% gel and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) using the wet transfer method for 1 h. After sealing with skimmed milk for 10 min, the membranes were incubated with primary antibodies for 15 h in a refrigerator at 4°C. On the next day, the membranes were washed and incubated with corresponding secondary antibody for 1 h at room temperature. Immunoreactive bands were finally exposed by the chemiluminescent substrate kit.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. χ^2-test and t-test were adopted for comparisons between less than three groups. Linear regression analysis was carried out to explore the relationship between the expressions level of miR-18a and proteins. $p<0.05$ was considered statistically significant.

Results

Guiding Significance of MiR-18a Expression Level in GC Tissues for the Prognosis of GC

Using ISH techniques, the expression of miR-18a in 90 pairs of GC tissues and para-cancer tissues was analyzed. It was found that miR-18a in GC tissues was stained in a moderate to high degree, with an average score of (7.56±0.45) points. Meanwhile, miR-18a was mainly located in the nucleus and cytoplasm. However, the weak to moderate staining of miR-18a was observed in para-cancer tissues, with an average score of (4.98±0.56) points. Statistical analysis revealed that the expression of miR-18a in GC tissues was notably higher than that in para-cancer tissues ($p<0.01$). The survival rate of patients with high expression of miR-18a is significantly lower, and there was a correlation between its expression level and survival time ($p<0.05$, Figure 1).

MiR-18a Promoted Cell Proliferation and Migration

The expression of miR-18a in GC cells was detected by qPCR. The results manifested that compared with GES-1 cells, the expression of miR-18a increased significantly in GC cell lines ($p<0.05$). This indicates that miR-18a expression may be related to the malignant phenotype of GC. Subsequently, AGS cells were selected to investigate the effect of miR-18a on the proliferation of GC cell lines. It was discovered that overexpression of miR-18a prominently facilitated the proliferation of AGS ($p<0.01$). Wound healing assay results demonstrated that the forced expression of miR-18a in AGS significantly enhanced cell migration when compared with cells in NC group ($p<0.01$) (Figure 2).

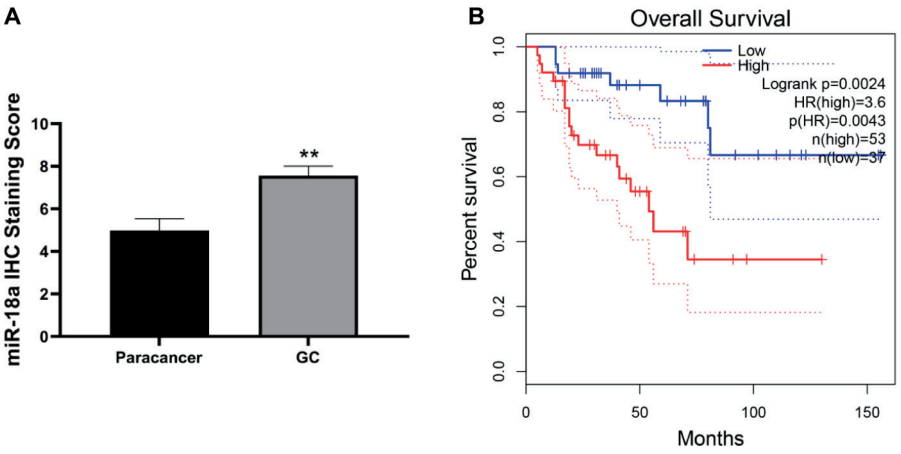

Figure 1. ISH analysis of miR-18a expression in GC tissues. **A,** MiR-18a expression in GC tissues. MiR-18a is highly expressed in GC tissues ($p<0.01$) (magnification: 200×). **B,** Assessment of the significance of miR-18a for prognosis of GC patients *via* Kaplan-Meier survival analysis. The survival time of patients with high expression of miR-18a is significantly shortened ($p<0.01$) ($^{**}p<0.01$).

