MicroRNA-29a inhibits cell migration and invasion via targeting Roundabout homolog 1 in gastric cancer cells

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Abstract. Deregulation of Roundabout homolog 1 (Robol) has been demonstrated to be associated with several types of human cancer, including gastric cancer. However, the detailed role of Robo1 and its regulatory mechanism in gastric cancer remain largely unclear. In the current study, it was demonstrated that the expression of microRNA (miR)-29a was frequently reduced in gastric cancer tissues, compared with their matched normal adjacent tissues. Similar results were additionally observed in AGS and SGC-7901 human gastric cancer cells. Overexpression of miR-29a led to reduced migration and invasion of AGS cells. To explore the targets of miR-29a in gastric cancer, bioinformatics analysis was conducted and Robol was identified as a putative target of miR-29a. Further western blotting and luciferase activity assay data confirmed that miR-29a was able to negatively regulate the protein expression of Robo1, through directly binding to the 3'-untranslated region of Robo1 mRNA in gastric cancer cells. In addition, it was demonstrated that Robol was frequently upregulated in gastric cancer tissues compared with their matched adjacent normal tissues, and a significant inverse correlation was identified between miR-29a and Robo1 expression. In addition, knockdown of Robo1 by small interfering RNA markedly inhibited the migratory and invasive capabilities of AGS cells, which the results obtained with overexpression of miR-29a. In conclusion, to the best of our knowledge the current study suggested for the first time, that miR-29a inhibits migration and invasion in part via direct inhibition of Robo1 in gastric cancer cells. Therefore, Robo1 and miR-29a may serve as diagnostic or therapeutic targets for gastric cancer.

Introduction

Gastric cancer is one of the most prevalent types of human cancer, and is the second most common cause of cancer-associated mortality (1). Previously, surgical resection combined with chemotherapy has helped save the lives of patients with gastric cancer at an early stage (2,3). However, the prognosis of patients with gastric cancer at a late stage remains poor, predominantly due to metastasis and recurrence (2,3). Therefore, studies into effective molecular targets for gastric cancer metastasis are necessary and important.

Roundabout homolog 1 (Robol), a member of the Robo family, has been observed to be expressed in various types of cells. It has been reported that Robol acts as a critical regulator in multiple biological processes, including proliferation, differentiation and migration (4,5). Robo1 has mononucleotide repeats in the coding exons, which may be mutation targets in cases of cancer with microsatellite instability (6). Je et al (7) reported five frameshift mutations in the repeats, and co-occurrences of mutation and loss of expression of the Robol gene were also observed in gastric cancer tissues, suggesting that Robo1 may serve an important role in gastric cancer. In addition, the protein expression levels of Robol were observed to be negatively regulated by microRNA (miR)-218, and miR-218 was able to inhibit invasion and metastasis of gastric cancer by inhibiting Robo1, indicating that Robol is associated with the invasion and metastasis of gastric cancer (7). It has been well established that one gene can be targeted by numerous miRs and that one miR has various target genes (8). Accordingly, additional miRs targeting Robol may also exist in gastric cancer, and serve crucial roles in the regulation of migration and invasion in gastric cancer cells.

Previously, deregulation of miR-29a has been demonstrated to participate in the initiation and development of several types of human cancer, including neuroblastoma, glioma, breast cancer and gastric cancer (9-12). Chen *et al* (11) revealed that miR-29a had an inhibitory effect on growth and invasion of gastric cancer cells via targeting vascular endothelial growth factor A (VEGF-A), suggesting that miR-29a acts as a tumor suppressor in gastric cancer. However, whether there is an association between miR-29a and Robo1 in gastric cancer remains to be fully elucidated.

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In the current study, the expression levels of Robo1 and miR-29a were investigated in gastric cancer tissues and cell lines. Subsequently, the roles of Robo1 and miR-29a in migration and invasion of the AGS and SGC-7901 gastric cancer cell lines were further investigated.

