Original Article
Expression of miR-551b and its effect on apoptosis in human gastric carcinoma

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Abstract: miR-551b has been reported to be involved in tumorigenesis, cell invasion, and metastasis in gastric cancer but the mechanism remains unclear. In this study, in an attempt to address this question, we examined expression of miR-551b in gastric tumors and adjacent normal tissue. We transfected SGC-7901 gastric cancer cells with miRNA non-sense sequences (NC group), miR-551b mimics (miR-551b mimic group), and miR-551b inhibitors (miR-551b inhibitor group) to investigate the role of miR-551b in autophagic apoptosis. In gastric tissue, our real-time PCR results revealed that expression of miR-551b was significantly downregulated and the relative expression of miR-551b (1.75 ± 0.13) was significantly lower than in normal tissue (2.47 ± 0.38) (*P*<0.05). In transfected SGC-7901 cells, compared with the NC and miR-551b inhibitor group, miR-551b expression level and apoptosis rate in miR-551b mimics group was significantly increased whereas proliferation and invasion rates were significantly decreased (*P*<0.05). Hoechst 33342 fluorescence staining showed that a large number of autophagosomes were detected in miR-551b mimic group, fewer in the NC group, and only a small number in miR-551b inhibitor group. Western blotting showed that expression levels of NF-κB, LC3 II, and Beclin 1 in miR-551b mimics group were significantly higher than in the NC group and miR-551b inhibitor group (*P*<0.05). Our findings suggest that miR-551b could inhibit proliferation, apoptosis, and invasion of gastric cancer cells and the action mechanism may be related to induction of gastric cancer cells autophagic apoptosis.

Keywords: miRNA, miR-551b, autophagic apoptosis, gastric cancer

Introduction

Gastric cancer is a type of digestive tract malignancy with a high mobility rate, as about 934 thousand patients are newly diagnosed worldwide and incidence of the disease increases every year [1, 2]. Malignant biological activities such as tumor proliferation, invasion, and metastasis are the primary causes of treatment failure and high mortality rate [3].

Autophagy [4] is a natural, regulated, destructive mechanism of the cell that plays an important role in disassembling unnecessary or dysfunctional components, structural reconstruction, cell growth and development, and maintaining balance of protein metabolism and stability of intracellular environment, etc. miRNAs are small non-coding RNA molecules containing 19-24 nucleotides, a type of newly discovered regulatory factor of oncogene or anti-oncogene. miRNA expression imbalance is involved in tumorigenesis of many cancer types. Previous studies have shown that miRNAs could influence the pathological process of tumors by regulating autophagy related protein expression [5].

In gastric tumors, miR-551 levels are more significantly downregulated than in normal tissue and decreased miR-551 expression has been found to promote cell invasion and metastasis in gastric carcinoma cell lines, indicating that miR-551 might play a role in regulating oncogene expression [6]. However, the mechanism of regulation is still unclear. Here, we investigated the role of miR-551b in autophagic apoptosis using human gastric carcinoma SGC-7901 cell line and provide the basis for finding new gastric cancer treatment targets.

Materials and methods

Sample collection

A total of 48 tumor tissues were collected between July 2014 and September 2015 from Affiliated Hospital of Hebei University. All of the patients were confirmed to have gastric cancer via pathological examination. Parts of the gastric cancer tissues were kept, for research purposes, during pathological assessments. As a control, adjacent normal tissues 10 cm apart from the edge of the tumors were also kept. Samples were rinsed several times with saline and stored in liquid nitrogen. This research was approved by our hospital's Ethics Committee and all patients provided written informed consent.

Cell lines and reagents

Human gastric carcinoma SGC-7901 cell lines were bought from Shanghai Institutes for Biological Sciences. MTT, DMSO, and fetal bovine serum were all purchased from Sigma. RPMI-1640 medium and trypsin (0.25%) were obtained from Hyclone and cell lysis buffer and BAC protein quantification kit were received from TianGen Biotech (Beijing). Antibodies of NF-κB, LC3 II, Beclin 1, and anti-human β-actin rabbit antibody were acquired from Boster Biological Technology. miR-551b mimics, muR-551b inhibitors, miR-551b anti-sense strand, Lipofectamine 2000 transfection reagent, and Annexin VFITC/PI cell apoptosis staining kit were obtained from Invitrogen. TRIzol extraction kit and real-time PCR reagent mix were from Takara Bio (Dalian). PCR primers were synthesized by Sangon Biotech (Shanghai). Hoechst 33342 staining kit was attained from Kindu Biotech and the Transwell chamber was delivered by Costar.

