RESEARCH ARTICLE

MiR-421 Regulates Apoptosis of BGC-823 Gastric Cancer Cells by Targeting Caspase-3

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Abstract

MicroRNAs might act as oncogenes or tumor suppressors in cancer. Recent studies have shown that miR-421 is up-regulated in human gastric cancer. Here, we found that miR-421 was over-expressed in gastric cancer tissues and cell lines. Bioinformatics analysis predicted that the caspase-3 gene was a target of miR-421. Caspase-3 was negatively regulated by miR-421 at the post-transcriptional level. Bax and Bcl-2 were also regulated by miR-421. Moreover, tumor necrosis factor receptor-I and -II, death receptors in the apoptosis pathway, were up-regulated by miR-421. The over-expression of miR-421 promoted gastric cancer cell growth and inhibited apoptosis of the BGC-823 gastric cancer cell line. These observations indicate that miR-421 acts as a tumor promoter by targeting the caspase-3 gene and preventing apoptosis of gastric cancer cells through inhibition of caspase-3 expression. These findings contribute to our understanding of the functions of miR-421 in gastric cancer.

Keywords: Gastric cancer - MiR-421 - caspase-3 - apoptosis

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Introduction

Gastric cancer is one of the major malignant diseases,which is the second most common and the leading cause of cancer mortality around world, with about 1,000,000 new cases every year (Yang 2006). However, it is estimated that 50% of gastric cancer cases especially in Asia, where China, Japan and Korea have the highest incidence in the world (Ferlay et al., 2010). (Saeki et al., 2013) revealed prostate stem cell antigen (PSCA) gene and Mucin 1 (MUC1) gene were related to human gastric cancer, additionally certain strains of H. pylori assisted by some of its virulence factors also play a critical role in gastric cancer development, which influence cellular proliferation signaling, the expression of cell proliferation regulating genes or the progression of gastric cancer (Kim et al., 2011), but successful therapeutic targets are limited. Thus, the molecular mechanisms of gastric cancer are of great importance and remain to be elucidated.

MicroRNAs (miRNAs) are small regulatory non-coding RNAs that bind to specific target mRNAs, leading to translational repression. MiRNAs act as negative regulators of gene expression, which are involved in the regulation of biological processes including cell growth, differentiation, and apoptosis in both physiological conditions and disease states such as tumors (He et al., 2004; Bartel 2009). Recently, the classical oncogenes and tumor suppressors have been expanded to include miRNAs, as miRNAs have emerged as significant protein regulators via the direct interaction with complementary sites in the 3'-untranslated region (3'UTR) (Lee et al., 2013). Ataxia-telangiectasia mutated (ATM) is a high molecular weight protein serine/threonine kinase, which plays a important role in the maintenance of genomic integrity by activating checkpoints of cell cycles or promoting the repair of DNA double-strand breaks. Additionally, multi-target anti-microRNA antisense oligonucleotide (MTg-AMOs) can specifically inhibit the expression of multiple miRNAs, and effectively antagonize proliferation and migration of gastric cancer cells promoted by oncomirs (Xu et al., 2012).

Some studies have reported cancer-specific miRNAs in numerous types of cancer, including cervical cancer (Peralta-Zaragoza O et al., 2010), lung cancer (Du et al., 2012), melanoma (Segura et al., 2012), colorectal cancer (Menendez et al., 2013), breast cancer (Liu 2009), and gastric cancer. The incresed or decreased miRNAs in cancers may play roles as oncogenes or tumor suppressors, respectively, and it has been demonstrated that miRNA genes are frequently located in cancer-associated genomic regions or fragile sites (Oulas et al., 2009). Furthermore, miRNA expression profiles provided a better classification of functions for poorly differentiated cancers compared with mRNA profiling assays (Peng et al., 2009). All

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these facts indicated that miRNAs play a critical role in cancers. Various miRNAs are differentially expressed in gastric cancer cells. miR-421, miR-21, miR-221/222, and miR-106a show increased expression in gastric cancer, while miR-124a, miR-128b, miR-148, and miR-129 show decreases in expression (Guo et al., 2009; Konishi et al., 2012). miR-421 target genes are down-regulated via miR-421 binding to the 3'UTR of ATM (Hu et al., 2010). Chen et al. reported a significant increase of miR-421 in nasopharyngeal carcinoma cells, which promotes the proliferative ability of nasopharyngeal carcinoma cells and prevents apoptosis (Chen et al., 2013). Therefore, miR-421 might be an oncogene or promoting factor in cancer.

