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Abstract: Background: miRNAs play an important role in cancer progression. Aberrant expression of miR-142-3p is related with various cancers. However, the function of miR-142-3p in gastric cancer (GC) remains poorly studied. We aim to investigate the biological roles and mechanisms of miR-142-3p in GC. Methods: miRNA-142-3p expression level in GC tissues and cell lines was determined using Real-Time PCR. Cell proliferation was analyzed by MTT. Cell apoptosis was analyzed by TUNEL assay. The target gene FOXO4 of miR-142-3p was predicted upon bioinformatics tool and verified by dual-luciferase reporter assay, Real-Time PCR, and Western Blot. The molecular mechanism-associated genes were measured using Real-Time PCR and Western Blot. Results: miR-142-3p is up-regulated in gastric cancer tissues and cell lines. MiR-142-3p promotes gastric cancer cell growth and suppresses cell apoptosis by binding to 3'UTR of FOXO4 and degrading the level of FOXO4 to decrease the expression of p21, p27, Bim, and FASL. Moreover, a significant negative correlation between miR-142-3p and FOXO4 was found in seven patients with gastric cancer. Conclusion: miR-142-3p acts as a tumor promoter by targeting FOXO4 to decrease the expression of p21, p27, Bim, and FASL in the progression of GC. miR-142-3p and FOXO4 may be potential therapeutic targets for gastric carcinoma patients.

Keywords: miR-142-3p, FOXO4, gastric cancer

Introduction

Gastric cancer (GC) exhibits the third leading cause of cancer-associated mortality worldwide and is the fourth most common cancer [1, 2]. In spite of recent advance in clinical and experimental oncology, the prognosis of gastric cancer is still unfavorable, with a 5-year survival rate of approximately 30% [3]. The progress of gastric cancer consists of multiple steps, in which key genes or pathways that control proliferation or apoptosis will be alternated. Thus, it is necessary to elucidate the molecular mechanisms in improving the diagnosis accuracy and treatment efficiency of GC [4]. In these decades, abundant evidences had shown that miRNAs played an important role in the evolution of GC [5].

miRNAs are a class of non-coding RNAs, whose sequence is about 22 nt. miRNAs decrease the expression level of target genes by complementarily binding the 3'UTR [6]. And it had been confirmed that miRNAs act as tumor suppressors or promoters in the progress of various cancers, and abnormal miRNAs expression level might be as a potential biomarker for GC diagnosis, prognosis and disease monitor, as well as therapeutic target [7]. Many miRNAs, such as, miR-337-3p, miR-203, and miR-320a have been proved to regulate tumor cell proliferation, migration, and invasion in GC [5, 8, 9].

MiR-142-3p plays different functional roles in different cancer. Recent studies also indicated that miR-142-3p characterizes as a potential tumor suppressor by targeting HMGB1 and TGFbetaR1 in non-small-cell lung carcinoma [10, 11], inhibits the migration and invasion of hepatocellular carcinoma [12]; and miR-142-3p also act as oncogene to promote cell proliferation and migration and to induce apoptosis in...
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In present study, we confirmed that miR-142-3p is up-regulated in GC tissues and cell lines. miR-142-3p can promote GC cells proliferation and inhibit cell apoptosis through directly targeting transcription factor FOXO4 to regulate the cell cycle and apoptosis-associated protein p21, p27, FASL, and Bim. Importantly, silencing FOXO4 can rescue the inhibitory effect of miR-142-3p. Collectively, our data ascertain the oncogenic role of miR-142-3p in GC, demonstrate the molecular mechanism how it regulates cell proliferation and apoptosis, and further contribute to offer the effective therapeutic targets for the diagnosis and treatment of GC.

Methods

Patients and specimens

45 normal donors and 116 patients with gastric cancer specimens were obtained from the First Affiliated Hospital, and college of Clinical Medicine of Henan University of Science and Technology between June 2013 and June 2014. None had received chemotherapy or radiotherapy before the surgery. Fresh specimens were collected and rapidly frozen in liquid nitrogen and stored at -80°C. Our study was approved by Institutional Ethics Committee of the First Affiliated Hospital, and college of Clinical Medicine of Henan University of Science and Technology.

