

RESEARCH COMMUNICATION

miR-181b as a Potential Molecular Target for Anticancer Therapy of Gastric Neoplasms

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Abstract

Objective: MicroRNAs (miRNAs) play important roles in carcinogenesis. The aim of the present study was to explore the effects of miR-181b on gastric cancer. **Methods:** The expression level of miR-181b was quantified by qRT-PCR. MTT, flow cytometry and matrigel invasion assays were used to test proliferation, apoptosis and invasion of miR-181b stable transfected gastric cancer cells. **Results:** miR-181b was aberrantly overexpressed in gastric cancer cells and primary gastric cancer tissues. Further experiments demonstrated inducible expression of miR-181b by *Helicobacter pylori* treatment. Cell proliferation, migration and invasion in the gastric cancer cells were significantly increased after miR-181b transfection and apoptotic cells were also increased. Furthermore, overexpression of miR-181b downregulated the protein level of tissue inhibitor of metalloproteinase 3 (TIMP3). **Conclusion:** The upregulation of miR-181b may play an important role in the progress of gastric cancer and miR-181b maybe a potential molecular target for anticancer therapeutics of gastric cancer.

Keywords: miR-181b - gastric cancer - TIMP3 - anticancer therapeutics

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Introduction

MicroRNAs (miRNAs) are evolutionarily conserved small non-coding RNAs that regulate gene expression (Bartel, 2004). Recent studies revealed significant roles of miRNAs in tumorigenesis and tumour progression (Garzon et al., 2009). MiRNAs can function as tumor suppressors or oncogenes and modulate tumor transformation, proliferation, invasion and metastasis (Kasinski and Slack, 2011). Numerous studies have reported aberrant expression of miRNAs in various cancer types. Therefore, miRNAs have recently been identified as attractive targets for therapeutic intervention. The rationale for using miRNAs therapeutics for anticancer application is based on the premise that miRNA expression is deregulated in cancer compared with normal tissues and that the cancer phenotype can be changed by correcting these miRNA deficiencies by either antagonizing or restoring miRNA function.

MiR-21 is one of the most intensively studied miRNAs whose metastasis-promoting role has been established (Asangani et al., 2008). Simultaneously, it exerts anti-apoptotic, pro-proliferative, and chemoprotectant effects in a wide variety of cancers. Studies showed that inhibition of miR-21 by antagomirs resulted in reversion of malignancy in various cancers (Krichevsky and Gabriely, 2009). PC3 cells, the highly aggressive prostate carcinoma cell line, naturally express high levels of miR-221/222.

Treatment of mice bearing established subcutaneous PC3 tumor xenografts with anti-miR-221/222 antagomirs significantly suppressed tumor growth with a long-term effect of tumor reduction, suggesting the potential clinical applicability of repression of miR-221/222 (Sun et al., 2011). Therefore, it could be a valuable approach for anticancer therapeutics to silence these miRNAs upregulated abnormally in cancers. Likewise, restoring tumoursuppressor miRNA by enforced expression in cancer is another strategy for miRNA-based therapeutics. Kota and colleagues recently showed that miR-26 expression was lost in human liver cancers, while it was expressed at high levels in normal tissue (Ji et al., 2009b). Ectopic expression of this miRNA in liver cancer cell lines was shown to induce cell-cycle arrest. The authors further introduced miR-26 by viral particles to an established Myc-dependent liver cancer mouse model (Lu et al., 2011). Intravenous injection of this miRNA resulted in the suppression of tumorigenicity by inducing tumour apoptosis and by repressing cell growth, without toxicity. This work is the first evidence that restoring the expression of a tumour suppressor miRNA blocks cancer progression in vivo.