Apoptosis, which is a form of programmed cell death, maintains the balance between mitosis and cell death in multicellular organisms (Thiel et al., 2013). Once the regulation of apoptosis interferes with this balance of factors, the disruption may lead to various cancers. Cysteinyl aspartate-specific proteinase-3 (caspase-3) is an apoptotic protease in the cytoplasm, which is mainly involved in cell growth, differentiation, and apoptosis of eukaryotic cells (Kania et al., 2003). Caspase-3 expression is significantly decreased in gastric cancer cells, and its expression shows a negative correlation with the invasion of gastric cancer cells or lymph node metastasis (Xiao et al., 2013).

If the specific mechanisms of miR-421 in gastric cancer are elucidated, they will provide us with more knowledge to identify novel and promising treatment strategies for the cure and treatment of gastric cancer.

Materials and Methods

Cancer tissues and cells

Frozen human gastric carcinoma and adjacent tissues were obtained from 50 patients at Xiangya Hospital (Central South University, ChangSha, China). Written informed consent was obtained from the patients. The tumor types were confirmed by pathological analysis. Human samples were used in accordance with the policies of the Institutional Review Board and the study was approved by the Ethics Committee of Xiangya Hospital. Human gastric cell lines, HGC-27, MGC-803, SGC-7901, and BGC-823, and normal gastric epithelial cell line GES-1 were purchased from the Cell Bank of the Shanghai Institute for Biological Sciences (Chinese Academy of Sciences, ShangHai, China).

Reagents

An RNA extraction kit, fluorescence quantitative PCR kit (SYBR Premix ExTaq), and reverse transcription kit (PrimeScript RT reagent Kit) were purchased from TianGen Biotechnology (BeiJing, China). An ImagenFect RNAi transfection kit was purchased from Naomi Biotech (WuXi, China) and a Cell Counting kit-8 (CCK-8) was purchased from Japanese Colleagues Chemical Institute (ShangHai, China). An snnexin V-FITC/PI detection kit was purchased from Yi Sen Biotechnology (ShangHai, China). Antibodies against caspase-3, Bax, Bcl-2, tumor necrosis factor receptor (TNFR)-I, TNFR-II, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Abcam (Cambridge, UK). MiR-421 mimics, miR-421 inhibitors, and negative control siRNA were synthesized by Zimmer Pharmaceutical Biotechnology (ShangHai, China).

Cell culture

Cell lines were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, USA), 100 U/mL penicillin, and 100 g/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO_2.

Primer design

The miR-421 sequence was obtained from the Sanger database. Specific stem-loop reverse transcription and PCR primers for miR-421 were synthesized by Zimmer Pharmaceutical Biotechnology as shown in Table 1.

PCR analyses

RT-PCR was performed with the Eppendorf PCR system (Eppendorf, Germany). Total RNA was isolated from gastric cancer cell lines or GES-1 cells using TRIzol reagent and quantified by a photometer. cDNA was synthesized by M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

SYBR Premix ExTaq (TaKaRa) was used for quantitative PCR according to the manufacturer's instructions. PCR cycling conditions were 95 °C for 10 min, 95 °C for 15 s, 60 °C for 1 min. Relative expression differences between normal and pericarcinoma and gastric cancer were calculated using the $2^{-\Delta\Delta Ct}$ method as follows. $\Delta\Delta Ct=\Delta Ct_{reference}-\Delta Ct_{sample}$, where ΔCt is the difference in the cycling threshold between the gene of interest and the "housekeeping" gene U6, ΔCt_{sample} is the Ct value for a given sample of pericarcinoma or gastric cancer normalized to the U6 gene, and $\Delta Ct_{reference}$ is the Ct value corresponding to control samples normalized to U6 expression. Duplicates of each sample were run, and mean Ct values were calculated.

For RT-PCR, 1 μL cDNA, 0.5 μL forward primer and 0.5 μL reverse primer, 2 μL 2.5 mM dNTPs, 0.5 μL DNA polymerase (10 U/μL), 4 μL 5× buffer, and 11.5 μL ddH2O were reacted for 35 cycles at 95 °C for 10 min, 95 °C for 15 s, and 60 °C for 1 min. PCR products were detected by 2.5% agarose gel electrophoresis.