Materials and methods

Clinical tissue collection. Written informed consent was collected from all patients with gastric cancer and the present study was approved by the ethics committee of The First Affiliated Hospital of Anhui Medical University (Hefei, China). The patients were aged between 43 and 68 and included 10 males and 7 females. The samples were collected between June and October 2012 by tumor resection. A total of 17 gastric cancer tissues and their matched adjacent normal tissues were obtained from the Department of General Surgery, The First Affiliated Hospital of Anhui Medical University (Hefei, China).

Cell culture. Human AGS and SGC-7901 gastric cancer cells, in addition to the human normal GES-1 gastric mucosal epithelial cell line were obtained from the Cell Bank of Central South University (Changsha, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen Life Technologies), 100 IU/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA) and 100 μ g/ml streptomycin sulfate (Sigma-Aldrich) at 37°C with 5% CO₂.

Transient transfection. The Robol small interfering RNA (siRNA), Robol plasmid, miR-29a mimic, miR-29a inhibitor and scramble miRNA were purchased from Nlunbio (Changsha, China). Cells in the logarithmic growth phase were seeded into 6-well plates. Transfection of cells with these oligonucleotides was performed using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells with TRIzol reagent (Life Technologies) according to the manufacturer's instructions. The relative expression of miR-29a was determined by RT-qPCR using the mirVana™ qRT-PCR microRNA Detection kit (Life Technologies) following the manufacturer's instructions. Specific primer sets for miR-29a and U6 (internal reference) were obtained from Life Technologies. Expression of Robo1 mRNA was determined by RT-qPCR using the standard SYBR Green RT-PCR kit (Takara Bio, Inc., Otsu, Japan) following the manufacturer's instructions. The specific primer pairs used were as follows: Robo1, sense 5'-CTTACACCCGTAAAAGTGACGC-3' and antisense 5'-TGGTCTCTCTAAGACAGTCAGC-3'; GAPDH as an internal control, sense 5'-CTGGGCTACACTGAGCACC-3' and antisense 5'-AAGTGGTCGTTGAGGGCAATG-3'. The following cycling conditions were used: 95°C for 10 min and 40 cycles of denaturation at 95°C for 15 sec and an annealing/elongation step at 60°C for 60 sec. Reverse transcription was performed at 16°C for 30 min, followed by an incubation step at 42°C for 30 min and enzyme inactivation at 85°C for 5 min. An ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used. The relative expression of Robo1 mRNA or miR-29a was quantified using GraphPad Prism software, version 4.0 (GraphPad Software, Inc., La Jolla, CA, USA) and the $2^{-\Delta\Delta Ct}$ method.

Cell migration and invasion assays. Cell migration and invasive capabilities were determined by performing a Transwell assay (Chemicon International, Inc., Temecula, CA, USA). For the migration assay, cells were plated in the upper chamber with the non-coated membrane. For the invasion assay, the chamber was coated with Matrigel (Becton Dickinson, Franklin Lakes, NJ, USA) and dried overnight, then cells were plated in the upper chamber with the Matrigel-coated membrane. For the two assays, DMEM supplemented with 10% FBS was used as a chemoattractant in the lower chamber. Following 24 h of incubation at 37°C, cells on the lower face of the membrane were stained with 0.4% crystal violet for 20 min and observed under a microscope (CX22; Olympus, Tokyo, Japan).

Western blotting. Total protein was extracted using radioimmunoprecipitation assay solution (Sigma-Aldrich). The protein concentration was determined by the Bradford DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). For determination of the protein level, proteins were separated with 10% SDS-PAGE and were blotted onto polyvinylidene difluoride (PVDF; Invitrogen Life Technologies) membranes, which were then incubated in Tris-buffered saline with Tween-20 (Sigma-Aldrich) with 50 g/l skimmed milk at room temperature for 4 h. Subsequently, the PVDF membranes were incubated with mouse anti-Robo1 (cat. no. ab201632; 1:50 dilution; Abcam, Cambridge, MA, USA) and mouse anti-GAPDH antibodies (cat. no. sc-365062; 1:50 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), respectively, at room temperature for 1 h. Following washing with PBST three times, the PVDF membranes were incubated with peroxidase-conjugated rabbit anti-mouse secondary antibody (cat. no. ab175743; 1:20,000 dilution; Abcam) for 1 h at room temperature. Chemiluminescent detection was performed with an ECL kit (Pierce Chemical Company, Rockford, IL, USA).

