MicroRNA-103a inhibits gastric cancer cell proliferation, migration and invasion by targeting c-Myb

Jinlong Liang^{*a}, Xianfeng Liu^{†a}, Hongpeng Xue[‡], Bing Qiu[‡], Bin Wei[§] and Kan Sun^{*}

^{*}Department of General Surgery, Heilongjiang Province Hospital, Harbin, Heilongjiang, 150000, China, [†]The 21 Ward of General Surgery, Daqing Oil Field General Hospital, Daqing, Heilongjiang, 163000, China, [‡]Department of Gastroenterology, Heilongjiang Province Hospital, Harbin, Heilongjiang, 150000, China and [§]The 19 Ward of General Surgery, Daqing Oil Field General Hospital, Daqing, Heilongjiang, 163000, China

Received 18 August 2014; revision accepted 23 September 2014

Abstract

Objectives: There have been no previous reports concerning functions of miR-103a in gastric cancer (GC) cells. Thus the aim of the study was to investigate its expression and role in development of this tumour.

Materials and methods: Real-time RT-PCR was performed to detect expression of miR-103a in GC cell lines and clinical cancer specimens. To further understand its role, we restored expression of miR-103a in MGC-803 cell line by transfection with miR-103a mimics or inhibitors. Effects of miR-103a on cell proliferation, migration and invasion on targets were also determined.

Results: miR-103a was down-regulated in both GC cell lines and clinical cancer specimens. Meanwhile, its level was closely associated with pM or pTNM stage of GC. Overexpression of miR-103a markedly suppressed proliferation, migration, and invasion of GC cells, while its inhibition significantly accelerated cell proliferation, migration and invasion. Moreover, c-Myb was identified to be a functional downstream target of miR-103a, ectopic expression of which partially reversed suppression of cell proliferation and invasion.

Conclusions: Thus our observations suggest that miR-103a functioned as a tumour suppressor by tar-

geting c-Myb. These findings indicate that miR-103a might play a significant role in pathogenesis of GC.

Introduction

Gastric cancer (GC), is the fourth most common cancer type in some areas, ranking second most frequent cause of cancers deaths there (1). Although advances in diagnostic techniques and therapeutic management have contributed to higher long-term survival of GC patients, it is still extremely low. Poor prognosis tends to be due to lack of early diagnosis, with insufficient biomarkers/effective therapeutic treatment. Most GC patients are already at advanced stages when initially diagnosed (2–4). It is thus of extreme importance to elucidate molecular mechanisms of GC and to identify novel molecular targets for early diagnosis and treatment.

MicroRNAs (miRNAs) compose an abundant class of small RNAs, approximately 22 nucleotides in length, which regulate gene expression post-transcriptionally by translational inhibition and destabilization of mRNA (5). Many important cell functions, such as proliferation, development, differentiation and metabolism, are regulated by miRNAs (6–8). Growing evidence has shown that miRNAs are involved in pathogenesis of most cancers, where some can function as tumour suppressors or as oncogenes (9,10). Aberrant miRNA expression has been reported in various tumours such as hepatocellular carcinoma, lung cancer, breast cancer, leukemia, and colon cancer, indicating close correlation between miRNAs and human malignancy (11–16). However, understanding involvement of miRNAs in carcinogenesis of GC remains to be resolved.

Accumulating evidence has suggested that miR-103 might serve to effect a novel oncogene in human cancers (17,18). For example, miR-103 can promote growth and

Correspondence: J. Liang, Department of General Surgery, Heilongjiang Province Hospital, No. 405 of Guogeli Street, Harbin, Heilongjiang 150000, China. Tel.: +86451 51980270; Fax: +86451 51980270; E-mail: liangjl111@126.com

^aThese authors equally to this work.

invasion of endometrial cancer cell lines (18). There is also some debate on whether miR-103 acts as tumour suppressor or as a promoter in GC development. Recently, Tchernitsa *et al.* (19) found that miR-103 acted as a promoter in gastric carcinogenesis and its expression was up-regulated, which was further confirmed by Li *et al.* (20). Also, regulation of miR-103 in human gastric cancer and its potential molecular mechanisms have not been investigated. However, further studies have reported, by real-time reverse transcription-polymerase chain reaction (RT-PCR) (20), that miR-103 is down-regulated in human gastric tumours. Here, we have comprehensively investigated function and molecular mechanism of miR-103a in GC. We observed its significant down-regulation in GC cell lines and clinical specimens. Overexpression of miR-103a inhibited cell proliferation, migration and invasion of GC cells. Moreover, c-Myb was indicated to be the direct target of miR-103a in GC. Thus, our results suggest a significant role of miR-103a in GC pathogenesis, indicating its potential application in gastric cancer diagnosis and therapy.