Table 1. Primers for RT-PCR

Genes names	Sequence	Product lenth
miR-421RT	RT 5' CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAG GCGCCCA 3'	
miR-421	R 5' TATGGTTGTTCTGCTCTCTGTGTC 3'	85bp
	F 5' CTCACTCACATCAACAGACATTAATT 3	
U6	R 5' GGAACGCTTCACGAATTTG 3'	70bp
	F 5' ATTGGAACGATACAGAGAAGATT 3'	

Table 2. Example target genes of miR-421 predicted by the Targetscan website

Target genes	Representative transcript	Gene names	Total context+ score
SCASP3	NM_004346	caspase 3, apoptosis-related cysteine peptidase	-0.44
BMF	NM_001003940	Bcl2 modifying factor	-0.21
PRKD1	NM_002742	protein kinase D1	-0.18
PAK2	NM_002577	p21 protein (Cdc42/Rac)-activated kinase 2	> -0.01
TPRG1	NM_198485	tumor protein p63 regulated 1	>-0.01
BCLAF1	NM_001077440	BCL2-associated transcription factor 1	-0.1
CDK6	NM_001145306	cyclin-dependent kinase 6	-0.11

Prediction of miR-421 target genes

Approximately 426 conserved human target genes of miRNA-421 were obtained from a website for miRNA target gene prediction (targetscan, http://www.targetscan.org/vert_61/).

Western blotting analysis of caspase-3

MGC-803, BGC-823, SGC-7901, HGC-27, and GES-1 cells were subjected to a protein extraction kit. Protein concentrations were measured by the Bradford method. Protein samples (50 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred to a polyvinylidene difluoride membrane. The membranes were then incubated with a rabbit anti-mouse caspase antibody, followed by an anti-rabbit horseradish peroxidase-labeled IgG as the secondary antibody.

Cell transfection

After BGC-823 cells were cultured in a 12-well plate for 24 h, the cells were washed with PBS and 500 μL OpitMEM was added to each well of the 12-well plate. Then, 20 pmol miR-421 mimics, miR-421 inhibitors, negative control siRNA, or a blank control were added to 50 μL OpitMEM and combined with 50 μL OpitMEM containing 5 μL IR that was heat shocked for 10 s at 37 °C. The mixed solutions were then added to the cells. After 6 h, the medium was replaced and the cells were cultured for 24 h. The transfected cells were observed by fluorescence microscopy.

Western blotting analysis of transfected cells and densitometry

The transfected BGC-823 cells were lysed with RIPA buffer. Following SDS-PAGE, the separated proteins were transferred to a nitrocellulose membrane. The membranes were incubated with primary rabbit monoclonal antibodies against caspase-3, Bax, Bcl-2, TNFR-I, TNFR-II, or GAPDH as the control at 4 °C overnight. Membranes were then incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG. Labeled proteins were detected by enhanced chemiluminescence and exposure to chemiluminescent film. LabWorks™ Image Acquisition and Analysis Software (UVP) was used to the quantify band intensities.

CCK-8 assay

Cell proliferation was assessed at days 1-6 of culture. Transfected BGC-823 cells were treated with 0.5 mg/ml CCK-8 at 37 °C for 3 h and then shaken for 20 min. The optical density was determined at 450 nm.

Apoptosis assay

Apoptosis of transfected BGC-823 cells was quantitatively determined by flow cytometry using an annexin V-FITC/PI apoptosis detection kit according to manufacturer's instructions. The stained cells were analyzed by a FACSCalibur flow cytometer and CellQuest analysis software.

Statistical analysis

All data were analyzed by SPSS16.0 software and are expressed as the mean±SEM of at least 3 independent experiments. Differences within each group were subjected to t-test or q-test. Statistically significant differences (*$p<0.05$) between the groups being compared are indicated by asterisks.

Results

MiR-421 expression in gastric cancer cells

To investigate the role of miR-421 in gastric cancer cells, the expression levels of miR-421 were first measured in 50 cases of human gastric cancer tissues, pericarcinoma tissues, and normal gastric tissues by quantitative PCR (Figure 1A). And to determine the levels of miR-421 expression in gastric cancer cell lines, miR-421 levels were detected by RT-PCR in four gastric cancer cell lines (MGC-803, BGC-823, SGC-7901, and HGC-27) and a normal gastric cell line (GES-1). Band intensities were scanned by Image J software and U6 was used for the control (Figure 1B). Above results show that miR-421 expression levels were significantly higher in gastric cancer tissues than those in normal gastric and pericarcinoma tissues, and Were also over-expressed in all gastric cancer cell lines.