Table 1. Primers for Real-Time PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>U6 F</td>
<td>CTCGCTTCGAGCACA</td>
</tr>
<tr>
<td>U6 R</td>
<td>ACGCTTCAGATTTGCCT</td>
</tr>
<tr>
<td>p21 F</td>
<td>AGTCATCCCTTGTGGAGCC</td>
</tr>
<tr>
<td>p21 R</td>
<td>CATGCGGTCTGAAGCACAGAT</td>
</tr>
<tr>
<td>p27 F</td>
<td>AAGAAGCCTGGCCTCAAGAG</td>
</tr>
<tr>
<td>p27 R</td>
<td>TTCATCAAGCAGTGATGTATCTGA</td>
</tr>
<tr>
<td>Bim F</td>
<td>CCTCCCTACAGACACAGAGCA</td>
</tr>
<tr>
<td>Bim R</td>
<td>GATAGGATGGGAGGCTGG</td>
</tr>
<tr>
<td>FASL F</td>
<td>GCACACAGCATCCTTGG</td>
</tr>
<tr>
<td>FASL R</td>
<td>GGACCTTGAGGGACCTTG</td>
</tr>
<tr>
<td>GAPDH F</td>
<td>GACTCATGACCACAGTCATGC</td>
</tr>
<tr>
<td>GAPDH R</td>
<td>AGAGGCAGGATGATGTCTG</td>
</tr>
</tbody>
</table>

RNA isolation, cDNA synthesis, and real-time PCR

Total RNA from gastric cancer and normal tissues and gastric cancer cell lines was isolated using Trizol reagent (Invitrogen, USA). cDNA synthesis was conducted with cDNA synthesis kit (Takara, Japan), whereas the cDNA for miRNA was synthesized with primer-specific cDNA synthesis process. Real-Time PCR was performed with SYBR Green PCR master mix (Roche, Indianapolis, IN). The miRNA expression levels were normalized to the endogenous U6 RNA. The sequence of the forward primer for miR-142-3p was 5'-TGT AGT GTT TCC TAC TTT ATG GA-3' and the reverse primer was provided by the miScript SYBR® Green PCR kit (Qiagen, Inc.). And the sequences of Real-Time PCR primers were listed at Table 1.

Western blot

To extract protein, cultured cell was lysed with RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 1% SDS, and 1% protease inhibitor cocktail), cocktail is bought from Roche. To analyze protein level, the prepared proteins were loaded into 10% sodium -dodecyl-sulfate polyacrylamide gel electrophoresis and then transferred to a nitrocellulose (NC) membrane. The primary antibody was rabbit anti-FOXO4 (1:1000 Santa Cruz), anti-p21 (1:1000, Abcam), anti-p27 (1:1000, Abcam), anti-FASL (1:1000, Abcam), anti-Bim (1:1000, Abcam), mouse anti-GAPDH (1:3000. Millipore), the secondary antibody was goat anti-rabbit IgG (Millipore). Immunoblot was manifested by ECL and X-Ray.
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Transfection of miRNA-142-3p inhibitor and mimics

According to the sequence of miRNA-142-3p (AGGUAUUUCAUCCUUUGUGAUGU), we designed the inhibitor 5'-UCCAUAAAGUAGGAAACAC-UACA-3', the negative control 5'-CAGUACUUUGUGUAGUACAA-3', and mimics of miRNA-142-3p. MGC-803 cells were transfected with the inhibitor/mimics or negative control.

RNA interference

RNA interference was performed with synthetic siRNA duplexes, which targeted to FOXO4, designed and synthesized by GenePharma (Shanghai, China). FOXO4 siRNAs oligonucleotide sequence was 5'-UCUCAACCCUCCAUUCUCCdTD-Td-3 and RNA duplex control was 5'-UUCUCCGAACCUCCGUGGACdTD-Td-3'. Cells was transected with 100 nmol siRNA/well in 6-well plates, using lipofectamine RNAIMAX (Invitrogen) as transfection reagents based on the manufacturer’s instructions.