Stomach cancer is the fourth most common cancer with about 800,000 deaths worldwide per year. There are an increasing number of studies showing the dysregulation of specific miRNA in gastric cancer (Wu et al., 2010; Wang et al., 2010b). More noticeably, Ueda et al. performed a

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comprehensive miRNA profiling on 353 gastric samples from two independent subsets of patients by microarray. They showed that many miRNAs are expressed aberrantly and correlate with tumorigenesis, progression, and prognosis of various haematological and solid tumours (Ueda et al., 2010). Among them, MiR-181 family members are the most upregulated miRNAs in gastric cancer. MiR-181 was initially identified as a hematopoietic lineage modulator. Ectopic expression in hematopoietic stem/progenitor cells led to an increased fraction of B-lineage cells in both tissue-culture differentiation assays and adult mice. The significant roles of miR-181 in cancer were then revealed by various studies. MiR-181 family members are significantly upregulated in hepatocellular carcinoma (HCC) and hepatocellular cancer stem cells (HSC) and directly target hepatic transcriptional regulators of differentiation (i.e., CDX2 and GATA6), an inhibitor of wnt/ β -catenin signaling (i.e., NLK) and tumor suppressors [i.e., RASSF1A, Tissue inhibitor of metalloproteinase 3 (TIMP3)] (Ji et al., 2009b; Wang et al., 2010a). Inhibition or depletion of miR-181b led a reduction in HCC cell quantity and tumor initiating ability in vitro and inhibited tumor growth of HCC cells in vivo. In HSCs, silencing of miR-181b led to a reduction in HSCs motility and invasion. MiR-181b also enhanced resistance of HCC cells to the anti-cancer drug doxorubicin. Taken together, it is logical to expect miR-181b as a promising molecular target for anticancer therapeutics in hepatocarcinoma.

To date, there was no detailed research about therapeutic effect of miR-181b in gastric cancer. In this study, we selected human gastric epithelial cell line GES-1, gastric carcinoma AGS and undifferentiated gastric carcinoma HGC-27 to define the expression of miR-181b. Furthermore, we studied the roles of miR-181 in the invasion and metastasis, as well as the effect of anti-miR-181 on the apoptosis which shed a new light on the therapeutic potential of miR-181b on gastric cancer.

Materials and Methods

Cell and H. pylori strain culture

All cell culture reagents and culture plastics were obtained from Gibco (Rockville, MD, USA) and BD Biosciences (San Diego, CA, USA), respectively, unless otherwise specified.

Gastric carcinoma cell lines, HGC-27 (undifferentiated adenocarcinoma), AGS (middle-differentiated adenocarcinoma) and human gastric epithelial cell line GES-1, come from the Cell bank of Chinese Academy of Sciences. They were maintained in Ham's F12 (AGS), MEM (HGC-27) and RPMI-1640 (GES-1) medium supplemented with 10% foetal bovine serum, 100 U ml⁻¹ penicillin and 100 μ g/ml streptomycin, in a humidified atmosphere of 5% CO₂ at 37 °C.

H. pylori strain NCTC 11637 was maintained on brain heart infusion agar medium containing 5% sheep blood incubated at 37 °C in 5% O₂ for a minimum of two and a maximum of four passages from frozen stocks. H. pylori bacteria were added to cultured gastric cancer cells at ratio of 1:10 and cocultured till total RNA was extracted at 12, 24, 48, 72 hr after H. pylori induction.

Samples

Patients were enrolled in the period between Jan 2009 and May 2011. Included in the study were 12 patients with gastric cancer who underwent wide excision and approved at the Cancer Chemotherapy Center, Yinzhou People Hospital, Ningbo. The patients had not received any therapy before admission. The matched specimens of gastric cancer and the corresponding adjacent non-neoplastic tissues obtained from the patients were preserved in Trizol Reagent (Invitrogen, USA) and liquid nitrogen within 5 min of excision, and stored briefly at -80 °C.

Construction of miR-181b expression plasmids

The expression vector of pre-miR-181b was constructed by amplification of the human genomic cDNA, with the following primer set: 5' GGCAAGCTTCCTGTGCAGAGATTATTTTT 3' and 5' GGCGAATTCAAGCGGGGCCACAGAGTTGC 3', and ligated into pcDNA3.1-EF1a at the EcoR I and Hind III sites (Takara).

