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Original Article

Level of circulated microRNA-421 in gastric carcinoma and related mechanisms

Guodong Zhao[1], Liang Xu[1], Limei Hui[2], Jianjun Zhao[3]

Departments of [1]General Surgery, [2]Obstetrics, [3]Urology, Affiliated Hospital of Hebei University of Engineering, Handan 056002, Hebei, China

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Abstract: As one of the most popular and deadly malignant tumors, gastric cancer still has difficulty in early-diagnosis. Recently the level of circulated DNA related with tumors can be used for diagnosis. MicroRNA-421 (miR-421) has been found to be up-regulated in tumor cells. Whether peripheral miR-421 can be used as a marker for diagnosis of gastric carcinoma, however, remains unclear. The expression level of miR-421 in both gastric cancer and normal people were firstly quantified. We then performed *in vitro* transfection of gastric carcinoma cell line to potentiate or silence miR-421 level. Cell apoptosis and apoptotic protein levels were quantified by flow cytometry and Western blotting, respectively. MiR-421 level in the peripheral blood of gastric cancer patients was significantly elevated. In gastric cancer cell line, the up-regulation of miR-421 significantly inhibited cell apoptosis. The silencing of miR-421 promoted cell apoptosis. Such anti-apoptotic role of miR-421 was accomplished by inhibiting caspase 3, up-regulating Bcl-2 and inhibiting Bax. MiR-421 was up-regulated in both tumor tissue and peripheral blood, and can modulate cell apoptosis. Circulated miR-421 can work as a serological marker for early diagnosis of gastric cancer.

Keywords: Gastric carcinoma, circulated microRNA-421, cell apoptosis, caspase 3, Bax and Bcl-2

Introduction

Gastric cancer is still one of the most popular and deadly malignant tumors in China, with its 5-year survival rate less than 10%. Early manifestations of gastric cancer are mostly insidious, and can be presented as vomiting, nausea or ulcer-like symptoms [1]. It is thus of difficulty to accomplish early diagnosis of gastric cancer. MicroRNA (RNA) is a type of non-coding RNA molecule for modulating protein synthesis and post-transcriptional level. Various studies have screened candidate miRNAs for gastric cancer using DNA array approach in tumor tissues, and have obtained a series of tumor-related miRNAs, some of which had unique values for diagnosis [2]. Recent studies also proved the tumor specificity of miRNAs in peripheral blood, and can exist in stable form [3, 4]. Therefore it can work as a tumor specific marker with potential values for diagnosis [5]. MiR-421 has been found to be significantly up-regulated in tumor cells, and have abnormal expressions in liver cancer and liver cirrhosis patients. Therefore it is possible that miR-421 may work a marker benefiting the diagnosis of gastric cancer.

In this study, we firstly tested the serum expression pattern of miR-421 in both normal and gastric cancer patients. *In vitro* experiment was then performed using gastric cancer cell line, on which miR-421 expression was either potentiated or depressed by the means of transfection. The correlation between miR-421 expression and cell apoptosis was also examined, along with possible mechanism. This study may illustrate the value of miR-421 as a marker for gastric cancer, and can also provide evidences for clinical diagnosis.

Materials and methods

Plasma DNA extraction

50 cases of gastric cancer patients were recruited in this study, along with 50 healthy individuals as the control group from Affiliated Hospital of Hebei University of Engineering. All controlled people had no history of liver dis-

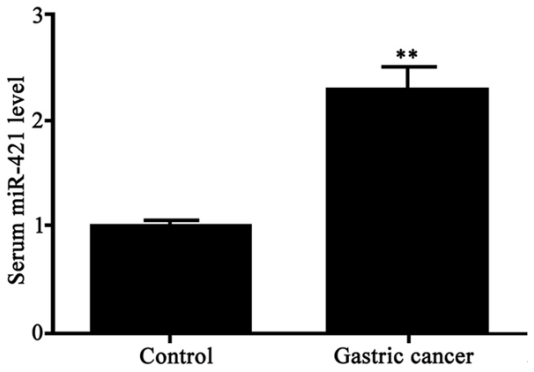

Figure 1. Serum miR-421 level. **P<0.05 compared to control people.