Material and methods

All patients participating in this investigation agreed to do so by written informed consent. Both the study and consent were approved by the Ethical Board of the Institute of Heilongjiang Province Hospital, and complied with the Declaration of Helsinki.

Human cancer tissues and samples

Fresh-frozen human gastric cancer tissue samples and matched normal gastric tissue samples (located >3 cm away from tumours) were obtained between 2009 and 2011 from 80 GC patients at the Heilongjiang Province Hospital. All of tumour types were confirmed by pathological analysis. None of the patients received radiotherapy or chemotherapy before surgery. Due to individual differences between patients, we lacked information concerning some clinicopathological data. Use of tissue samples for all experiments was approved by each of the patients and by the Ethics Committee of Heilongjiang Province Hospital. Patient characteristics are described in Table S1.

Cell culture and cell transfection

The following human GC cell lines were used in this study: MKN-45 (poorly differentiated signet cell carcinoma), HGC-27 (undifferentiated carcinoma, metastatic lymph node), MGC-803 (poorly differentiated mucinous gastric cancer), and SGC-7901 (moderately differentiated adenocarcinoma), and GES (normal gastric mucosal cell

line). MGC-803 and GES cells were obtained from Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). These were propagated in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (GIBCO, Grand Island, NY, USA) and streptomycin (100 µg/ml) and penicillin (100 U/ml). HGC-27, MKN-45 and SGC-7901 cell lines were provided by American Type Culture Collection (ATCC; Manassas, VA, USA) and were maintained in RPMI 1640 (PAA) supplemented with 10% FBS (GIBCO) and streptomycin (100 µg/ml) and penicillin (100 U/ml). miR-103a mimics, inhibitor and scramble mimics (non-homologous to the human genome) were synthesized by GenePharma (Shanghai, China) and transfected into the cells to a final oligonucleotide concentration of 10 nmol/l. Transfection was performed using Lipofetamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Cells were trypsinized, counted and seeded in plates on the day before transfection to ensure suitable cell confluence. Oligonucleotides and plasmids were used at final concentrations of 10 nmol/L, both in antibiotic-free Opti-MEM medium (Invitrogen).

TaqMan RT-PCRs for miRNA expression

Total RNA was extracted from tissue samples and cell lines using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. The TaqMan stem-loop real-time PCR method was used to assess expression of miR-103a with kits from Applied Biosystems (Foster City, CA, USA). cDNA synthesis was carried out with 1 µg total RNA in 12 µl final volume containing 10 mM dNTP Mix (Invitrogen) and 2 M stem-loop primer. The mix was plated at 65 °C for 5 min, then mixed with 5xRT buffer, 0.1 M DTT, 200 U/µl MultiScribe reverse transcriptase and 40 U/µl RNase inhibitor (Invitrogen), at 37 °C for 55 min, 70 °C for 15 min, then held at –20 °C. Real-time PCR was performed according to standard TaqMan PCR protocols. 20 µl PCR reactions included 1 µl RT product, 1 Universal TaqMan Master Mix and 1x TaqMan probe/primer mix (Invitrogen). All RT reactions including no-template controls were run in triplicate. All miRNA quantification data were normalized to U6, and mRNA quantification data were normalized to GAPDH (21) (Table S2).

Cell proliferation

Cells were incubated in 10% CCK-8 (Dojindo; Kumamoto, Japan) diluted in normal culture media at 37 °C, until visible colour conversion occurred. Proliferation levels were determined 0, 24, 48 and 72 h after transfection. All experiments were performed in quadruplicate.