Bioinformatical analysis. TargetScan (Release 6.2, www. targetscan.org) was used to predict the putative target genes of miR-29a.

Dual luciferase reporter assay. SGC-7901 cells were cotransfected using Lipofectamine 2000 with the reporter constructs Robo1-3' untranslated region (UTR)-psi-CHECK2 (containing the 3'-UTR of Robo1 including the miRNA-29a binding sites) or mutant-Robo1-3'UTR-psi-CHECK2 (containing the corresponding mutated sequence of 3'-UTR of Robo1), while miR-29a mimics or scramble miRNA were used as the negative control. Luciferase activity was determined after 48 h using the Dual-Glo Substrate system (Promega Corporation, Madison, WI, USA) and the LD400 Luminometer (Beckman Coulter, Inc., Brea, CA, USA). Data are presented as the ratio of *Renilla* luciferase to Firefly luciferase.



Figure 1. miR-29a is downregulated in gastric cancer. (A) The relative expression levels of miR-29a were determined by RT-qPCR in 18 paired human gastric cancer tissues (T) in addition to their corresponding normal adjacent tissues (N) and were normalized against an endogenous U6 RNA control. (B) The relative expression levels of miR-29a were determined by RT-qPCR in three human gastric cancer cell lines (SNU-1, AGS and SGC-7901) and a normal gastric mucosal epithelial cell line (GES-1). Two-tailed Student's t-test was used to analyze the significant differences. **P<0.01 vs. GES-1. miR-29a, microRNA-29a; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.



Figure 2. Overexpression of miR-29a inhibits migration and invasion of gastric cancer cell lines. (A) Transfection of miR-29a mimics to AGS cells upregulated the expression level of miR-29a determined by reverse transcription-quantitative polymerase chain reaction. NC, negative control, AGS cells transfected with scramble miRNA. **P<0.01 vs. AGS. (B) The inhibitory effect of miR-29a on the migration and invasion of AGS cells. Two-tailed Student's t-test was used to analyze the significant differences. **P<0.01 vs. AGS. miR-29a, microRNA-29a.

Statistical analysis. Data are expressed as the mean \pm standard deviation. Differences between two groups were determined using Student's t-test. Analysis was performed using SPSS software, version 15.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of miR-29a is downregulated in gastric cancer tissues and cell lines. The endogenous expression of miR-29a was determined in human gastric cancer tissues and their adjacent normal tissues using RT-qPCR. As demonstrated in Fig. 1A, the relative expression of miR-29a was frequently downregulated in 77.8% (14/18) of gastric cancer tissues compared with the corresponding adjacent normal tissues. Similarly, it was identified that expression of miR-29a was significantly reduced in AGS and SGC-7901 gastric cancer cell lines, compared with that of the GES-1 human gastric mucosal epithelial cell line (Fig. 1B). It is suggested that downregulation of miR-29a may be associated with the development and progression of gastric cancer.



Figure 3. miR-29a negatively regulates Robol gene expression. (A) miR-29a inhibited the mRNA expression of Robol in gastric cancer AGS cells. Two-tailed Student's t-test was used to analyze the significant differences. **P<0.01 vs. AGS. (B) miR-29a inhibited the protein expression of Robol in gastric cancer AGS cells. Two-tailed Student's t-test was used to analyze the significant differences. **P<0.01 vs. AGS. (C) The mRNA level of Robol was commonly increased in gastric cancer tissues (T) compared with their matched adjacent normal tissues (N). (D) Correlation of miR-29a and Robol expression in gastric cancer tissues (two-tailed Pearson's correlation analysis; R²=0.6379, P<0.0001) by determining the mRNA expression. miR-29a, microRNA-29a; Robol, Roundabout homolog 1; AGS, AGS cells without any transfection.