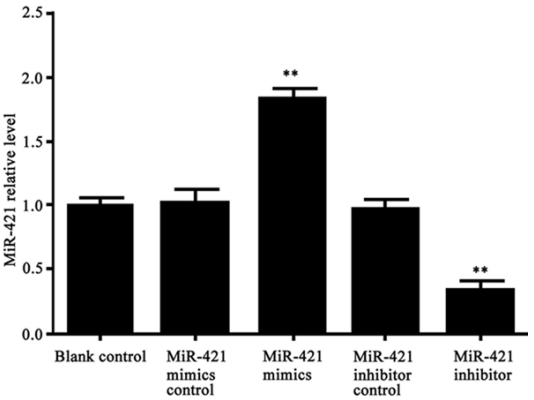

Figure 2. MiR-421 expression levels after transfection. **P<0.05 compared to blank control (healthy people).

ease, diabetes, heart disease, or hypertension. No anti-tumor medication or other therapy has been received in any of those cancer patients. Blood samples were collected and centrifuged to separate serum. DNA was extracted using tissue DNA extraction kit (Qiagen, US) following manual instruction.

The experimental protocol has been pre-approved by the ethical committee of Affiliated Hospital of Hebei University of Engineering and written consents have been obtained from all patients and healthy volunteers.

Cell culture and transfection

Gastric carcinoma cell line BCG-823 (Cell Bank of Chinese Academy of Science, Shanghai, China) was incubated using DMEM medium (Hyclone, US) containing 10% fetal bovine serum (FBS, Hyclone, US) in a humidified chamber with 5% CO_2 at 37°C. All cells were digested by trypsin and seeded into 6-well plate before transfection until reaching a confluence of 60%~70%.

MiR-421 mimics and inhibitor, along with negative controlled scramble RNA sequences were commercially purchased (Gimma, China). Before transfection, miR-421 mimics or inhibitor was diluted in Opti-MEM (Invitrogen, US), and was mixed with equal volume of Opti-MEM containing Lipofectamine 2000 (Invitrogen, US). After 20-min incubation, the transfection mixture was added into 6-well plate containing cells at confluence 60%~70%. After 4-hour incubation, culture medium was changed, followed by real-time quantitative PCR for miR-421 expression level.

Cell flow cytometry

24 hour after transfection, cells were digested by trypsin and washed three times using serum-free DMEM. AV and PI were added to stain cell for 5 min, followed by flow cytometry assay.

Total RNA extraction

Cells were lysed in Trizol solution (Invitrogen, US) in 5-min incubation. Chloroform (0.2 mL) was then mixed with tissue lysate. After 15-min incubation and 10000 g centrifugation (15 min), supernatants were saved and mixed with 0.5 mL isopropanol to precipitate RNA. RNA pellet was collected by 10-min incubation followed by 10000 g centrifugation (15 min), and was washed in 75% ethanol. RNA was re-suspended in 50 μL DEPC-treated water.

Real-time quantitative PCR

Real-time PCR was performed [6] using SYBR Green PCR Master Mix (Invitrogen, US) in a 20 μL reaction system containing specific primers (Forward: 5'-CTCAA CTGGT GTCGG AGTCG GCAAT TCAGT TGAGG CGCCC A-3'; Reverse: 5'-CTCAC TCACA TCAAC AGACA TTAAT T-3', as previously reported [7]; U6 internal reference, forward: 5'-GGAAC GCTTC ACGAA TTTG-3'; reverse: 5'-ATTGG AACGA TACAG AGAAG ATT-3') and cDNA template. The reaction conditions were: 95°C 10 min, followed by 40 cycles each containing 95°C denature (15 sec), 60°C annealing (1 min) and 72°C elongation (1 min).

