Targeting of GSK-3β by miR-214 to facilitate gastric cancer cell proliferation and decrease of cell apoptosis

H.-L. LI1, S. LIANG2, J.-H. CUI3, G.-Y. HAN1

1The 2nd Department of General Surgery, Hongqi Hospital Affiliated to Mudanjiang Medical University, Mudanjiang, Heilongjiang, China
2Department of Radiology, Hongqi Hospital Affiliated to Mudanjiang Medical University, Mudanjiang, Heilongjiang, China
3Department of Gastroenterology, Hongqi Hospital Affiliated to Mudanjiang Medical University, Mudanjiang, Heilongjiang, China

CONCLUSIONS: MiR-214 decreases GSK-3β expression and promotes the pathogenesis of gastric cancer. The inhibition of miR-214 reduces the proliferation of gastric cancer cells via upregulation of GSK-3β and suppression of Wnt/β-catenin signal pathway, which provides fundamental support for the future therapy of gastric cancer.

Key Words: MiR-214, GSK-3β, Wnt/β-catenin pathway, Gastric cancer, Cell proliferation, Apoptosis.

Introduction

Gastric cancer (GC) is a type of malignant tumor commonly found in digestive tract worldwide1. GC relatively presents insidious onset without significant symptoms at early stage, but progresses rapidly at terminal stage. Due to high malignancy, potency of invasion and metastasis, GC severely threatens patient's health and life quality2. β-catenin represents a critical protein in canonical Wnt/β-catenin signal pathway, and plays an important role in the activation of Wnt/β-catenin signal pathway. Of note, up-regulation of β-catenin induces abnormal activation of Wnt/β-catenin signal pathway, and is closely correlated with onset, progression and prognosis of multiple tumors such as breast cancer3, pancreatic carcinoma4, colon cancer5 and endometrial carcinoma6. Besides, β-catenin, glycogen synthase kinase-3β (GSK-3β) belongs to the Wnt/β-catenin signal pathway, in which GSK-3β can phosphorylate and degrade β-catenin protein to suppress the activation of...
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Wnt/β-catenin signal pathway, serving as tumor suppressor in tumor pathogenesis. Previous studies showed that the decrease of GSK-3β was closely correlated with onset, progression and drug resistance of colon cancer, prostate carcinoma, and breast cancer. Also, the reduction of GSK-3β was found to be closely correlated to GC pathogenesis, tumor growth and progression. MicroRNA is a kind of single stranded small molecule non-coding RNA with the length of 22-25 nucleotides, and is widely distributed in multiple tissues and cells. MicroRNA is involved in cell proliferation, differentiation, tissue development and organ formation. The role of microRNA in tumor pathogenesis and progression has become research interests. Multiple studies found that miR-214 expression in GC tissues was significantly elevated, and it was closely correlated with tumor progression, patient’s treatment sensitivity and prognosis. In silico analysis by bioinformatics showed complementary binding sites between miR-214 and GSK-3β. This study aimed to study the role of miR-214 in affecting GC cell proliferation or apoptosis.

Materials and Methods

Major Reagent and Materials

Human GC cell line MKN-28 and normal human gastric mucosal epithelial cell line GES-1 were purchased from Jilin Baili Biotechnology Co., Ltd. (Changchun, Jilin, China). Roswell Park Memorial Institute-1640 (RPMI-1640) medium was from HyClone (South Logan, UT, USA). Fetal bovine serum (FBS) was bought from Gemini Bio-Products (West Sacramento, CA, USA). Penicillin streptomycin was from Gibco (Rockville, MD, USA). Total RNA extraction kit EasyPure RNA Kit and fluorescent quantitative PCR kit TransScript Green One-Step qRT-PCR SuperMix were provided from TransGen Biotech (Beijing, China). Lipofectamine 2000 was used to transform DH5α competent cells. Positive clones with correct sequences were screened out by sequencing and were named as pGRE-GSK-3β-wt and pGRE-GSK-3β-mut. Lipofectamine 2000 was used to co-transfect GRE-GSK-3β-wt and pGRE-GSK-3β-mut. Lipofectamine 2000 was used to transfect DH5α competent cells. Positive clones with correct sequences were screened out by sequencing and were named as pGRE-GSK-3β-wt and pGRE-GSK-3β-mut. Lipofectamine 2000 was used to co-transfect GRE-GSK-3β-wt (or pGRE-GSK-3β-mut) and miR-214 mimic (or miR-NC) into HEK293T cells. After 48 h incubation, dual luciferase activity was measured by LightSwitch™ Luciferase Assay System.