Experimental procedures

miR-551b expression test: Stored gastric cancer tissue and normal tissue were taken out of liquid nitrogen and ground thoroughly. TRIzol (1 mL/100 mg) and 150 μL of phenol chloroform were then added and mixed followed by centrifugation at 15,000 rpm for 10 minutes. The upper layer was transferred into a new tube and mixed with 200 μL of isopropanol, samples were centrifuged again for 10 minutes at 15,000 rpm, then the upper layer was discarded and precipitate was washed using 75% ethanol. For each sample, 5 μg of total RNA was used to measure miR-551b expression level via real-time PCR. Sequences of forward and reverse primer were 5'-CTGAGCGACCCATACTTGG-3' and 5'GTGCAGGGTCCGAGGT-3', respectively, and the reaction mixture contained 7 μL of 10× PCR buffer, 0.5 μL of Taq polymerase, 5 μL of dNTP, 5 μL of template, and 2 μL of primers. Deionized water was used to fill reaction mix to the total volume of 50 μL. PCR program was set as 94°C for 3 minutes, 30 cycles of 94°C 30 seconds, 62°C 40 seconds, 72°C 1 minute, followed by a 5 minute extension period at 74°C. U6 was used as internal reference and the basis for calculating relative expression of miR-551b.

SGC-7901 cell transfection: SGC-7901 cells were thawed and placed in RPMI-1640 medium with 10% fetal bovine serum at 37°C and 5% CO_2. Cells at the exponential growth stage were collected and washed 3 times with serum-free medium and resuspended in medium without antibiotics. Cell density was adjusted to 1×10^6 cells per well and incubated in 6 well plates. For the negative control group (NC), cells were treated with medium containing 5 μL of miRNA anti-sense strand and 2 μL of Lipofectamine 2000. In miR-551b mimics groups (Mimic), 5 μL of miR-551b mimics (concentration: 20 nmol/L) and 2 μL of Lipofectamine 2000 were added to 200 μL of medium and the mixture was used to treat cells in 6 well plates. Lastly, for the miR-551b inhibitors group (Inhibitor), 5 μL of miR-551b inhibitors (20 nmol/L) and 2 μL of Lipofectamine 2000 were used. After transfection, all samples were kept in the incubator for another 5-6 hours at 37°C. The medium was then swapped with the ones containing fetal bovine serum and antibiotics and incubated for an additional 24 hours.

MTT cell proliferation assay: Transfected cells from all three groups were inoculated into 96 well plates with concentration of 1×10^7 cells/L followed by addition of 10 μL of 5 g/L MTT solution to each well at 12, 24, 36, 48, 60, and 72 hours and left to incubate for 4 hrs before discarding the medium. After transferring 150 μL DMSO to each well, a microplate reader was used for all samples to measure absorbance at 570 nm.