Transfection and establishment of stable miRNA expressing gastric cell lines

All the three gastric cells were seeded into 6-well plate and transfected with 2 μ g pcDNA3/miR-181b using lipofectamine 2000. After 48 hr transfection, the transfected cells were selected at 750 mg/ml G418 for 15 days, and the presence of the transgene copy in stable G418-resistant foci was confirmed by RT-PCR. Cell lines were named HGC-27/181b, AGS/181b and GES1/181b.

RNA extraction, quantitative reverse transcription-PCR analysis and Northern blot

Total RNA, inclusive of the small RNA fraction, was extracted from cells using Trizol Reagent according to the manufacture's protocol. The RNA concentration was quantified using a Spectrophotometer. cDNA was synthesized from 1 μ g of total RNA with M-MLV Reverse Transcriptase (Promega, USA) in a 25 μ l volume {2 μ g total RNA, 400 μ M reverse transcription primer [oligo (dT)18 for mRNA genes, random primers for U6 rRNA and miRNA specific primers (Bulge-LoopTM miRNA qPCR Primers from RiboBio, China) for miRNA], 4 U/ μ l M-MLV, 1 U/ μ l inhibitor, 0.4 mM dNTP mix}. Real time PCR was carried out with the reagents of a Sybr green I mix (Takara, China) in a 20 μ l reaction volume (10 μ l Sybr green I mix, 200 μ M forward and reverse primer, 2 μ l cDNA template) on a MJ Opticon Monitor chromo4 instrument (Bio-Rad, USA) using the following protocol: 95 °C for 30 s; 40 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s. Data analysis were performed using the 2 ^{$\Delta\Delta$ Ct} method.

Northern blot was performed as previously described (Connolly et al., 2008).

Synthesis and transfection of miRNA mimics and miRNA inhibitors

miRNA mimics and miRNA inhibitors were designed and synthesized by Guangzhou RiboBio (RiboBio, China). miRNA inhibitors were all nucleotides with 2-O-methyl

modification. 24 hrs prior to transfection, cells were plated onto a 24-well plate at 40-60% confluence. Transfection was performed with Lipofectamine 2000 according to the manufacturer's protocol. The medium was replaced 4-6 hrs after transfection with new culture medium.

Cell proliferation assay

Stable transfected cells and transfection of the anti-miR-181b oligonucleotide or the control was performed in 96-well plates in quadruplicate. Cell culture medium was replaced 24 hr after transfection. Cell viability was measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Cells were plated into a 96-well microplate and then incubated with different agents. 100 μ l of MTT (Sangon, China) solution (0.5 mg/ml) was added to each well, and the cells were further incubated at 37 °C for 3 hours. The supernatant was replaced with dimethyl sulphoxide (DMSO) to dissolve solid residues. The absorbance at wavelength of 570 nm was measured with a microplate reader. The ratio of the absorbance of mifepristone treated cells relative to that of ethanol treated cells was calculated and expressed as a percentage of living cells.

Apoptosis assay

Staurosporine (Sigma-Aldrich, St. Louis, MO, USA) was added to the cell media at a final concentration of 1 μ M 48 hr after transfection with anti-miR-181b oligonucleotide. After 16 hr incubation, cells were collected and assayed with an Annexin V FITC kit on a BD FACSCalibur™ system (Becton Dickson, San Jose, CA, USA) following the manufacturer's instructions.

Cell invasion and migration assay

Matrigel invasive assays were performed using 24-well matrigel-coated chambers (8 μ m pore size) from BD Biosciences (Bedford, MA, USA) as described in the manufacturer's protocol. The lower chamber was filled with 0.5 ml of medium containing 10% FBS. AGS cells were serum-starved overnight, and then resuspended in medium without FBS. Cell suspension (100 μ l) containing 1×10^5 cells was added to the upper chamber. After 24 hr incubation, noninvaded cells on the upper surface of the membrane were scraped off by cotton tip. The migrant cells attached to the lower surface were fixed in 90% alcohol and followed by crystal violet stain. The number of migrated cells on the lower surface of the membrane was counted under a microscope in 10 fields with magnification of 400 \times .

