RETRACTED ARTICLE: miR-200a-3p plays tumor suppressor roles in gastric cancer cells by targeting KLF12

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ABSTRACT

Background: The role of miR-200a-3p in gastric cancer (GC) remain unclear.

Materials and methods: miR-200a-3p expression in 65 paired GC and adjacent tissues (AT) were evaluated by quantitative real-time PCR (qRT-PCR) and Western blot. Cell proliferation, cell cycle, and cell migration were assessed by cell growth counting assay, cell cycle analysis, and transwell assay, respectively. The target of miR-200a-3p was analyzed by dual-luciferase reporter assay.

Results: miR-200a-3p in GC tissues was significantly reduced compared with AT. miR-200a-3p expression was closely associated with clinicopathological features (P < .05). SGC-7901 cell line demonstrated the lowest level of miR-200a-3p. Cell proliferation and colony formation was significantly inhibited by miR-200a-3p overexpression, but increased by miR-200a-3p knockdown (P < .05). miR-200a-3p upregulation increased the G1/S cell ratio. The 3'-UTR of KLF12 directly interacted with miR-200a-3p. Furthermore, increased levels of KLF12 expression was detected in GC tissues. A correlation analysis suggested a negatively correlation between miR-200a-3p and KLF12 mRNA expressions.

Conclusion: miR-200a-3p was down-regulated in GC tissues and was correlated with clinicopathological features. miR-200a-3p overexpression inhibits GC cell proliferation, cell cycle, and cell migration. Furthermore, miR-200a-3p might act as a tumor suppressor in GC by targeting KLF12.

Introduction

Gastric cancer (GC) is the fourth most common human malignancy and the third leading cause of cancer-related mortality worldwide, resulting in over 300,000 deaths each year [1,2]. Nearly 42% of new GC cases worldwide occur in China [3]. Although standard clinical management including complete surgery and chemotherapy has been improved and widely used, the five-year survival rate of GC patients is still poor, which is only 35–40% [4]. Therefore, it is very important to find novel targets for improved understanding of the pathogenesis and pathological mechanisms for gastric cancer.

miRNAs (miRs) are a class of non-coding small RNAs with 18–24 nucleotides in length that play important roles in post-transcriptional regulation by targeting the 3'-untranslated region (3'-UTR) of the mRNAs in various biological processes. Growing evidence indicates that certain miRs act as oncogenes or tumor suppressors, influencing cancer development, differentiation and progression [5,6]. Rawlings-Goss et al. [7] reported that miR-647 is associated with GC and may act as a biomarker for GC. miR-9 has been reported to play as a tumor suppressor by targeting KLF12 and MYO1C for GC [8].

The miR-200a-3p is encoded by a gene on chromosome 1 and is a member of the miR-200 family. Notably, the miR-200 family has been specifically proposed as a tumor regulator with respect to tissues. They are decreased in hepatocellular carcinoma and renal cell carcinoma [6,9] while being upregulated in colorectal cancer, and bladder cancer [10,11]. Chang et al. [12] reported that miR-200a-3p is downregulated in GC and may potentially act as a useful prognostic predictor of GC. However, the exact mechanism of miR-200a-3p in GC remains unclear. Therefore, the purpose of this study was to investigate the effects of miR-200a-3p on cell proliferation, cell cycle, and cell migration and the potential mechanism pathway in GC cell line.
Materials and methods

Ethics statement

This study was approved by the Ethics Committee of the Henan Provincial People’s Hospital and all patients provided informed consent.

Clinical specimens, cell culture, and cell transfection

Sixty-five pairs of GC samples and adjacent tissue (AT) samples were collected between January 2015 and October 2017 from the Henan Provincial People’s Hospital with an average age of 56.4 years, including 50 males and 15 females. Human GC SGC-7901 and BGC-823 cell lines and human gastric epithelial cells (HGEpics) were obtained from American Type Culture Collection (Manassas, VA, USA). All cell lines were maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS; HyClone, USA) and cultured at 37°C in a humidified incubator with 5% CO₂. The culture medium was renewed every 2 days. The negative control, miR-200a-3p mimic and miR-200a-3p inhibitor (GenePharma, China) were transfected into the cell using Lipofectamine 2000 (Thermo Fisher Scientific, Inc., USA), according to the manufacturer’s instructions. Transfected cells were collected for further experimental analysis at 24 h post-transfection.

RNA extraction and quantitative real-time PCR

RNA extraction kit (Beijing ComWin Biotech Co., Ltd., China) was used to extract total RNA from cell lines and GC tissue samples. The expression level of miR and relevant reference gene was verified by qRT-PCR using a SYBR green mix (GeneCopoeia, China) on illumine Ecosystem. The primer of miR-200a-3p was supplemented by the kit. U6 was used as an internal control. The expression levels of genes were quantified using the 2⁻ΔΔCₚ method. The primers of KLF12 was as follow: forword: TGGCAAAGCACAAATGGAC; reverse: CTAAATGGTGAAATTGAACAAGG. All procedures were performed in triplicate.