Clinical Information

A total of 28 GC patients (15 males and 13 females) who received treatment in Hongqi Hospital Affiliated to Mudanjiang Medical University from May 2016 to November 2016 were recruited. GC tissues were collected during surgery. Another 22 normal gastric mucosal tissues were collected during gyroscope as control group.

This study was approved by Ethics Committee in Hongqi Hospital Affiliated to Mudanjiang Medical University and all the enrolled objects had signed informed consent.

Cell Culture

MKN-28 and GES-1 cells were all cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 1% streptomycin-penicillin in a 37°C chamber with 5% CO₂.

Dual Luciferase Reporter Gene Assay

Using HEK293T genomic DNA as the template, full-length fragment of wild type or mutant forms of 3'-UTR of GSK-3β gene was amplified and were cloned into pGRE-luc plasmid. Recombinant plasmid was used to transform DH5α competent cells. Positive clones with correct sequences were screened out by sequencing and were named as pGRE-GSK-3β-wt and pGRE-GSK-3β-mut. Lipofectamine 2000 was used to transfect GRE-GSK-3β-wt (or pGRE-GSK-3β-mut) and miR-214 mimic (or miR-NC) into HEK293T cells. After 48 h incubation, dual luciferase activity was measured by LightSwitch™ Luciferase Assay System.

Construction of GSK-3β Over-Expression Plasmid

CDS domain fragment of GSK-3β gene was amplified and determined for the length by gel electrophoresis. After dual enzymatic digestion, the fragment was ligated into pSicoR-GFP plasmid for transforming bacteria. Positive clones were amplified to extract recombinant plasmids...
containing targeted fragments. Sequencing was performed to confirm correct insertion of target fragments. Those plasmids with correct insertion were named as pSicoR-GSK-3β. Empty plasmid pSicoR-blank was used as the control group.

**Cell Transfection and Grouping**

*In vitro* cultured MKN-28 cells were assigned into five transfection groups: miR-NC transfection group, miR-214 inhibitor transfection group, pSicoR-blank transfection group, pSicoR-GSK-3β group, and miR-214 inhibitor + pSicoR-GSK-3β group. At 72 h after transfection, assays were performed.

**qRT-PCR for Gene Expression**

TransScript Green One-Step qRT-PCR SuperMix was used to test gene relative expression level using RNA extracted by EasyPure RNA kit. In a 20 μL reaction system were added 1 μ template RNA, 0.2 μM forward and 0.2 μM reverse primer, 10 μL 2XTransStart Tip Green qPCR SuperMix, 0.4 μL One-step RT Enzyme Mix, 0.4 μL Passive Reference Dye II and RNase-free water. qRT-PCR conditions were: 45°C for 5 min, 95°C 30 s, followed by 40 cycles each containing 94°C 5 s and 60°C for 30 s. ABI 7500 Real-time fluorescent quantitative PCR cycler was used to measure gene expression (Thermo Fisher Scientific, Waltham, MA, USA). Primer sequences were: miR-214 P F: 5'-GGACA GGACG CA CAG TCA-3'; miR-214 P R: 5'-CAGAC GAGGC TCCGT GGT-3'; U6 P F: 5'-ATTGG AACGA TACAG AGAAG ATT-3'; U6 P R: 5'-GGAAG CTTTC ACGAA TTTG-3'; GSK-3β P F: 5'-TG GTC GCCAT CAAGA AAGTA TTG-3'; GSK-3β P R: 5'-GCGTC TGTTT GGCTC GACTA T-3'; β-catenin P F: 5'-CATCT ACACA GTTTG AT GCT GCT-3'; β-catenin P R: 5'-GCAGT TTTGT CAGTT CAGGG A-3'; survivin P F: 5'-AGGAC CACC CGATCA CATCA TAC-3'; β-actin P F: 5'-GAGACC CTAAG GCCA A C-3'; β-actin P R: 5'-TGTCG CGAGA ACAGT TTCC-3'.

**Western Blot**

Proteins were extracted by radioimmunoprecipitation assay (RIPA) lysis buffer, and 50 μg samples were separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After transferring to the polyvinylidene difluoride (PVDF) membrane, the membrane was blocked by phosphate-buffered saline and tween-20 (PBST) containing 5% defatted milk powder for 60 min at room temperature. Primary antibody (GSK-3β at 1:300, β-catenin at 1:200, survivin at 1:200, and β-actin at 1:800) was added for 4°C overnight incubation. Horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000) was added for 60 min incubation at room temperature. After 3 times of PBST rinsing, enhanced chemiluminescence (ECL) reagent was added for development.