Western blot analysis

The cells and tissues lysed in lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.02% sodium azide, 100 μ g/ml PMSF, 1 μ g/ml aprotinin, and 1% Triton X-100). The proteins were probed with rabbit anti-Timp3 antibody (1:200, sc-514, Santa Cruz Biotechnologies, USA). Equivalence of protein loading was assessed using mouse anti- β -actin antibody 1:2000, Sigma).

Statistical analysis

Statistical analysis was performed using SPSS 13.0 and

analyzed with the test of ANOVA or two-tailed Student t test, with $p < 0.05$ indicative of a significant difference. Results are expressed as mean \pm SD. Values were expressed as means \pm SEM.

Results

miR-181b was Aberrantly overexpressed in human gastric cancer cell lines

In order to ascertain whether miR-181 family is relevant to gastric carcinogenesis, we began by determining the expression of miR-181 family in a panel of human gastric cell lines by real-time PCR. Figure 1A showed that miR-181a, miR-181b, miR-181c and miR-181d were all upregulated in gastric cancer cells than nonmalignant gastric cell line GES-1. Among of them, miR-181b was found to be highly expressed in gastric carcinoma cell lines, indicating that miR-181 family,

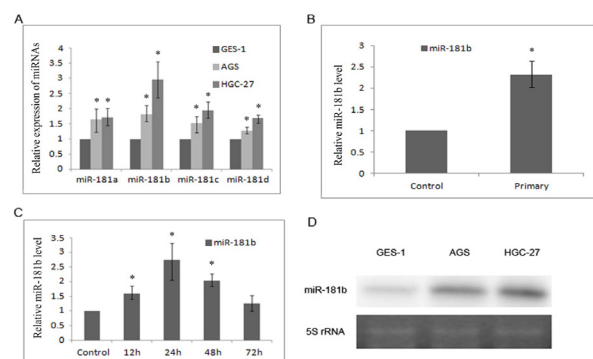


Figure 1. The Expression of miR-181b was Closely Related with Gastric Cancer. A) miR-181a, miR-181b, miR-181c and miR-181d were all upregulated in gastric cancer cells than nonmalignant gastric cell line GES-1, among of them, miR-181b was upregulated to nearly 3 folds in HGC-27 cells as compared with GES-1 cells; B) miR-181b was highly expressed in primary gastric cancer tissue as compared with adjacent normal tissue; C) *H. pylori* Infection resulted in the upregulation of the expression of miR-181b at 12h, 24 and 48h returned to normal level at 72h; D) Northern blot analysis showed the upregulation of miR-181b in HGC-27 and AGS cells than GES-1 cells. * $p < 0.05$

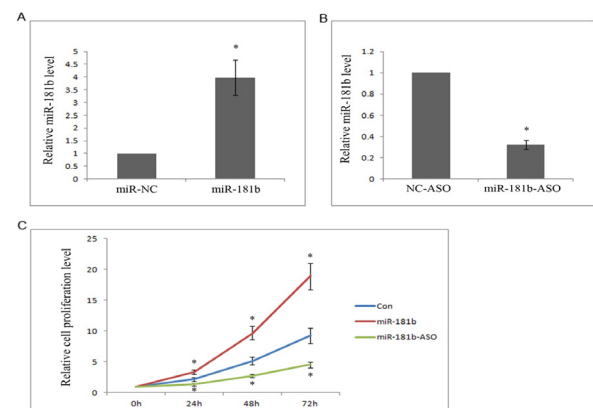


Figure 2. miR-181b Regulated the Cell Proliferation in AGS Cells. A) showed the overexpression of miR-181b by stable transfection of miR-181b; B) showed the downregulation of miR-181b by transiently transfection of miR-181b-ASO; C) The cell proliferation was promoted and suppressed by miR-181b and miR-181b respectively. * $p < 0.05$