Caspase-3 protein expression in gastric cancer cell lines

The caspase-3 gene (CASP-3) is a target gene of miR-421, indicating that miR-421 may suppress its expression through translational repression or degradation of transcripts. Caspase-3 protein was decreased in MGC-803, BGC-823, SGC-7901, and HGC-27 cells, suggesting that down-regulation of caspase-3 protein may contribute to the abnormal proliferation of gastric cancer cells (Figure 2).

Target genes of miR-421

A total of 426 conserved target genes of miR-421 were predicted by the targetscan website, of which seven target genes are involved in the cell cycle, apoptosis, and other biological activities (Table 2).

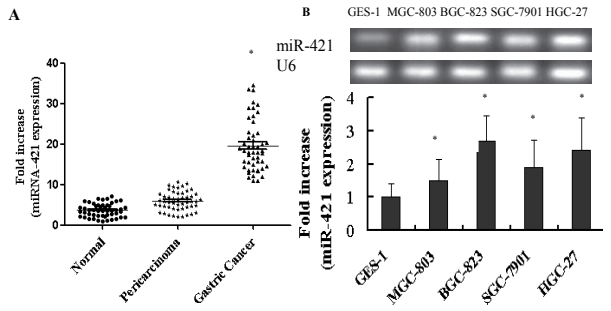

Figure 1. Analysis of the Differential Expression of Mir-421 in Gastric Cancer Tissues or Gastric Cancer Cell Lines. A: The expression levels of miR-421 in 50 cases of gastric cancer tissues, pericarcinoma tissues, and normal gastric tissues were detected by quantitative PCR. **B**: RT-PCR results of miR-421 expressed in four gastric cancer cell lines;U6 was used as the control (mean±SEM, three independent experiments). *p<0.05.

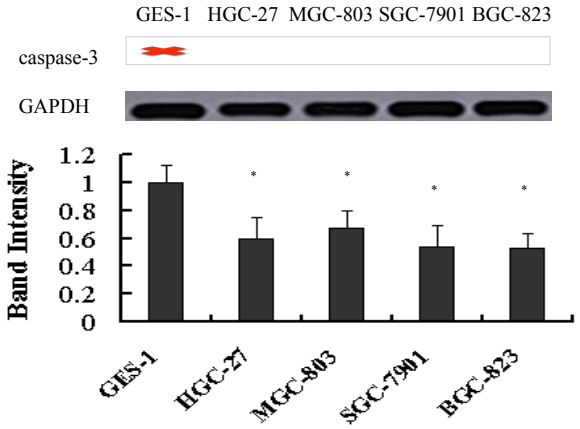

Figure 2. Caspase-3 Protein Expression in Four Gastric Cancer Cell Lines and Normal Gastric Cells. A: Western blotting results. GAPDH was used as the control; **B**: Statistical analysis (mean±SEM, three independent experiments). *p<0.05.

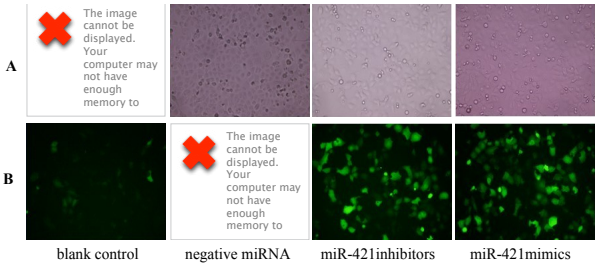

Figure 3. Immunofluorescence Microscopy of BGC-823 Gastric Cancer Cells Transfected with siRNAs (×40). A: Untransfected BGC-823 cells; **B**: BGC-823 cells transfected with siRNAs. Experiments were repeated 3 times.

BGC-823 cells transfected with siRNAs

BGC-823 cells were transfected for 24 h with siRNAs including miR-421 mimics, miR-421 inhibitors, or a negative control siRNA using an ImagenFect RNAi kit. Then transfection BGC-823 cells were observed under a fluorescence microscope to assess the transfection efficiency. Most BGC-823 cells emitted green fluorescence, indicating that the siRNAs were successfully transfected into BGC-823 cells (Figure 3).