**Flow Cytometry for Cell Apoptosis**

Following manual instruction, cells were re-suspended in 100 μL Annexin V Binding Buffer. 5 μL FITC Annexin V were firstly added, followed by the addition of 10 μL propidium iodide (PI). After room temperature incubation for 15 min, 400 μL Annexin V Binding Buffer were added. EPICS XL-MCL flow cytometry was used to measure cell apoptosis (Beckman Coulter, Brea, CA, USA).

**Flow Cytometry for Cell Proliferation**

EdU Flow Cytometry Kit was used to test cell proliferation. Cells were incubated in culture medium containing 10 μM EdU at 37°C for 2 h. Cells were inoculated into 60 mm culture dish and were incubated for 72 h. Cells were then digested and collected, and were fixed in paraformaldehyde. 500 μL test buffer containing phosphate-buffered saline (PBS), catalyst solution, 6-FAM azide and buffer additive were added for 30 min dark incubation at room temperature. After centrifugation and re-suspending in 500 μL wash reagent, EPICS XL-MCL flow cytometry was used to test cell proliferation (Beckman Coulter, Brea, CA, USA).

**Statistical Analysis**

SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Measurement data were presented as mean±standard deviation (SD). Student *t*-test of Mann-Whitney U test was used for comparing measurement data between groups. A statistical significance was defined when *p* < 0.05.

**Results**

**Up-regulation of miR-214 and β-catenin, and Down-Regulation of GSK-3β in GC Tissues**

qRT-PCR results showed that, compared to normal gastric mucosal tissues, the levels of
miR-214 and β-catenin in GC tissues were significantly elevated while GSK-3β mRNA level was statistically reduced (Figure 1A-C). Similarly, Western blot detection also indicated that the expression of GSK-3β protein in GC tissues was decreased than that in normal gastric mucosal tissues, whilst β-catenin protein expression was increased (Figure 1D).

**MiR-214 Expression in GC Cells was Correlated with Lower GSK-3β and Higher Survivin Expression**

Flow cytometry revealed that the proliferation potency of MKN-28 cells was significantly higher than that of GES-1 cells (Figure 2A), whilst the basal apoptotic rate was lower comparatively (Figure 2B). qRT-PCR results showed that, compared to GES-1 cells, the expressions of miR-214, β-catenin, and survivin mRNAs in MKN-28 cells were significantly higher, whilst GSK-3β mRNA expression was remarkably decreased (Figure 2C). Concomitantly, Western blot results unraveled significantly lower GSK-3β protein expression in MKN-28 cells compared to GES-1 cells, along with the increase of β-catenin protein expression (Figure 2D). In silico target gene prediction with microRNA.org showed the existence of binding sites between miR-214 and 3’-UTR of GSK-3β mRNA (Figure 2E). Additionally, dual luciferase reporter assay illustrated that, after the transfection with miR-214 mimic, the relative luciferase enzymatic activity in HEK293 cells was significantly decreased (Figure 2F), demonstrating the targeted regulation between miR-214 and 3’-UTR of GSK-3β mRNA.

**Figure 1.** miR-214 and β-catenin expression, and lowered GSK-3β expression in gastric cancer tissues. **A**, qRT-PCR for miR-214 expression in GC and normal gastric mucosal tissues. **B**, qRT-PCR for β-catenin mRNA expression in GC and normal gastric mucosal tissues. **C**, qRT-PCR for GSK-3β mRNA expression in GC and normal gastric mucosa. **D**, Western blot for GSK-3β and β-catenin protein expression in GC and normal gastric mucosa. *p < 0.05 compared to normal gastric mucosa.
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In order to further validate the mechanism of miR-214 on the proliferation of gastric cancer cells, we evaluated the expressions and apoptosis by the transfection of miR-214 inhibitor and/or pSico-GSK-3β. Our data indicated that, after the transfection, significantly rising expression of GSK-3β, downregulation of β-catenin and survivin expressions in MKN-28 cells were observed in MKN-28 cells (Figure 3A). More-
over, the increase of cell apoptosis and weakened proliferation potency were shown after the treatment of miR-214 inhibitor and/or pSico-GSK-3β (Figure 3B, 3C).