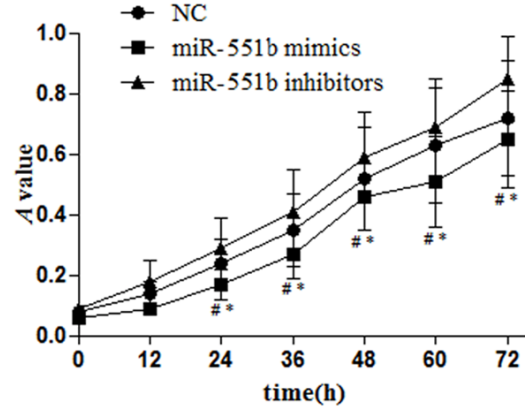*Transwell migration and invasion assay:* Serum-free medium was used to adjust cell
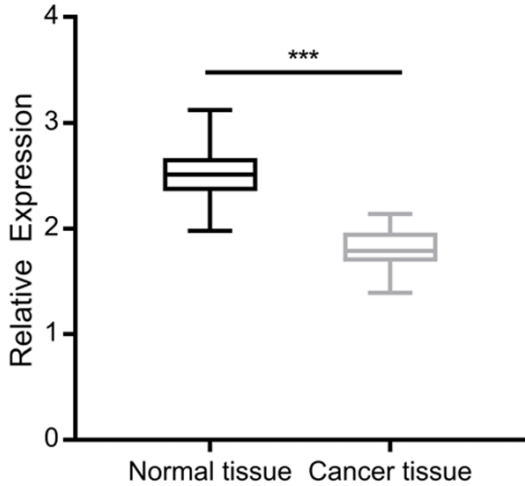
Figure 1. Expression levels of miR-551b in normal and gastric cancer tissues. ***: $P<0.001$.
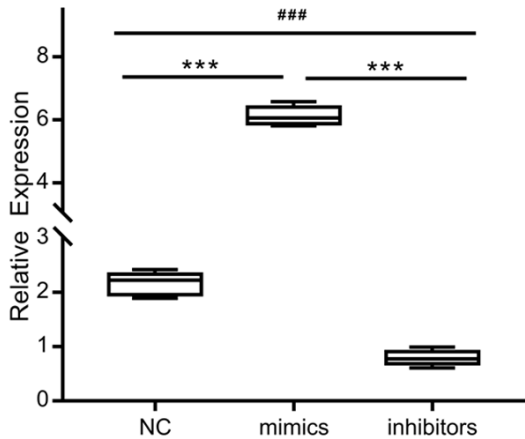
Figure 2. Expression levels of miR-551b in transfected cells of miR-551b mimics, miR-551b inhibitors group, and normal control group. ***: $P<0.001$. ###: $P<0.001$.

Figure 3. Proliferation ability changes in each cell group. Compared with miR-551b inhibitors group, #$P<0.05$, compared with NC group, *$P<0.05$.

concentration to 1×10^9 cells/L and 150 μL was placed into every well in upper Transwell chamber whereas 600 μL of RPMI-1640 medium with 20% fetal bovine serum was used to fill up the bottom part. Every sample group had 6 repeating wells set up. After incubation for 48 hours, 4% paraformaldehyde was used to fix the membrane. The inserts were then stained with crystal violet solution for 10 minutes followed by 3 repeated rinses using PBS. Subsequently, membranes were examined under light microscope where migrated cells were counted and cell invasion rate was calculated.

*Apoptosis analysis using flow cytometry:* Cells were collected 24 hours after transfection and centrifuged at 5,000 rpm for 5 minutes. They were then fixed in 75% ethanol at 4°C for 24 hours. The following day, centrifugation was carried out at 1,500 rpm for 5 minutes, ethanol was discarded, and precipitate was washed with PBS solution. Cells were prepared according to the protocol of Annexin VFITC/PI cell apoptosis staining kit. Flow cytometry analysis was performed and apoptosis rate was calculated.

Hoechst 33342 staining: Cells under exponential growth were inoculated into 6 well plate and incubated for 6 hours. They were then fixed using fix solution at 4°C overnight. Hoechst staining solution was later added and left for 10 minutes, then photos were taken under fluorescence microscope.

Western blotting: Protein expression of NF-κB, LC3 II, and Beclin 1 was analyzed using Western blot. Cells were lysed using 100 μL of cell lysis buffer containing 1% PMSF followed by 20 minutes of centrifugation at 4°C (15,000 rpm). Upper layer was then transferred to the new tube and boiled to 100°C for 5 minutes. SDS-PAGE was used to separate proteins and they were transferred onto PVDF membrane. Current was set at 100 mA and lasted 40 minutes. Skim milk (5%) was applied to the membrane to block for 2 hours. Primary antibodies of NF-κB, LC3 II, Beclin 1, and β-actin were added in a ratio of 1:1000, 1:1000, 1:500, and 1:50, respectively, and then incubated overnight at 4°C. The following day, the membrane was washed 3 times with PBS and secondary antibody was added in 1:1000 ratio. The membrane was observed using chemiluminescent HRP substrate. Gray