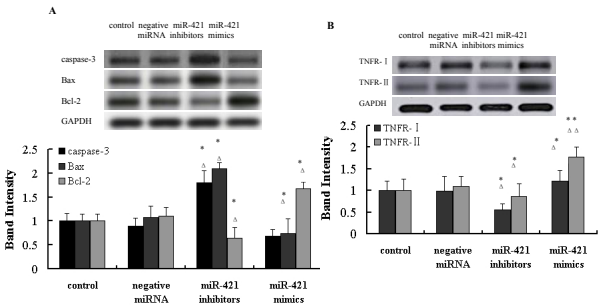

Figure 4. Analysis of Proteins Related to Apoptosis in BGC-823 Cells Transfected with Various siRNAs. A: Western blotting results of caspase-3, Bax, and Bcl-2 proteins. **B**: Analysis of TNFR-I and TNFR-II protein expression by western blotting. GAPDH was used as the control, and Quantification of results was analyzed by SPSS 16.0 (mean±SEM, three independent experiments). *p<0.05 vs control; $^{\Delta}p$<0.05 vs negative control

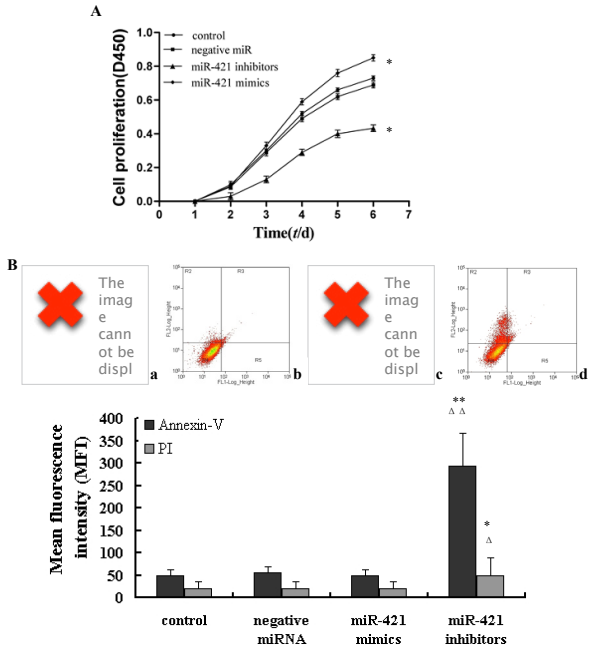

Figure 5. Analysis of the Proliferative Ability or Apoptosis of Bgc-823 Cells Transfected with Mir-421 Inhibitors or Mimics. A: CCK-8 assay results of BGC-823 cell numbers. B: Apoptosis of BGC-823 cells transfected with miRNA-421 mimics or inhibitors as detected by flow cytometry. (mean ± SEM, n = 3). *p<0.05 vs control; **p<0.01 vs negative control; $^{\Delta}p$<0.05 vs control; $^{\Delta\Delta}p$<0.01 vs negative control.

Analysis of apoptosis-related proteins

After BGC-823 cells were transfected with miR-421 mimics, miR-421 inhibitors, or negative control siRNA for 24 h, caspase-3, Bax, and Bcl-2 proteins were analyzed by western blotting and densitometry (Figure 4A). TNFRs are also important proteins for the apoptosis of cancer cells, so the expression of cell surface receptor proteins TNFR-I and TNFR-II was analyzed in transfected BGC-823 cells by western blotting (Figure 4B). Results indicated that Caspase-3 and Bax protein expression

was increased significantly, while Bcl-2, TNFR-I and TNFR-II expression was decreased in BGC-823 cells transfected with miR-421 inhibitors. The expression levels of caspase-3, Bax and Bcl-2, TNFR-I and TNFR-II showed an opposing trend in BGC-823 cells transfected with miR-421 mimics.

MiR-421 promotes the growth of BGC-823 cells in vitro

The proliferative ability of transfected BGC-823 cells was analyzed by a CCK-8 kit (Figure 5A). Additionally, we analyzed the asymmetry and permeability of the cell membrane by annexin V-FITC and PI staining with flow cytometry(Figure 5B). All data stated that the number of BGC-823 cells transfected with miR-421 inhibitors was significantly lower than that of cells transfected with miR-421 mimics or negative control siRNA, and BGC-823 cells transfected with miR-421 mimics showed apoptosis or necrosis. These results indicated that miR-421 may be a tumor promoter in BGC-823 gastric cancer cells.