**MTT**

After transfection, 1000 cells were seeded in 96 wells plates and cultured for day 0-4. 20 µL MTT (3-(4,5-dimethylthi-azol-2-yl)-2,5-diphenyltetrazolium bromide, 5 mg/ml, Sigma Aldrich) was added into each well, and cells were incubated for 4 h at 37°C. Then the supernatants were removed and 150 µL DMSO was added. Absorbance value was obtained at the wavelength of 490 nm with a micro-plate re-ader (Bio-Rad Laboratories). The data was from three independent experiments and shown as mean ± SEM.

**Luciferase report assay**

Human FOXO4 3'UTR was cloned into the firefly luciferase expressing vector pMIR-report (Promega), forming FOXO4-3'UTR wt. Mutation of the miR-142-3p binding site was cloned into pMIR-report, forming FOXO4-3'UTR mut. The luciferase activity signal was measured by firefly/Renilla ratio.

**TUNEL**

2.5×10⁵ cells/well was seeded into 12-well plate and transfected with miRNA-142-3p inhibitor/mimics or control for 48 h. After transfection, the cells were washed with PBS, fixed with 4% formalin for 30 min at room temperature. Then the cells were washed with PBS once, and incubated with 0.1% Triton-100 for 2 min on the ice. Next, the cells were blocked with 0.3% H₂O₂ in methanol for 20 min, washed and incubated with 1× TdT labeling mixture (TdT enzyme + TUNEL detection buffer + fluorescence labeling buffer) for 1 h at 37°C in a dark room. Finally, the labeled cells were washed three times and stained with DAPI. The images were captured at 40× lenses on Leica scanning confocal microscope.
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Figure 2. miR-142-3p promoted gastric cancer cell growth and suppressed the cell apoptosis. A. QRT-PCR analysis of miR-142-3p transfection efficiency after the miR-142-3p mimics, or miR-142-3p inhibitors transfection in gastric cancer cell lines. B. The MTT assay analysis was used to evaluate the proliferation of gastric cancer cell lines after transfection with the miR-142-3p mimics, inhibitor or controls. C. TUNEL assay analysis of gastric cancer cell lines after treatment with miR-142-3p mimics, inhibitor or controls. D. Western Blot analysis of p21, p27 and FASL and Bim proteins after transfection of miR-142-3p mimics or inhibitors or controls. E. Column charts were used to display the changes of protein expression. F. Gray value was analyzed by Image J Software. GAPDH was used as an internal control. Data are mean ± SEM (n=3). *P<0.05, **P<0.01 vs controls.
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Statistical analysis

The statistical analyses were performed with SPSS19.0 software. All the data were presented as the mean ± SEM, and differences between each group were analyzed by the Student t-test using two-side, and significance defined as P<0.05 P<0.01. The relationship between miR-142-3p and FOXO4 was performed with Correlation.

Results

Up-regulated expression of miR-142-3p in GC tissues and cell lines

To investigate the potential role of miR-142-3p expression in GC development, we first examined the expression of miR-142-3p in tissues from 45 normal donors and 161 patients, and the normal gastric cell line and GC cell lines. The expression of miR-142-3p was up-regulated in GC tissues and cell lines compared to normal tissues and cell lines (Figure 1A and 1B), which showed initial evidence that miR-142-3p may play an important role in the development of GC. In order to further address the relationship between miR-142-3p and GC, we analyzed the expression of miR-142-3p in different grades from GC patients. Real Time-PCR demonstrated that higher expression of miR-142-3p is positively correlated with GC clinical grades (Figure 1C). All the above results indicated that miR-142-3p was up-regulated in GC, and we further convinced that possible link between miR-142-3p up-regulation and progression of human GC.