NC   miR-551b mimics   miR-551b inhibitors
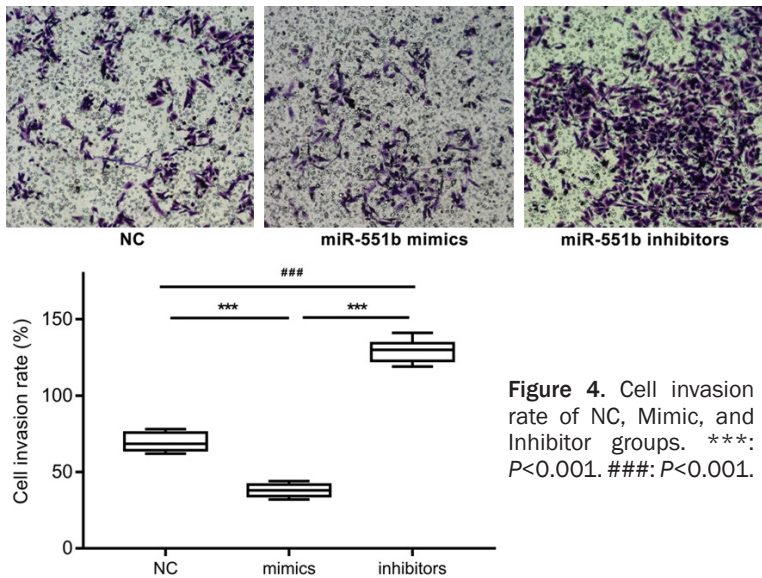
Figure 4. Cell invasion rate of NC, Mimic, and Inhibitor groups. ***: P<0.001. ###: P<0.001.

Alteration of proliferation ability in different cell groups

Proliferation ability of cells in Mimic group was significantly suppressed in contrast to the NC and Inhibitor groups, between 24-72 hours ($P<0.05$), see **Figure 3**.

Cell invasion ability alteration in different groups

Cell invasion rates of NC, Mimic, and Inhibitor group were (52.01 ± 2.01)%, (45.37 ± 1.72)%, and (94.47 ± 3.07)%, respectively. Cells in the Mimic group had significantly lower invasion rate than that of NC and Inhibitor group ($P<0.05$) whereas invasion rate was greatly increased in Inhibitor group ($P<0.05$), see **Figure 4**.

value of 3 target protein bands was measured and compared with that of β-actin, then finally relative protein expression was calculated.

Cell apoptosis rate of each group

Cell apoptosis rate of each group differed greatly. NC group had a rate of (44.05 ± 16.21)%, (72.31 ± 22.79)% for Mimic group, and (35.30 ± 11.27)% for the Inhibitor group. Compared with the NC and Inhibitor group, the apoptosis rate was dramatically elevated with increased expression of miR-551b ($P<0.05$) whereas the rate was much lower in Inhibitor group ($P<0.05$), see **Figure 5**.
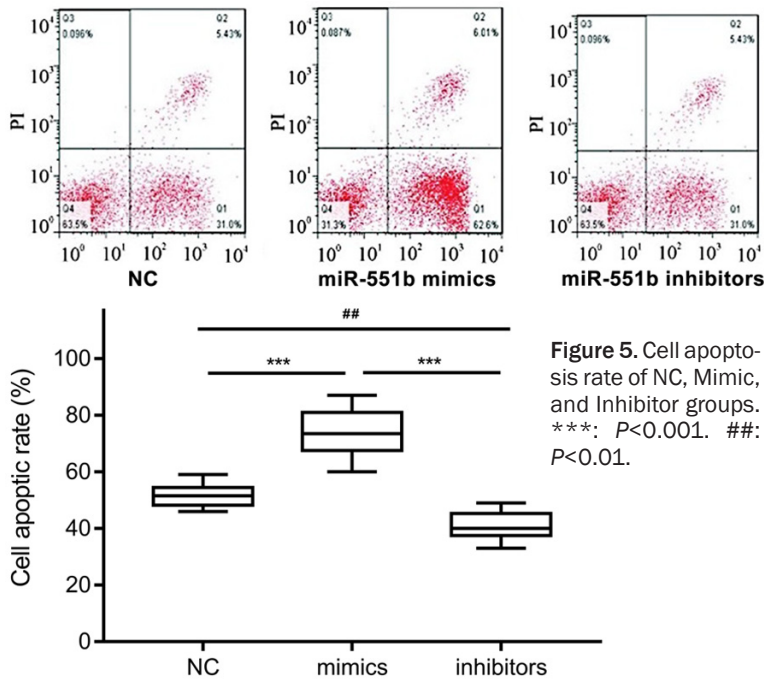
Morphological changes of cell autophagy of each cell group