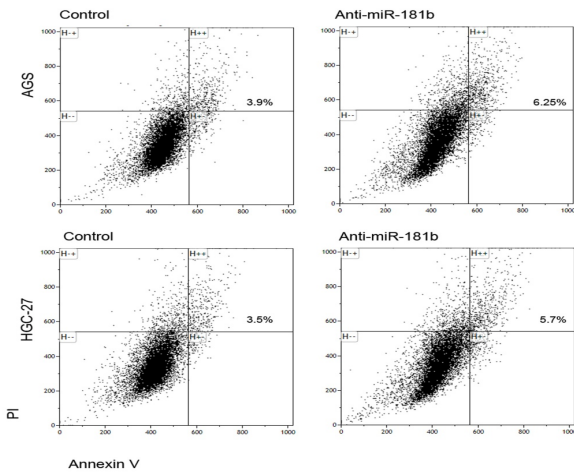


Figure 3. Anti-miR-181b Could Enhance the Apoptosis of AGS and HGC-27 Cells. The percent of apoptotic cells as determined by flow cytometry. In AGS and HGC-27 cells, the percent of apoptotic cells increased to 6.25% and 5.7% respectively by transiently transfection of miR-181b-ASO

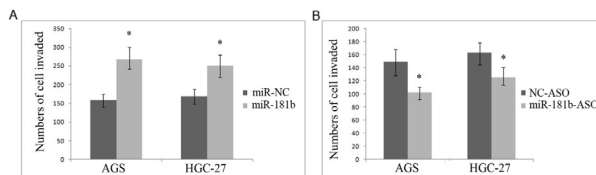


Figure 4. miR-181b Regulated Cell Invasion and Migration of Gastric Cancer in Vitro. A) Transwell invasion assay of AGS and HGC-27 gastric cancer cells in stable transfected with control or miR-181b; B) represent the invasion assay of transiently transfection with control antisense oligonucleotide (NC-ASO) or anti-miR-181b antisense oligonucleotide (miR-181b-ASO). *p<0.05

especially miR181b may be involved in gastric cancer development. Northern blot analysis further confirmed these results (Figure 1D). Moreover, we further detected the expression of primary gastric cancer tissue and found the high expression of miR-181b in cancer tissue as compared with adjacent normal tissue (Figure 1B). This consistency provided the opportunity to address the significance of the increased level of miR-181b in gastric cancer. In the following sections we focus on miR-181b to study the role of miRNAs that are upregulated in gastric cancer.

H. pylori Infection resulted in the Upregulation of miR-181b in AGS Cells

H. pylori infection is the most important factor for gastric cancers. We detected the expression of miR-181b expression in AGS cells induced by *H. pylori*. The expression of miR-181b increased at 12h, 24 and 48h of *H. pylori* induction and returned to normal level at 72h after *H. pylori* treatment (Figure 1C) which suggested that overexpression of miR-181b in gastric cancer cells might be responsible for *H. pylori* infection.

miR-181b regulated the cell proliferation in AGS cells

We studied the potential oncogenic activity of miR-181b. Aberrant cell proliferation is a hallmark of cancers. First, we tested miR-181b expression in AGS/

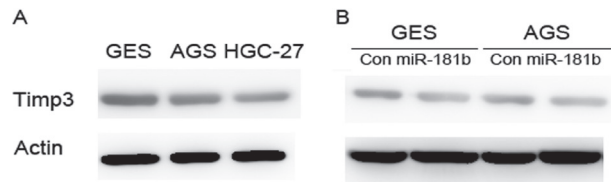


Figure 5. miR-181b Regulated the Protein Expression of Timp3. A) The expression of Timp3 in GES, AGS and HGC-27 gastric cancer cell lines; B) represented the downregulation of miR-181b in miR-181b stable expressed GES and AGS cells

miR-181b and anti-miR-181b transfected cell lines using real-time PCR which showed the overexpression and downregulation of miR-181b respectively (Figure 2A, B). We observed a significant increase in proliferation in stable miR-181b expressing cells and dramatic decrease in anti-miR-181b transfected cells (Figure 2C) which indicated that gastric cancer cell proliferation could be significantly regulated by miR-181b.