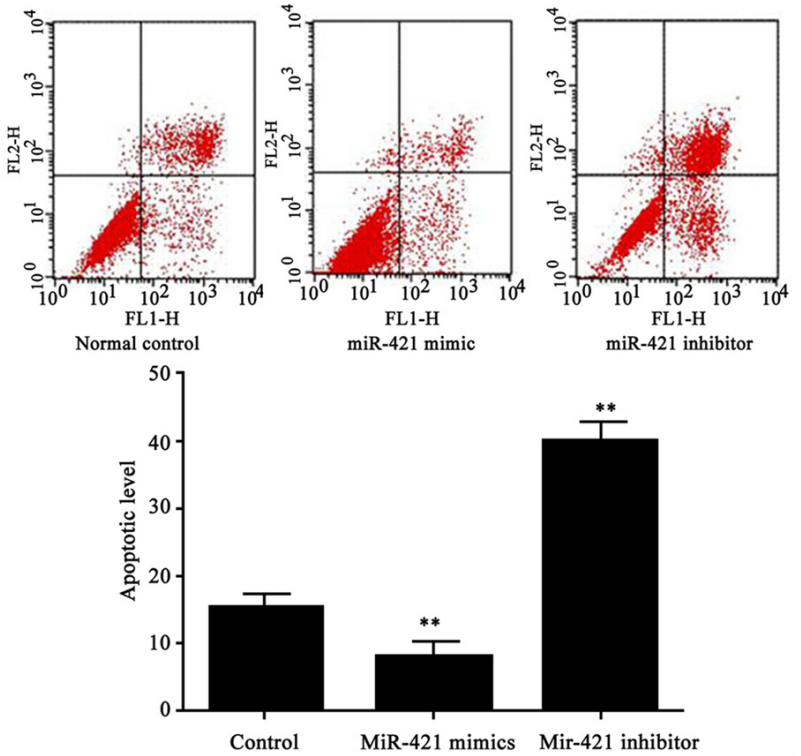

Figure 3. Cell apoptosis after transfection. **P<0.05 compared to control group.

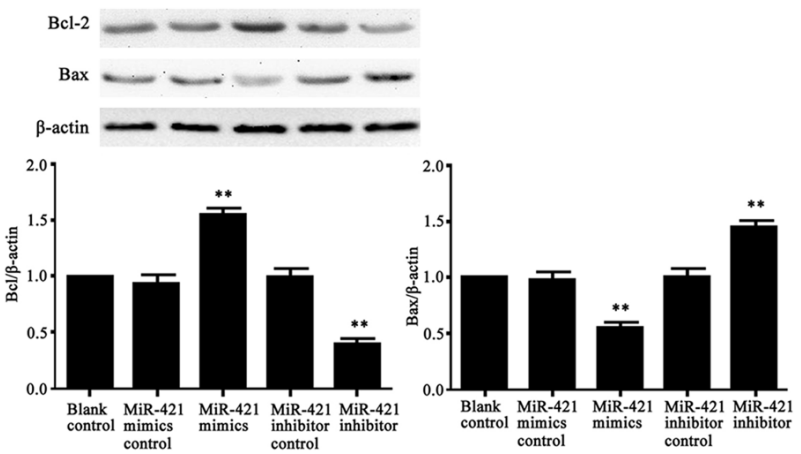

Figure 4. Bcl-2 and Bax expression in transfected cells. **P<0.05 compared to blank control group.

The relative expression level was determined by $2^{-\Delta\Delta Ct}$ method.

Western blotting

Transfected cells were rinsed in PBS three times, followed by RIPA lysis buffer (Sigma, US) for 5 min incubation. Protein was then extracted by 10000 g centrifugation for 5 min. After quantification by BCA method, proteins were loaded onto 8% SDS-PAGE for separation and were transferred to PVDF membrane. The membrane was the blocked in 5% defatted milk powder, and was incubated with primary antibody against Bcl-2, Bax or activated caspase-3 (1:1000,Proteintech, China) for overnight incubation. After washing in TTBS for three times, secondary antibody (1:1000, Invitrogen, US) was applied for 1-hour incubation. The membrane was developed by ECL method.

Statistical analysis

SPSS 18.0 software was used to process all collected data, which were presented as mean ± standard deviation (SD). Student t-test was used to compare means between two groups. Analysis of variance (ANOVA) was then in multi-group comparison. A statistical significance was defined when P<0.05.

Results

Plasma circulated miR-421 level

We tested serum free miR-421 levels in both normal and gastric cancer individuals. Results found significantly elevated miR-421 level in gastric cancer patients (P<0.05, **Figure 1**).

MiR-421 in transfected gastric carcinoma cells

To investigate the role of miR-421 in gastric cancer, we transfected cell line using miR-421 mimics or inhibitor, and found significantly ele-

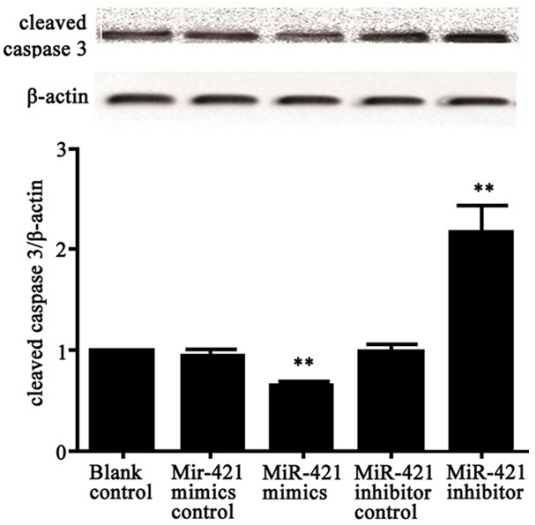

Figure 5. Active caspase-3 levels after transfection. **P<0.05 compared to blank control group.

vated miR-421 level in mimics transfected group, and depressed miR-421 level in inhibitor transfected group (P<0.05 compared to control group, **Figure 2**).