According to Hoechst 33342 fluorescent staining, the Mimic group had a large amount of autophagosome (bright blue) whereas only a limited number of autophagic vacuoles could be observed in the miR-551b inhibitor group (**Figure 6**).

Protein expression of NF-κB, LC3 II, and Beclin 1 in different cell groups

In comparison with NC and Inhibitor groups, NF-κB, LC3 II, and Beclin 1 expression were significantly upregulated in Mimic group ($P<0.05$) and their expression was suppressed in Inhibitor group when compared with NC ($P<0.05$), see **Figure 7**.

Statistical analysis

SPSS 19.0 software was used to analyze experimental data. All quantitative values are shown as mean ± standard deviation (x ± s). Means of multiple groups were compared using one-way ANOVA and multiple group comparisons were made using Student-Newman-Keuls method. Statistical significance was defined as $P<0.05$.

Results

miR-551b expression in gastric cancer and normal tissues

miR-551b was downregulated in gastric cancer tissue. The relative expression level was 1.75 ± 0.13, significantly lower than that of normal gastric tissue (2.47 ± 0.38, $P<0.05$) (**Figure 1**).

miR-551b expression in transfected cells of miR-551b mimics and miR-551b inhibitors group

Compared with NC and Inhibitor groups, significant higher expression level of miR-551b in the Mimic group was observed ($P<0.05$) whereas lower level of miR-551b was detected in Inhibitor group (**Figure 2**). This indicates that transfection was successful as expected, miR-551b mimics and miR-551b inhibitor functioned well.

Figure 5. Cell apoptosis rate of NC, Mimic, and Inhibitor groups. ***: *P*<0.001. ##: *P*<0.01.

Figure 6. Cell autophagy morphological changes of different cell groups with Hoechst 33342 fluorescent staining.

miR-551b expression leading to faster cancer development [11]. In our current study, we examined the expression level of miR-551b in gastric cancer and normal tissues. The results show that miR-551b is downregulated in tumors and has significantly lower expression level than that of normal tissue, indicating that miR-551b could serve as a negative regulator during gastric cancer development. Chen et al. had similar observations after testing 40 tumors and normal tissues, levels of miR-551b dropped significantly in tumors and its expression was negatively correlated to tumor size, severity of invasion, and metastasis. He also suggested that miR-551b may be involved in invasion and metastasis of gastric cancer, as a cancer suppressive factor [12].

According to previous studies, miRNAs are closely related to a number of cell functions such as tumor proliferation, drug resistance, invasion, and metastasis. For example, miR-124 was found to be involved in gastric cancer development and miR-15a possibly plays a role in cancer cell proliferation and invasion [13, 14]. In our research, we took SGC-7901 cell line as the object of study and transfected cells with miR-551b mimics, miR-551b inhibitors, and miR-551b anti-sense strands, separately. Real-time PCR showed increased expression of miR-551b in the Mimic group and decreased level in Inhibitor group, proving that transfection was successful. Further analysis revealed that by comparing with the NC and the Inhibitor group, cells in the Mimic group had significantly lower proliferation and invasion rate with increased percentage of apoptosis. Thus, our findings confirm that miR-551b plays the role of tumor suppressor in gastric cancer cells. Ding et al. showed that in colon tumors, miR-551b was also significantly downregulated compared with normal tissue and its expression level was negative
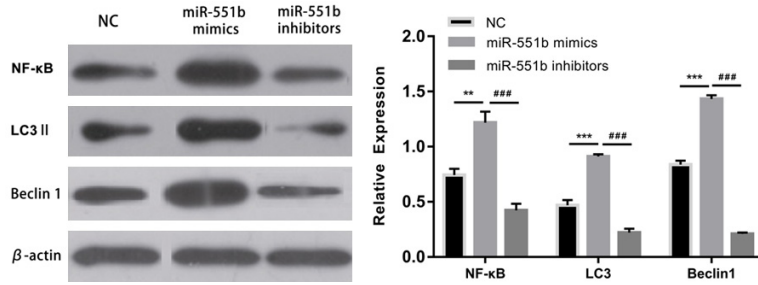
Discussion