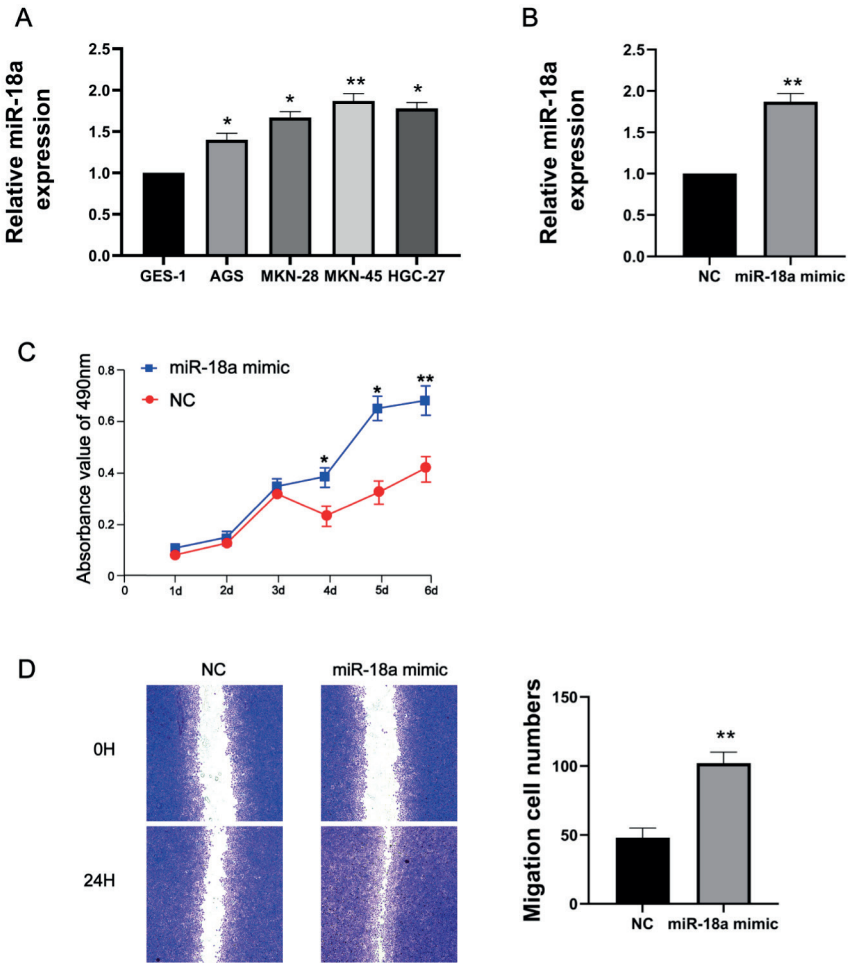

Figure 2. Effects of miR-18a on cell proliferation and migration. **A,** Expression of miR-18a in GC cell lines. In comparison with GES-1, miR-18a is highly expressed in four GC cell lines ($p<0.05$). **B,** MiR-18a expression in AGS cells transfected with miR-18a mimic and NC. The expression of miR-18a in miR-18a group is notably higher than that in NC group ($p<0.01$). **C,** Effect of miR-18a expression on cell proliferation. OD value in miR-18a mimic group is significantly higher than that in NC group ($p<0.01$). **D,** Effect of miR-18a expression on cell migration. In comparison with NC group, the proportion of migrating cells in miR-18a mimic group increases remarkably ($p<0.01$) ($^{*}p<0.05$, $^{**}p<0.01$) (magnification: 200×).

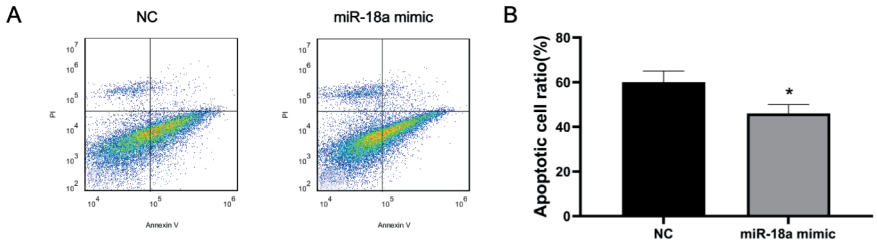

Figure 3. Effect of miR-18a on cell apoptosis. **A-B,** In contrast to that in NC group, the proportion of apoptotic cells in miR-18a mimic group decreases significantly ($p<0.05$) ($^{*}p<0.05$).

MiR-18a Inhibited Cell Apoptosis

Flow cytometry was adopted to examine the apoptosis changes of cells after transfection with miR-18a mimic and NC. The results showed that the proportion of apoptotic cells in miR-18a mimic group was remarkably reduced after 48 h of cell culture. Therefore, the proportion of apoptotic cells markedly declined after miR-18a up-regulation in GC cells (Figure 3).