miR-142-3p promotes GC cell growth and suppresses cell apoptosis

In order to examine the biological roles of miR-142-3p in GC cell lines, we investigated the effect of miR-142-3p on cell viability and proliferation. Firstly, real time PCR results confirmed that transfection of miR-142-3p mimics dramatically increased the miR-142-3p level. Meanwhile, transfection of miR-142-3p inhibitor decreased the miR-142-3p level, comparing with the transfection of control (Figure 2A). Then MTT assays were performed to detect the cell proliferation ability. Consistent with real time PCR results, MTT assays showed that the GC cell proliferation was significantly increased when the GC cell lines were transfected with miR-142-3p mimics after 48 h. Meanwhile, the cell proliferation was inhibited when the cell was transfected with miR-142-3p inhibitor (Figure 2B). Because apoptosis is a contributing factor to cell growth inhibition, we performed immunofluorescence analysis of GC cell apoptosis after transfection with miR-142-3p mimics, inhibitor and control. We found that miR-142-3p inhibitor could obviously increase the apoptosis rate, while miR-142-3p mimics resulted in lower apoptosis rate (Figure 2C, 2D). Taken together, these results indicated that miR-142-3p promoted cell growth and suppressed the apoptosis in GC cell lines. To better understand the underlying molecular mechanism of miR-142-3p, we examined the protein level and mRNA level of potential proliferation and apoptosis-associated genes when the disruption of differential miR-142-3p levels. Indeed, miR-142-3p mimics suppressed the expression of p21, p27,
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FASL, and Bim, whereas, miR-142-3p inhibitor increased the expression of these cell cycle and apoptosis-associated proteins, including p21, p27, FASL, and Bim (Figure 2E shows the level of protein, Figure 2F shows the level of mRNA).

**FOXO4 is a direct target of miR-142-3p in GC**

As a group of regulatory factors, miRNAs achieve their biological function relies on their regulating target gene. Therefore, we predicted the possible target genes of miR-142-3p using miRBase, a comprehensive database on miRNA. We found FOXO4 was one of the target genes that were predicted to be bound by miR-142-3p, which was shown as Figure 3A. As predicted, miR-142-3p mimics transfected in MGC-803 cells obviously decreased the protein level of FOXO4, whereas miR-142-3p inhibitor increased the protein level of FOXO4 (Figure 3B).

Base on the predicted-result (Figure 3A), we set up a dual-luciferase reporter system to ascertain whether FOXO4 was the direct target gene of miR-142-3p. The 3’UTR of FOXO4, including the miR-142-3p binding sites, was established and cloned into the firefly luciferase expressing vector pMIR-report, forming FOXO4-3’UTR wt; whereas, corresponding point mutations within the miR-142-3p seed of these predicted sites, forming FOXO4-3’UTR mut. The MGC-803 was co-transfected with reporter vector and ectopic expression of miR-142-3p.

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**Figure 4.** Loss-of function of FOXO4 mimicked impact of miR-142-3p on gastric cancer cell proliferation. A. The MTT assay analyzes proliferation ability of gastric cancer cell lines after transfected miR-142-3p inhibitor or co-transfected si-FOXO4 or controls. B. Western blot and QRT-PCR analysis of FOXO4 protein and miR-142-3p expression in gastric cancer tissues. C. Scatter plots show the relationship between FOXO4 and miR-142-3p (n=7). *P<0.05, and **P<0.01 vs controls.
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The luciferase activity in GC cells was decreased with FOXO4-3'UTR wt construct when transfection with miR-142-3p mimics; whereas, miR-142-3p inhibitor significantly increased the luciferase activity in GC cells with FOXO4-3'UTR wt construct. But, ectopic expression of miR-142-3p failed to affect the luciferase activity in GC cells with FOXO4-3'UTR mut (Figure 3C). Collectively, these results suggested that miR-142-3p recognized the binding sites on FOXO4 mRNA 3'UTR and degraded the FOXO4 level.

FOXO4 and miR-142-3p own the opposite function in GC

To investigate whether miR-142-3p promote GC cell proliferation through FOXO4, we determined the expression and role of FOXO4 in GC cell lines. MTT assay showed that si-FOXO4 significantly promoted cell proliferation attenuated by miR-142-3p inhibitor in MGC-803 cells (Figure 4A). Then we tested the expression of miR-142-3p and FOXO4 in seven patient's tissues with GC by real time PCR and Western Blot analysis. The results showed that the higher expression level of miR-142-3p in the lower expression of FOXO4 protein (Figure 4B). Figure 4C showed that the expression of miR-142-3p and FOXO4 was negative correlation in GC tissues (Figure 4C). These results demonstrated that miR-142-3p promoted the proliferation in GC cells by targeting FOXO4.