Figure 4. Robol 3'-UTR is a target of miR-29a. (A) Diagram of the luciferase reporter plasmids with the WT or MUT Robol 3'-UTR. (B) The relative luciferase activity in AGS cells was determined after the plasmid with WT or MUT Robol 3'-UTR was cotransfected with miR-29a mimics, respectively. Two-tailed Student's t-test was used to analyze the significant differences. **P<0.01 vs. control. Robol, Roundabout homolog 1; UTR, untranslated region; miR-29a, microRNA-29a; WT, wild-type; MUT, mutant.

Overexpression of miR-29a inhibits the migration and invasion of gastric cancer cell lines. Based on the results obtained, whether miR-29a serves a role in the regulation of migration and invasion of gastric cancer cells was further investigated. AGS human gastric cancer cells were transfected with miR-29a mimics or the negative control, respectively. As indicated in Fig. 2A, transfection of miR-29a mimics resulted in a significant increase in miR-29a expression, when compared with the negative control transfected with scramble miRNA. In addition, it was further identified that restoration of miR-29a led to reduced migration and invasion of gastric cancer cells, when compared with the control cells (Fig. 2B). Accordingly, it is suggested that miR-29a acts as a tumor suppressor and contributes to inhibition of migration and invasion of gastric cancer cells.

miR-29a negatively regulates Robol gene expression. Targetscan prediction software was further used to identify potential targets for miR-29a. Robol was identified as a putative target gene for miR-29a, which has been reported to participate in the regulation of cell migration and invasion (13). To further confirm this prediction, the mRNA



Figure 5. Knockdown of Robol reduces migration and invasion in gastric cancer cells. (A) Transfection of Robol siRNA inhibited the mRNA expression of Robol in AGS cells. (B) Transfection of Robol siRNA inhibited the mRNA and protein expression of Robol in AGS cells. (C) Downregulation of Robol by siRNA suppressed migration and invasion in AGS cells. Two-tailed Student's t-test was used to analyze the significant differences, *P<0.01 vs. AGS. Robol, Roundabout homolog 1; siRNA, snall interfering RNA; NC, AGS cells transfected with non-specific siRNA; AGS, AGS cells without any transfection.

and protein expression levels of Robo1 were determined in AGS cells transfected with miR-29a mimic. It was observed that the mRNA and protein expression of Robo1 was significantly downregulated following overexpression of miR-29a, compared with the negative control (Fig. 3A and B). Furthermore, the mRNA levels of Robo1 in gastric cancer and adjacent normal tissues were determined with RT-qPCR, then the significance of the miR-29a/Robo1 correlation was assessed in gastric cancer. As presented in Fig. 3C, the mRNA expression of Robo1 was frequently increased in gastric cancer tissues, compared with the matched adjacent normal tissues. Furthermore, as demonstrated in Fig. 3D, a significant inverse correlation was observed between miR-29a and Robo1 mRNA expression. Accordingly, the data suggest that miR-29a negatively regulates Robol gene expression and Robol is a potential target gene of miR-29a.

Robol 3'-UTR *is a target of miR-29a*. To verify whether Robol 3'-UTR is a target of miR-29a, a luciferase reporter assay was conducted. The vectors containing the wide type of Robol 3'UTR as well as the mutant type of Robol 3'UTR were constructed (Fig. 4A). As demonstrated in Fig. 4B, cotransfection of AGS cells with Robol-3'-UTR/pmirGLO and miR-29a mimics resulted in a significant reduction in the luciferase activity, when compared with the negative control (P<0.05). This inhibitory effect was abrogated by point mutations in the core binding sites of the Robol 3'-UTR. All of these observations indicated that miR-29a exerts suppressive effects on Robol expression via binding to the 3' UTR of Robol.

Knockdown of Robol reduces migration and invasion in gastric cancer cells. To verify the effect of Robol on gastric cancer cell migration and invasion, the expression of Robol was observed to be downregulated by Robol siRNA. As presented in Fig. 5A and B, the mRNA and protein expression levels of Robo1 were notably downregulated following knockdown of the Robo1 gene in AGS cells. Consistently, downregulation of Robo1 markedly inhibited migration and invasion in AGS cells (Fig. 5C), which resembled the suppressive effects of miR-29a. These observations suggest that miR-29a may inhibit gastric cancer cell migration and invasion via targeting Robo1.