MiR-18a Directly Down-regulated the Expression of RUNX1 Protein in GC Cells

The target of miR-18a was searched using TargetScan biological software, and RUNX1 was found to be its potential target through GO analysis. Subsequently, WT or MUT RUNX1 3'UTR sequences were amplified and cloned into Luciferase reporter vectors. Luciferase activity in WT group was overtly reduced probably because the binding of miR-18a to RUNX1 3'UTR suppressed Luciferase expression ($p<0.05$). However, MUT RUNX1 3'UTR in 293T cells was not affected. Immunohistochemistry results also revealed that RUNX1 protein was lowly expressed in GC cells with high expression of miR-18a ($p<0.05$) (Figure 4). The above experimental results suggest that miR-18a directly regulates the expression level of RUNX1 protein.

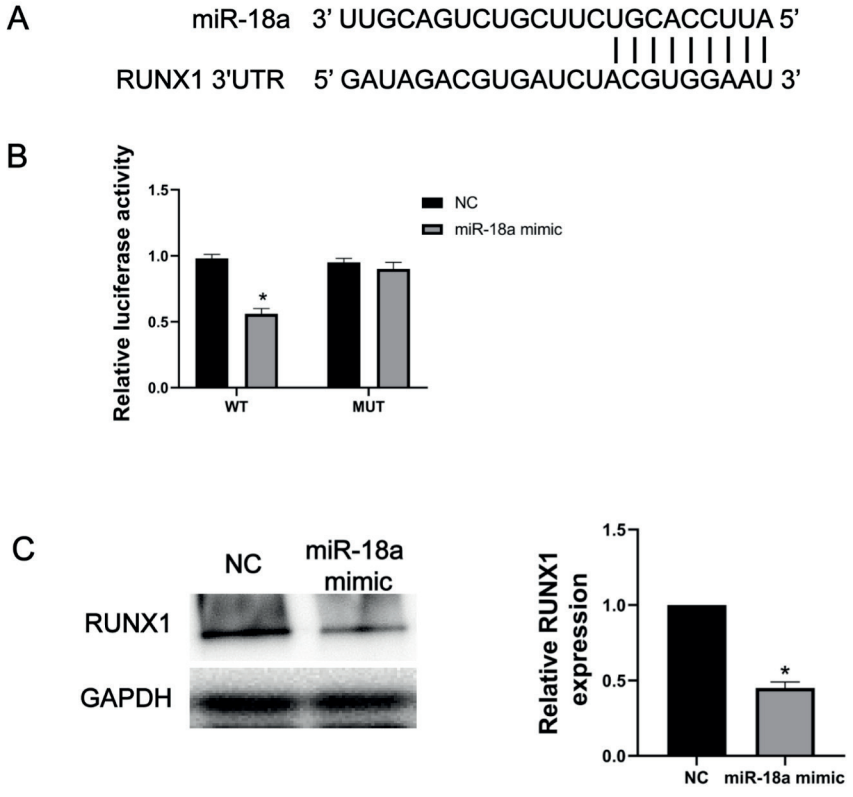

Figure 4. RUNX1 is the direct target of miR-18a. **A,** Binding site of miR-18a and RUNX1 3'UTR. **B,** Luciferase activity changes in 293T after transfection with Luciferase vectors. In comparison with cells transfected with MUT RUNX1 3'UTR, those transfected with WT RUNX1 3'UTR have overtly reduced Luciferase activity ($p<0.05$). **C,** RUNX1 protein expression detected by Western blotting. RUNX1 protein expression is prominently reduced in miR-18a mimic group when compared with mimic control group ($p<0.05$) ($^{*}p<0.05$).

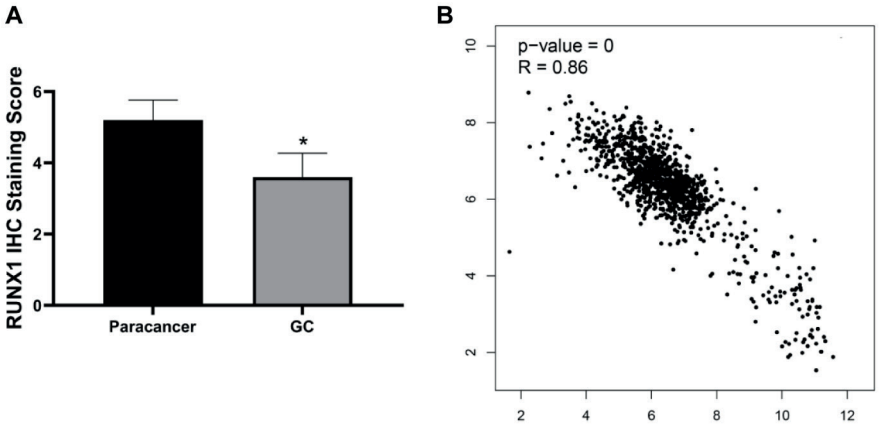

Figure 5. RUNX1 expression is remarkably negatively correlated with miR-18a expression. **A,** Staining results of RUNX1 protein in tissues. RUNX1 exhibits an evidently low expression in GC tissues ($p<0.05$) (magnification: 200×). **B,** RUNX1 expression is negatively associated with miR-18a expression ($r=0.86$, $p<0.001$) (*$p<0.05$, ***$p<0.001$).