miRNAs [7, 8] are a type of newly discovered small non-coding RNAs that regulate target protein expression at the transcription and translation level. Although short in length and limited in number, it effects large amounts of gene expression and has been proven to play an important regulatory role in tumorigenesis and tumor development [9]. Several miRNAs such as miR-29a, miR-15a, miR-124, and miR-551b have been discovered and found to be associated with gastric cancer. Xin Wen et al. found that miR-551b expression in gastric cancer tissue was abnormal and it could be used as a potential biomarker for cancer diagnosis and prognosis [10]. Min Ye et al. speculated that miR-551b may be involved in gastric cancer development with functions that were similar to anti-oncogenes, with suppressed

Figure 7. Expression levels of NF-κB, LC3 II, and Beclin 1 in different cell groups. Western blot was used to determine the expression level of related proteins in each group. **: $P<0.01$. ***: $P<0.001$. ###: $P<0.001$.

correlated with clinical staging and lymphatic metastasis [15]. Wei et al. transfected miR-551b mimics into gastric cancer cells and discovered that it could restrain tumor cell invasion and metastasis by suppressing PRL-3 expression [16].

Autophagy is a type of degradation and recycling system that exists in eukaryotic cells. The main process involves engulfing cytoplasmic constituents by isolating them from the rest of the cell within the autophagosome and degrading and recycling the contents after fusing with lysosomes. Autophagy has been seen as an adaptive response to stress and could be induced by factors such as starvation and oxidation [17]. Its detailed mechanism is still unclear but it is speculated to be related to the imbalance of cell metabolism. Previous experiments have indicated that clinical treatments like chemotherapy and radiotherapy can activate autophagy activity and increase adaptability to the environment stimuli [18] but, like a double-edged sword, excessive autophagy could also lead to autophagic apoptosis. Therefore, studying autophagy related signaling pathways is vitally important. Research has proven that by regulating autophagy related protein expression, miRNA has the ability to influence the pathological process of tumors [19]. Our results revealed that cells of the Mimic group had a considerable amount of autophagosome whereas only a limited number of autophagosome were detected in Inhibitor group. Cell membrane structure in the Mimic group was vague and apoptosis rate was high, therefore, miR-551b can evidently trigger gastric cancer cells to enter autophagic apoptosis.

NF-κB is a protein complex that controls transcription of DNA. Once activated, it could par-ticipate in programmed cell death of tumor cells. Ho et al. showed that upregulation of NF-κB expression can be induced by chemo and radio therapy and rate of cell death could also be promoted [20]. LC3 II protein is located at the inner membrane of autophagosome. Activating LC3 precursor protein leads to the formation of LC3 I, then the exposed glycine residue of LC3 I binds to phosphatidyl ethanolamine on the inner membrane of autophagosome to form LC3 II. Therefore, the quantity of LC3 II can be used in turn to reflect the amount of existing autophagosome [21]. Beclin 1 is an important protein in cell autophagy regulation [22]. By binding to its corresponding receptor PI3K3C to form the Beclin 1/PI3K3C complex, it activates autophagy related signaling pathways and regulates cell autophagy process. Our work has shown that in miR-551b mimics group, expression level of NF-κB, LC3 II, and Beclin 1 were all increased, indicating that NF-κB may be involved in cell autophagic apoptosis by regulating the expression of autophagy-related factors.

Overall, miR-551b has the ability to suppress a number of biological characteristics such as proliferation, apoptosis, and invasion in gastric tumor cells, possibly by promoting autophagical apoptosis. This discovery may provide a new target for future gastric cancer treatments.

Acknowledgements

Disclosure of conflict of interest

None.

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