Discussion

Initiation and progression of cancer has been demonstrated to involve deregulation of various genes, including upregulation of oncogenes and downregulation of tumor suppressor genes (14). Robo1 has been reported to be involved in gastric cancer. Tie et al (7) demonstrated that as a target of miR-218, Robo1 contributed to gastric cancer metastasis (7). In addition, dysfunction of miR-29a has been reported to be involved in gastric cancer (11). However, the downstream target gene of miR-29a in gastric cancer remains largely unclear. In the current study, it was observed that miR-29a/Robo1 signaling serves a key role in the regulation of migration and invasion in gastric cancer cells. The expression levels of miR-29a were frequently reduced in gastric cancer tissues, when compared with matched adjacent normal tissues. Furthermore, miR-29a was additionally downregulated in gastric cancer cell lines compared with the normal gastric mucosal epithelial cell line. These observations suggest that miR-29a may serve a role in the malignant progression of gastric cancer. It was further demonstrated that restoration of miR-29a and knockdown of Robo1 may significantly inhibit the migratory and invasive capabilities of gastric cancer cells, and Robo1 was identified as a novel target of miR-29a. These

observations suggest that Robol may act as an oncogene while miR-29a is hypothesized to be a tumor suppressor in gastric cancer. Thus, it is suggested that their deregulation may promote gastric cancer metastasis.

It is widely accepted that deregulated expression levels of certain miRNAs directly participate in the development and progression of numerous types of human cancer. In addition to its role in cancer, miR-29a has also been demonstrated to be associated with osteoblastic differentiation (15), myogenesis (16), sclerosis (17), fibrosis (18), HIV-1 replication (19), diabetes (20) and Alzheimer's disease (21). In the current study, it was identified that overexpression of miR-29a markedly suppressed the migratory and invasive capacities of gastric cancer cells. Chen et al (11) reported that miR-29a was notably downregulated in gastric cancer, consistent with the results of the current study. In addition, Chen et al (11) demonstrated that miR-29a had an inhibitory effect on cell proliferation and invasion in gastric cancer cells, at least in part via targeting VEGF-A. As VEGF-A acts as a key regulator in angiogenesis, it was further identified that miR-29a was able to suppress the tumor microvessel density in gastric cancer (11). Cui et al observed that miR-29a inhibited cell proliferation and induced cell cycle arrest through the downregulation of p42.3 in gastric cancer (22). Taking these previous observations together with the results of the current study, it is suggested that miR-29a is able to suppress malignant phenotypes of gastric cancer cells, including cell proliferation, cell cycle progression, migration, invasion and tumor angiogenesis, highlighting the importance of miR-29a in gastric cancer.

Furthermore, the molecular mechanisms underlying the inhibitory effect of miR-29a on gastric cancer were investigated. As one miR has multiple target genes, other miR-29a targets are likely to exist, which are involved in the development of gastric cancer. Putative targets of miR-29a were searched using a bioinformatics approach, and Robol was predicted to be a novel target of miR-29a. To verify this prediction, western blotting and a luciferase activity assay were performed, and it was identified that miR-29a was capable of inhibiting the protein expression levels of Robol, by directly binding to the 3'-UTR of Robo1 mRNA in gastric cancer cells. In addition, as Robol has been demonstrated to act as a key migration and invasion factor in cancer cells, it is suggested that downregulation of Robo1 induced by miR-29a overexpression may contribute to inhibition of migration and invasion in gastric cancer cells. In addition, it has been previously reported that extracellular signal-regulated kinase (ERK) signaling and matrix metalloproteinase 9 (MMP-9) were downstream effectors of Robo1 (13). Upregulation of ERK signaling has been identified to contribute to the development and progression of various human malignancies including gastric cancer (23-26). MMP-9 additionally serves a critical role in the regulation of cancer cell invasion through degradation of the extracellular matrix and promotion of cancer metastasis (27-29).

In conclusion, to the best of our knowledge the present study suggests for the first time, that miR-29a inhibits gastric cancer cell migration and invasion via inhibition of Robo1. Therefore, miR-29a and Robo1 may serve as diagnostic or therapeutic targets for gastric cancer.

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