RUNX1 Protein Was Negatively Related to MiR-18a Expression in GC Tissues

To further evaluate the relationship between miR-18a and RUNX1 in GC, immunohistochemical method was adopted to detect RUNX1 expression in TMA. According to the results, the staining intensity of RUNX1 protein in GC TMA was markedly lower than that in para-cancer tissues ($p<0.05$). Besides, RUNX1 protein was lowly expressed ($p<0.05$), while miR-18a was highly expressed in GC tissues ($p<0.01$). Furthermore, there was a negative correlation between the expressions of miR-18a and RUNX1 ($r= 0.86$) ($p<0.001$) (Figure 5).

Discussion

MiRNAs are a series of small non-coding RNAs that naturally exist, which exert a regulatory role through decreasing the expression of corresponding target genes[13-15]. MiRNAs participate in many biological processes, including the regulation of metabolism, immunity, cell growth and differentiation. Aberrantly expressed miRNAs have been found involved in the occurrence and development of multifarious malignant tumors, including GC[16,17]. This implies its potential importance for biomarkers and therapeutic targets specific to diseases. Recently, miR-18a has been discovered to play a vital role in suppressing ataxia-telangiectasia by modulating PIAS3 expression and STAT3 activity and targeting neogenein[3,18]. In addition, miR-18a is highly expressed in various malignant tumors, including nasopharyngeal carcinoma[19], colorectal cancer[3] and glioblastoma[4]. Zheng et al[20] have proved that miR-18a may hinder angiogenesis in GC. The above findings indicate that miR-18a has complex functions in facilitating tumor formation. In this study, the expression of miR-18a in GC tissues and para-cancer tissues was verified. Meanwhile, miR-18a was found to have a high predictive value for the prognosis of GC patients. *In vitro* functional experiments manifested that up-regulation of miR-18a facilitated the malignant biological phenotype of tumor cells, such as proliferation and migration.

In recent years, there are studies on the mediation of the post-transcriptional regulation of RUNX1 by miR-9-144, miR-21527, miR-13945 and other miRNAs. However, whether miR-18a can regulate RUNX1 protein has not been reported so far. In this study, it was discovered that Luciferase activity decreased significantly in cells transfected with WT RUNX1 3'UTR. It was also found that miR-18a could target the regulation of RUNX1. RUNX1, also known as acute myeloid leukemia 1 protein, is a transcription factor composed of 453 amino acids. It cooperates with another protein called core binding factor β (CBFβ) to form a CBF complex, thereby binding to the core part of enhancers and promoters. Besides, RUNX1 is able to modulate the differentiation of hematopoietic stem cells into functional blood cells[21]. In fact, RUNX1 often mutates in sporadic myeloid and lymphoid leukemia through point mutation[22], translocation[23] or amplification[24]. These findings all indicate that RUNX1 functions in the carcinogenesis of blood cancer. However,

RUNX1 is considered as a tumor suppressor gene in other solid cancer types, like GC[25-27]. It exhibits a low expression in corresponding tumor tissues. In this work, it was found that the expression of RUNX1 in GC tissues was significantly lower than that in para-cancer tissues ($p<0.01$), indicating that RUNX1 might be a tumor suppressor gene in GC. Meanwhile, the correlation between miR-18a and RUNX1 expression levels in the same TMA was statistically analyzed. The data revealed that miR-18a displayed a remarkable negative correlation with RUNX1, which also supported the speculation that miR-18a was capable of directly regulating RUNX1.

This research provides *in vitro* evidence that miR-18a targets RUNX1 to participate in the proliferation and apoptosis of GC cells. Nevertheless, the potential role of miR-18a in GC tumorigenesis *in vivo* has not fully been elucidated. Therefore, more *in vivo* experiments are still required for verification, which will deepen the key theory that miR-18a participates in GC occurrence. In addition, it is also crucial to identify other targets of miR-18a or other miRNAs targeting RUNX1 in GC.

Conclusions

The novelty of this study was that miR-18a modulates the proliferation and apoptosis of GC cells by targeting RUNX1. Furthermore, miR-18a/RUNX1 can be applied as a molecular target for innovative drugs in the treatment of GC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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