Discussion

Wnt/β-catenin signal pathway is one of the highly conserved pathways during the evolution, and is closely correlated with tissue/embryonic development, body growth, immune response, and tumor formation16. Canonical Wnt/β-catenin signal pathway is featured with cytoplasmic deposition, stable expression and nuclear translocation of β-catenin. In contrast to β-catenin, GSK-3β negatively regulates Wnt/β-catenin signal pathway, and hydrolyzes β-catenin protein via phosphorylation17,18. Survivin is the most potent apoptotic inhibitor in IAPs family, and can retard cell apoptosis via suppressing activity of Caspase-3 and Caspase-719. Survivin also plays an important role in facilitating mitosis20. Several studies showed the involvement of survivin as an important target gene in Wnt/β-catenin signal pathway21,22. GSK-3β negatively various signal pathways including PI3K/AKT, NF-κB, ERK1/2-MAPK besides Wnt/β-catenin pathway, thus affecting various cellular processes such as cell metabolism, proliferation, apoptosis, differentiation and motility23. Down-regulation of GSK-3β or up-regulation of β-catenin facilitates occurrence of various tumors4,5,7,8. MiR-214 plays an essential role in the onset and development of multiple tumors via the cause of alternation in key proteins and signaling pathways24. In this work, we found that, compared to those in normal gastric mucosal tissues, the expressions of miR-214, β-catenin expression and GSK-3β were significantly altered, indicating the possible role of miR-214, β-catenin and GSK-3β in GC pathogenesis. Volinia et al25 showed significantly elevated miR-214 expression in GC patient tumor tissues compared to normal gastric mucosa. Ueda et al26 also found higher miR-214 expression in GC tumor tissues compared to adjacent tissues, and demonstrated close correlation between miR-214 up-regulation and tumor infiltration depth, lymph node metastasis and clinical phase, which can be treated as predictive index for unfavorable prognosis. Wang et al27 found that GC tissues had about 7.13-fold higher miR-214 expression than adjacent tissues, and miR-214 was significantly correlated with infiltration depth, vein invasion, lymph node metastasis and TNM stage. Yang et al28 found that those GC patients with higher miR-214 expression presented worse prognosis, indicating tumor facilitating role of miR-214 in GC pathogenesis. This study also showed abnormally elevated miR-214 expression in GC tissues, suggesting its correlation with GC pathogenesis, as consistent with Volinia et al and Ueda et al. Hirakawa et al29 observed lower GSK-3β expression in GC tissues compared to normal gastric mucosal epithelium. In this study, GSK-3β expression level was abnormally decreased, indicating tumor suppressing role of GSK-3β in GC pathogenesis, as similar with Hirakawa et al29. Yang et al28 found more than 4-fold increase of miR-214 expression in GC cell lines such as SGC-7901, BGC-823. Xin et al30 demonstrated significantly elevated miR-214 expression in highly metastatic GC cell lines GC9811-P and MKN-28M compared to low metastatic GC cell lines GC9811 or MKN-28NM, indicating the correlation between miR-214 up-regulation and tumor metastasis.

Figure 3. miR-214 inhibition increased GSK-3β expression, facilitated MKN-28 cell apoptosis and inhibited their proliferation. A, Western blot for protein expression. B, Flow cytometry for cell apoptosis. C, EdU staining for cell proliferation.
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ulation and malignant biological features of GC cells. Xin et al.\(^10\) showed that over-expression of miR-214 could facilitate proliferation, migration and clonal formation of low metastatic GC cells GC811 via targeted inhibition of tumor suppressor gene PTEN, thus enhancing its malignant biological features. The down-regulation of miR-214 expression in highly metastatic GC9811-P cells remarkably elevated PTEN expression and weakened malignant biological features. Yang et al.\(^28\) showed targeted regulation between miR-214 and PTEN in GC cell lines SGC-7901 and BGC-823. Ko et al.\(^10\) found that knockout of GSK-3β expression remarkably accelerated GC tumor growth, enhanced HIF-1 and VEGF expression for angiogenesis, thus facilitating GC progression. Zhou et al.\(^31\) enhanced HIF-1 and VEGF expression for angiogenesis remarkably accelerated GC tumor growth, and the clinical effect of miR-214 requires further validation with animal model.

**Conclusions**

Our data demonstrated that the down-regulation of miR-124 suppresses the proliferation of gastric cancer cells by inducing GSK-3β expression and inhibiting Wnt/β-catenin signal pathway, which offers academic basis for a future therapy for gastric cancer.

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**Conflict of Interest**

The Authors declare that they have no conflict of interests.

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