Anti-miR-181b could enhance the apoptosis of AGS and HGC-27 cells.

We further investigated the effect of miR-181b on apoptosis. The results demonstrated that apoptosis was dramatically increased (Figure 3) in AGS and HGC-27 cells 72 hr after transfection with anti-miR-181b, suggesting that miR-181b may function as a strong antiapoptotic factor in human gastric cancer cells.

miR-181b regulated cell invasion and migration of gastric cancer in vitro

In an in vitro cell invasion assay, we observed that cell invasion and migration was significantly strengthened by 70% and 52% in miR-181b stable overexpressing AGS and HGC-27 cells (Figure 4A), while was suppressed by 46% and 59% after transfection of anti-miR-181b inhibitor (Figure 4B).

miR-181b exerted its role on tumor progress by regulating the protein expression of Timp3

Previous study demonstrated that Timp3 is the direct target of miR-181b in HCC. We also detect the expression of Timp3 in different gastric cancer cells. The results of western blot showed that Timp3 expression was downregulated in gastric cancer cells than nonmalignant gastric cell line GES-1 which was negative correlated with miR-181b (Figure 5A). Moreover, in miR-181b stable transfected cells, the protein level of Timp3 was downregulated than wild type cells (Figure 5B). These results elucidated that miR-181b acted its effect on gastric carcinogenesis by regulating Timp3 expression.

Discussion

The roles of miR-181 family have been gotten a lot of attention in cancer which suggested the incompatible effect on different cancer (Ji et al., 2009b; Chen et al., 2010; Wang et al., 2010a; Wang et al., 2011). Accordingly, these miRNAs are upregulated in breast, hepatic, colon and pancreatic tumors, but are downregulated in gliomas. Three recent studies showed significant increase in the

level of miR-181b in NASH patients as well as HCCs, further confirming that this microRNA plays important role in hepatocarcinogenesis by directly regulating its target-Timp3. However, the roles of miR-181b in gastric cancer have not been reported. We firstly studied the expression of miR-181b in primary and cell lines of gastric cancer which demonstrated the significantly decrease in the gastric cancer tissues compared with the corresponding adjacent non-neoplastic tissues, which suggested that the loss of miR-181b may correlate with the carcinogenesis of gastric cancer.

Timp3 is an inhibitor of metalloproteases (a group of peptidases involved in degradation of the extracellular matrix) and was shown to induce apoptosis and inhibit growth of tumor cells and is, thus, considered to be a tumor suppressor (Mohammed et al., 2004; Wang et al., 2010a). Recently, the role of Timp3 methylation was analyzed in esophageal and gastric cancers which showed the downregulation of Timp3 expression has been linked to Timp3 gene methylation in adenocarcinomas (Leung et al., 2005; Gu et al., 2008; Kim et al., 2009). We also analyzed the Timp3 expression in different gastric cancer cell lines and primary tissue, showing the correlation of reduction of Timp3 expression and the progress of gastric cancer. Furthermore, the transfected miR-181b also downregulated the protein level of Timp3, elucidating that miR-181b exerted its effect on tumorigenesis and tumour progression by directly regulating the expression of Timp3.

Deregulated cell proliferation and apoptosis is a key mechanism for neoplastic progression. Our MTT assay and apoptosis detection indicate that miR-181b is associated with significant apoptosis inhibition in gastric cancer cells at different degrees. These findings also confirm the previous result of qRT-PCR. Moreover, In the present study, anti-miR-181b transfection led to obvious downregulation of miR-181b expression and a significant inhibition of invasive and migration of the gastric cancer cells. Anti-miR-181b may thus have a suppressor function in gastric cancer. It is logical to conceive that miR-181b functions as an oncogenic miR and gene therapy targeting miR-181b should be investigated further as a potential alternative therapeutic strategy for gastric cancer.

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