Discussion

Gastric cancer is one of the most highly lethal malignancies in the world, which is usually detected at a late stage. Consequently, the five-year survival rate is low, ranging from 5 to 15% (Yu et al., 2009; Hsu et al., 2012). In 2008, the incidence rate of gastric cancer (0.738 million mortalities and 0.98 million new cases) ranked it as the second leading cause of cancer all over the world (Jemal et al., 2011). The incidence rate of gastric cancer is comparatively high in Eastern Asia and the majority of gastric cancer cases occur in developing countries with a wide variation of gastric cancer incidences in different regions (Ali et al., 2012). To a certain extent, the low survival rates of gastric cancer are due to the poor understanding of the mechanism of the cancer, so it is important to identify the mechanism that participates in the tumorigenesis of gastric cancer. MiRNA-421 inhibits the protein expression of target genes through its 5' end binding the 3'-UTR region of target genes. Additionally, one miRNA may have various target genes (Brennecke et al., 2005). Previous studies reported that overexpression of miR-106b shortened the G0/G1 phase and accelerated cell cycle progression, while reducing p21 and E2F5, without any significant effects on the capacity for migration and invasion of gastric cancer cells, suggesting miR-106b may promote cell cycling of gastric cancer cells through regulation of p21 and E2F5 target gene expression (Yao et al., 2013). There were 426 conserved genes predicted as targets of miRNA-421, such as CASP-3, Bcl-2 modifying factor (BMF), protein kinase D1 (PRKD1), p21 protein (Cdc42/Rac)-activated kinase-2 (PAK2), tumor protein p63 regulated-1 (TPRG1), BCL2-associated transcription factor-1 (BCLAF1), and cyclin-dependent kinase-6 (CDK6). These genes are mainly involved in the cell cycle and apoptosis.

Caspase-3 protein plays an important role in Fas/FasL signaling that induces apoptosis of tumor cells through a series of kinase activations or substrate cleavages (Krajewska et al., 1997). Compared with gastric carcinoma and primary cancer cells, caspase-3 is over-expressed in invasive gastric cancer, which is closely related to the degree of malignancy and differentiation (Hoshi et al., 1998). MiRNA mimics are miRNAs synthesized by a chemical method to simulate endogenous miRNAs, which enhance the function of endogenous miRNAs, whereas miRNA inhibitors are modified by a chemical method to inhibit target miRNAs (Tang et al., 2013). It is assumed that up-regulated miRNAs in cancers may function as promoters and vice versa (Liu et al., 2011). Considering that the increase of caspase-3 expression in gastric cancer cells was related to over-expression of miR-421, we speculated that miR-421 may be an inhibitor of caspase-3 expression and promote the growth of gastric cancer cells. We found that the expression level of miR-421 was significantly higher in gastric cancer tissues compared with that in adjacent tissues and normal gastric tissues. Furthermore, miR-421 expression was increased markedly in gastric cancer cell lines, while caspase-3 protein expression was decreased in gastric cancer cell lines. However, caspase-3 protein expression was enhanced in BGC-823 cells transfected with miR-421 inhibitors, whereas miR-421 mimics had the opposite effect.

Bax and Bcl-2 play essential roles in the induction of apoptosis. It is well known that most apoptosis-inducing factors eventually cause apoptosis through the caspase-mediated signal transduction pathway (Samali et al., 1999; Hu et al., 2009; Wei et al., 2011) and caspase-3 is the main effector of apoptosis. Therefore, the expression of Bax and Bcl-2 was determined in BGC-823 cells transfected with miR-421 inhibitors or mimics. Bcl-2 expression was decreased significantly in BGC-823 cells transfected with miR-421 inhibitors, while there was an increase of Bax expression. In addition, activation of TNF-α/TNFR-I signaling in the tumor microenvironment promotes gastric cancer development and contributes to maintaining the cancer cells in an undifferentiated state (Oshima et al., 2013). The positivity rates of TNFR-I and TNFR-II in human gastric cancer tissues were significantly higher than those in the adjacent mucosal and normal mucosal tissues, and their expression was significantly higher in the surrounding mucosa than in the normal tissues. This result indicated that the expression of TNFR-I and TNFR-II is a valuable indicator of malignancy to predict the differentiation grade of gastric cancer (Kong et al., 2012). Our results indicated that miRNA-421 also enhanced the expression of TNFR-I and TNFR-II in BCG-823 cells and influenced the apoptotic signaling to induce apoptosis of cancer cells.

Finally, analyses of cell proliferation and apoptosis showed that miRNA-421 promoted the proliferation of BGC-823 cells and inhibited their apoptosis. These findings indicate that up-regulated miR-421 in gastric cancer contributes to gastric cancer cell proliferation, and that miR-421 may be a therapeutic target for gastric cancer. Therefore, our study provides further elucidation of the molecular mechanism of gastric cancer initiation and progression.

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