Transwell assay

SGC-7901 cell lines were transfected with miR-200a-3p mimic and inhibitor, and about 4 × 10⁴ cells and 24-well, 8.0-μm pore membranes (BD Biosciences, USA) were placed in the upper chamber with 200 μl RPMI 1640. The lower layers were filled with mixed cultures including 90% RPMI 1640 and 10% FBS. Twenty-four hours after incubation, the transfected cells were removed with a cotton swab. The cells were transferred to the surface of the film and stained with 0.1% crystal violet stain for about 30 min. Cell counting was carried out by a microscope system and was performed in triplicate to obtain an average value.

MTT assays and colony formation

Cell proliferation assays were performed using MTT assays. SGC-7901 cells (3 × 10⁴ cells/well) were plated in 96-well plates and grown for 1–5 days. Then, cells were incubated in darkness with 20 μl 5 mg/ml MTT solution (Sigma-Aldrich; USA) in each well for 4 h. After removal of the supernatants, 150 μl DMSO (Sigma-Aldrich; USA) was added. The absorbance at 490 nm was measured with a microplate reader (Bio-Tek, USA).

For the colony formation assay, SGC-7901 cells were plated in 6-well plates at a density of 10⁵ cells/well and incubated for 2–3 weeks. Next, at room temperature, the cells were fixed with 4% methanol for 30 min and stained with 0.5% crystal violet for 10 min. Only the colonies containing more than 50 cells were counted.

Cell cycle assays by flow cytometry

After 48 h transfection, SGC-7901 cells were harvested and washed twice by PBS and then fixed in ice-cold 70% ethanol at 4°C for 1 h. Next, the cells were incubated in PBS combined with 0.1 mg/ml RNase A (Sigma-Aldrich, USA) for 30 min, prior to being stained with 0.05 mg/ml propidium iodide (PI; Sigma-Aldrich, USA) for another 30 min. The cell cycle was then analyzed with flow cytometry (BD-C6, USA).

Dual-luciferase reporter assay

The amplified sequences of wild type (wt) and mutant type (mut) of KLF12 were synthesized chemically and cloned into pmirGLO Dual-Luciferase miRNA vector (XhoI/Sac I) (Youbio (Shanghai Gene Pharma Co., Ltd., China). The wt or mut 3’-UTR vectors together with miR-200a-3p mimic, or miRctrl were transfected into 293 T cell line. Following transfection for 48 h, luciferase activity was determined using the Dual-Glo Luciferase assay kit (Promega, USA), according to the manufacturer’s protocol.

Western blotting

Total protein was extracted using a radioimmunoprecipitation assay buffer (Thermo Scientific, USA) and samples were resolved by using SDS-PAGE analysis (TGX Gels, Bio-Rad Laboratories Inc., USA). Then, the resolved protein was transferred to PVDF membrane (Millipore, Billerica, MA) that was blocked with 5% non-fat milk for 2 h at room temperature. Antibodies including anti-KLF12 and anti-β-actin (Abcam, UK) were applied to membranes. After the membranes had been washed three times, protein expression was assessed by LI-COR model 3600 (LI-COR, USA).

Statistical analysis

All Statistical analyses were performed using SPSS 18.0 software. All data are presented as the mean ± standard deviation. All experiments were repeated at least three times independently. Statistical comparisons between groups were performed using the Mann-Whitney U test, Student’s t-test, or one-way analysis of variance, followed by Tukey’s post hoc test. P < .05 was considered statistically significant.
Results

miR-200a-3p was upregulated in human GC tissues and GC cell lines

As shown in Figure 1(A), the expression of miR-200a-3p in GC tissues was significantly reduced than that in AT according to qRT-PCR. We further analyzed the relationship between the expression of miR-200a-3p and clinicopathological features in GC samples (Table 1). It was revealed that the expression of miR-200a-3p was closely associated with pathological differentiation, venous invasion, depth of invasion, lymphatic metastasis and distal metastasis (all \( P < .05 \)). However, there was no association of the expression of miR-200a-3p with gender or age (Table 1). Similarly, compared with normal HGEpic, the human GC cell lines (SGC-7901 and BGC-823) presented significantly lower levels of miR-200a-3p (\( P < .05 \)); furthermore, SGC-7901 cell line demonstrated the lowest level of miR-200a-3p. Taken together, these findings indicated that miR-200a-3p is downregulated in GC tumor tissues and cell lines.

miR-200a-3p inhibit GC cell proliferation, cell cycle and cells migration

To investigate whether miR-200a-3p influences GC cell behavior, SGC-7901 cell line was generated by transfecting with a miR-200a-3p mimic/inhibitor that generated a stable miR-200a-3p-overexpression/silencing cell line. The results of the MTT and colony formation assays showed that overexpression of miR-200a-3p in SGC-7901 cells significantly inhibited cell proliferation and colony formation \( (P < .05) \); whereas, downregulation of miR-200a-3p could significantly increase cell proliferation and colony formation \( (P < .05) \). The effect of miR-200a-3p on cell cycle progression was detected using flow cytometry. As shown in Figure 2(C), overexpression of miR-200a-3p could increase the percentage of G1 phase cells and decrease the percentage of S phase cells, whereas, downregulation of miR-200a-3p inhibited the cell cycle \( (P < .05) \). We then detected the migration capability using Transwell assays. The results indicated that overexpression of miR-200a-3p could inhibit the migration of GC cells, while downregulation of miR-200a-3p could significantly promote the migration of GC cell \( (P < .05) \).