Discussion

To date, miR-142-3p has been shown to own the opposing biological function in different cancer. It has also been reported that miR-142-3p is down-regulated in HCC [12], breast cancer [15], thyroid cancer [16], and human acute lymphoblastic leukemia [17]. Wu and his colleagues proved that miR-142-3p over-expression suppressed the migration and invasion by targeting RAC1 [12]. Isobe et al reported that miR-142-3p regulated the tumorigenicity of breast cancer stem cells through the wnt signaling pathway [15]. On the other hand, miR-142-3p is up-regulated in NPC [14], renal cell carcinoma [13], human T-cell acute lymphoblastic leukemia (T-ALL) [18] and NSCLC [11]. Lv et al showed that miR-142-3p alleviated the suppressive effect of PKA in T-ALL [18]. miR-142-3p represses TGF-beta-induced growth inhibition by decreasing the level of TGF-betaR1 in NSCLC [11]. Consistent with the latter observation, we found that miR-142-3p is up-regulated in GC tissues and cell lines; and the expression of miR-142-3p is associated with the grade of GC. Over-expression of miR-142-3p can promote the proliferation and inhibit apoptosis in GC cell line. And knockdown of miR-142-3p inhibited the proliferation and induced apoptosis in GC cell line. These results show that miR-142-3p exerts the oncogenic function in GC. However, the molecular mechanism of the miR-142-3p promoting cell proliferation needs to be solved.

Real-Time PCR and Western Blot analyzed the expression level of cell cycle inhibitor p27 and p21, and apoptosis inducers Bim and FASL. The results indicated that miR-142-3p mimics decreased the level of p21, p27, Bim, and FASL, whereas miR-142-3p inhibitor can increase the level of the four proteins, including p21, p27, Bim, and FASL. Next, the gap between miR-142-3p and the abovementioned proteins remain to be considered. We predicted the potential target of miR-142-3p and found out FOXO4 using bioinformatics tool. Luciferase assays indicated that miR-142-3p directly binds to 3'UTR of FOXO4 and dramatically decreases the level of FOXO4 mRNA and protein expression. This provided that FOXO4 is regulated by miR-142-3p. FOXO4 is a member of Forkhead FOXO transcription factor family, which includes FOXO3a, FOXO1, and FOXO4. And FOXO4 is recognized as a key tumor suppressor in the regulation of tumor genesis [19]. Previous study had shown that FOXO4 plays important roles in cell cycle progression [20, 21], apoptosis [22], and DNA repair [23]. Yang et al confirmed that FOXO4 can induce accumulation of p27 by directly binding to the promoter of p27 and inducing the transcription of p27 [24] and regulating the p27 post-transcription [25]. Pascale F, and his colleagues proved that Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1 [26]. Based on the fact that FOXO4 can regulate the level of p21, p27, Bim and FASL [27, 28], we hypothesized that miR-142-3p degrades the level of FOXO4 by binding to 3'UTR of FOXO4, thus reduces the level of p21, p27, Bim, and FASL, and finally leading to cell growth and apoptosis inhibition.

FOXO4 was regulated by several miRNAs in different cancers. Li et al found that miR-150 promoted cell growth and survival by targeting
FOXO4 in cervical cancer [27]. miR-664 was ascertained to promote osteosarcoma cell proliferation via down-regulating FOXO4 [29]. MiR-1247a accelerated cell proliferation and migration by directly targeting FOXO4 in GC [30]. In this paper, we found that FOXO4 is regulated by miR-142-3p to promote cell proliferation and inhibit cell apoptosis in GC.

In addition to, we detected the expression of miR-142-3p and FOXO4 in gastric carcinoma patients and found the negative correlation between miR-142-3p and FOXO4 in seven patients with GC. Li et al showed that FOXO4 and FOXD3 are predictors of prognosis in gastric carcinoma patients by analyzing the data from The Cancer Genome Atlas (TCGA) [31]. Given the importance of mir-142-3p and FOXO4 in cancer signaling pathway, strategic change on miR-142-3p and FOXO4 should be useful in GC. Our research provides the theoretical foundation for diagnosing and curing the GC in clinic.

Conclusion
MiR-142-3p acts as a tumor promoter by targeting FOXO4 to decrease the expression of p21, p27, Bim and FASL in the progression of GC. miR-142-3p and FOXO4 may be potential therapeutic targets for gastric carcinoma patients.

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Disclosure of conflict of interest
None.

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