Cell apoptosis after transfection

To investigate the effect of miR-421 on cell apoptosis, we utilized serum-free induced apoptosis model. Results showed that cells transfected with miR-421 mimics had significantly depressed apoptosis level, while those cells with miR-421 inhibitor expression had elevated apoptosis (P<0.05, **Figure 3**).

Bcl-2 and Bax expression after miR-421 transfection

As two representative anti- and pro-apoptotic proteins, Bcl-2 and Bax expression levels were determined in transfected cells by Western blotting. After transfecting miR-421 mimics, Bcl-2 level was significantly increased while Bax level was decreased (P<0.05, **Figure 4**). The transfection of miR-421 inhibitor, however, depressed Bcl-2 level and increased Bax expression (P<0.05, **Figure 4**). These results collectively suggested the correlation between miR-421 and apoptotic proteins.

Active caspase-3 level

Caspase-3 is an apoptosis related protein and can be activated at the time of apoptosis [8], to study the participation of caspase-3 in miR-421 induced apoptosis, we detected the level of active caspase-3 and found significant depression after miR-421 mimics transfection (P<0.05, **Figure 5**), and elevation of caspase-3 level with miR-421 inhibitor (P<0.05).

Discussion

As the most common malignant tumor in clinics [9], gastric cancer has been suggested to be related with abnormal expression of various miRNAs, which may regulated tumor cell's proliferation and/or apoptosis. This results suggested us that miRNA might affect the expression of oncogene or tumor suppressor gene at post-transcriptional level, thus modulating proliferation and apoptosis of tumor cells.

Previous studies have revealed the significant difference of expression pattern of miR-421 between tumor tissues, adjacent tissues and healthy gastric tissues [10, 11]. MiR-421 has been found to potentiate the proliferative ability of nasopharyngeal carcinoma cells and retard its apoptosis [12]. Meanwhile, up-regulation of miR-421 has also been found in serum of liver cancer or cirrhosis patients [13]. MiR-421 can also modulate mitochondrial breakage and cardiac infarction by targeting Pink1 gene [14]. As certain miRNAs had tumor specificity and tissue compatibility regarding its peripheral expression, the potency of miR-421 as a novel biomarker for gastric cancer is worth further study. In this study, we thus investigated expressional profiles of miR-421 in both gastric cancer and healthy individuals, and found elevated miR-421 level in cancer patients.

Synthetic mimics and inhibitors of miR-421 [15, 16] can target miRNA for up- or down-regulating miRNA level, and are useful tools in miRNA functional assays. To investigate the correlation between miR-421 expression and cell apoptosis, we transfected gastric cancer cell line BCG-823 with miR-421 mimics or inhibitors, and used flow cytometry to quantify apoptotic level of transfected cells. Results showed significantly depressed apoptosis by miR-421 up-regulation and vice versa, suggesting the potency of miR-421 as one novel target for induced tumor cell apoptosis.

Mature miRNA can directly affect the expression of downstream genes by complete or partial base-pairing against 3'-untranslated region

of target mRNA [17]. In various miRNAs participating post-transcriptional modulation, some miRNA molecules have been found to be closely related with tumor pathogenesis [12]. Via literature review, possible functional targets of miR-421 have been identified including FOXO4 [12], angiotensin convertase 2 [18], farnesol X receptor [19] and caspase-3 [20]. In this study, classical apoptotic protein, caspase-3, anti-apoptotic protein, Bcl-2 and anti-apoptotic protein Bax were quantified. Results established the inhibitory role of miR-421 on cell apoptosis by depressing caspase-3 and Bax, and upregulating Bcl-2.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jianjun Zhao, Department of Urology, Affiliated Hospital of Hebei University of Engineering, 81 Congtai Road, Congtai District, Handan 056002, Hebei, China. Tel: +86-310-3130800; Fax: +86-310-3130800; E-mail: zhfjianjun@sina.com

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