KLF12 is a potential target of miR-200a-3p

According to the biological information online analysis software (TargetScan and StarBase), KLF12 was predicted as a potential target of miR-200a-3p (Figure 4(A)). To confirm potential miR-200a-3p binding sites in the 3’-UTR of KLF12, a dual-luciferase reporter assay was applied. Luciferase reporter assay showed that the 3’-UTR of KLF12 could directly interact with miR-200a-3p (Figure 4(B)). The qRT-PCR assay suggested that there was a negative correlation of KLF12 with miR-200a-3p in mRNA expression level. Western blot analysis demonstrated that the expression of KLF12 was significantly downregulated in SGC-7901 cells transfected with miR-200a-3p mimic and upregulated in cells transfected with miR-200a-3p inhibitor \( (P < .05) \). Additionally, we detected KLF12 mRNA and protein expression in GC and AT tissues, indicating that GC tissues presented increased levels of KLF12 mRNA and protein (Figure 5(A) and (B)). A correlation analysis showed that the expressions of miR-200a-3p and KLF12 mRNA were negatively correlated in GC tissues \( (P = .0003) \).

Discussion

GC is the third leading cause of cancer-associated mortality around the world [13]. Despite the recent improvements in GC diagnosis, surgical methods, perioperative management, and the concept of chemotherapy, GC patients still have a very low 5-year overall survival [14]. Thus, it is crucial to investigate the molecular mechanism underlying the development and progression of GC.

Numerous studies have demonstrated that miRs expression plays different roles in a variety of cancers and are involved in cell growth and signaling pathways by targeting multiple target genes. Although various studies have indicated that miRs play vital roles in the development of GC, knowledge of the abnormal expression and function of miRs still remains unclear. Therefore, identification of underlying
miRs and their targets is crucial for understanding their roles in the tumorigenesis and may be helpful for developing novel targets for GC treatment.

Published evidence has shown that miR-200a-3p acts as a potential tumor suppressor by regulating epithelial to mesenchymal transition and cancer initiation [15]. However, the biological role and function of miR-200a-3p in RC is rarely reported. To our knowledge, only two studies have demonstrated the correlations of miR-200a-3p with GC [4,16], but none have investigated the molecular mechanism of miR-200a-3p in GC.

In the current study, we investigated the function of miR-200a-3p in GC. The results indicated that miR-200a-3p was significantly decreased in GC tissues and cell lines. There were significant correlations between miR-200a-3p expression and clinicopathological features, including pathological...
Figure 3. miR-200a-3p inhibits the migration of SGC7901 gastric cancer cell lines.

Figure 4. KLF12 is a target of miR-200a-3p. (A) Predicted miR-200a-3p target sequence in the 3′-UTR of KLF12; (B) dual-luciferase reporter assay was performed in 293 T cells and showed that the 3′-UTR of KLF12 luciferase activity was significantly inhibited by miR-200a-3p expression dependently; (C) qRT-PCR showed that miR-200a-3p could reduce the expression of KLF12 mRNA in GC cells; (D) western blot analysis confirmed that miR-200a-3p could reduce the expression of KLF12 protein in GC cells. *P < .05.
differentiation, venous invasion, depth of invasion, lymphatic metastasis, and distal metastasis. Furthermore, overexpression of miR-200a-3p could inhibit the proliferation, cell cycle and migration of GC cells. Dual-luciferase reporter assay and western blot analysis presented KLF12 as a target of miR-200a-3p. Taken together, these findings demonstrated that miR-200a-3p acted as a tumor suppressor by targeting KLF12 in GC and likely served as a potential therapeutic target for GC treatment.

Many reports have shown that KLF12 plays roles in various kinds of cancers involving in proliferation, apoptosis, differentiation, migration and pluripotency [17]. KLF12 is a member of KLFs’ family with a highly conserved family of zinc finger transcription factors [18]. Giefing et al. showed KLF12 gene might be a potential tumor suppressor for salivary gland tumor [19]. Xu et al. have reported that miR-382 could inhibit the growth and chemosensitivity in osteosarcoma by targeting KLF12 and HIPK3 [20]. Hunten et al. found that KLF12 was identified as the target of P53 using next-generation sequencing (NGS) [21]. In the present study, the findings indicated that the expression of KLF12 was negatively related to miR-200a-3p, suggesting it might function as an oncogene in human GC.

Conclusions

In summary, we found that miR-200a-3p was down-regulated in GC tissues and was correlated with clinicopathological features, including pathological differentiation, venous invasion, depth of invasion, lymphatic metastasis, and distal metastasis. Overexpression of miR-200a-3p could inhibit the capabilities of proliferation, cell cycle, and cell migration in GC cell lines. Furthermore, miR-200a-3p might act as a tumor suppressor in GC by targeting KLF12.

Disclosure statement

No potential conflict of interest was reported by the authors.

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