CCK-8 solution (10 μ l) was added to each well, and absorbance at 450 nm was measured after incubation for 2 h at 37 °C, to calculate numbers of viable cells.

Cell migration and invasion

Transfected cells were seeded in 24-well plates. When grown to confluence, cultures were scratched with a pipette tip (200 μ l); cells were then cultured under standard conditions for 24, 48 and 72 h. Plates were washed twice in fresh medium to remove non-adherent cells, then photographed. Cell migration was evaluated by counting cells that had migrated from the 'wound' edges.

Invasion assays were evaluated by examining ability of cells to pass through Matrigel-coated membrane matrix (BD Biosciences, Qume Drive, San Jose, CA, USA). Cells were seeded on Matrigel-coated membrane matrix in 24-well culture plates, after transfection. Foetal bovine serum was added to non-invading cells which were removed. Invasive cells located on lower surfaces of chambers were stained with 0.1% crystal violet (Sigma, Sigma-Aldrich, Oakville, ON, Canada), and were then counted.

Dual luciferase assays

c-Myb 3'UTRs containing predicted miR-103a binding sites were amplified by PCR from human cDNA using primers, and inserted into pMIR-REPORT luciferase reporter vectors (Ambion, Austin, TX, USA); to obtain constructs containing wild-type c-Myb3'UTR (c-Myb-WT), c-Myb-MUT contained sequences with mutations in the first putative binding site of c-Myb3'UTR. Predicted seed regions in these mRNA sequences were created, using primers including mutated sequences. Recombination constructs, pRL-TK (Promega, Madison, WI, USA) and miR-103a, or control mimic, were co-transfected into MGC-803 cells using lipofectamine 2000 (Invitrogen). Plasmids of pRL-TK containing Renilla luciferase were used as internal control. Firefly and Renilla luciferase activity were measured using Dual Luciferase Assay (Promega) according to the manufacturer's instructions, 24 h after transfection. All transfection assays were carried out in triplicate.

Western blotting

Western blotting was performed according to standard methods (22). Cells were lysed in ice-cold cell lysis buffer (50 mM pH 8.0 Tris, 120 mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM phenylmethylsulphonyl fluoride (PMSF), 20 μ M sodium orthovanadate, 1x protease

inhibitors, 1x phosphatase inhibitors). Total protein was isolated from tumour samples and corresponding normal tissues or cells. Concentration was measured using a BCA protein assay kit (Pierce, Meridian Rd, Rockford, IL, USA). Proteins were separated with the aid of 10% SDS-PAGE, then transferred to PVDF membranes (Amersham, Buckinghamshire, UK). Membranes were blocked with 5% non-fat dried milk and incubated overnight in anti-c-Myb antibody (Abcam, Cambridge, UK) and anti-GAPDH antibody (Proteintech, Chicago, IL, USA). After washing three times in TBST, membranes were incubated for 2 h in goat anti-rabbit antibody (Zsfg-bio, Beijing, China).

Histology

Histological diagnosis was made according to the triple-site gastric biopsy method. Tissues were fixed overnight in buffered formalin, embedded in paraffin wax, sectioned at 3- μ m and stained with haematoxylin and eosin (H&E).

Statistical analysis

Each experiment was repeated at least three times. Statistical analysis was performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Data were presented as mean $\pm$ SD. Statistical analyses were performed with either analysis of variance (ANOVA) or Student's *t*-test, and significance level was set at $\alpha = 0.05$ (two-sided).

Results

MiR-103a was down-regulated in human GC cell lines and tissues

Using real-time RT-PCR, we detected expression of miR-103a in the four human GC cell lines-MKN-45 (poorly differentiated signet cell carcinoma), HGC-27 (undifferentiated carcinoma, metastatic lymph node), MGC-803 (poorly differentiated mucinous gastric cancer), and SGC-7901 (moderately differentiated adenocarcinoma), as well as in normal gastric epithelial cell line-GES-1. We found that expression of miR-103a was down-regulated in all four GC cell lines compared to GES-1 (Fig. 1b). We further examined miR-103a expression in 80 primary GC tissues in comparison to their adjacent non-tumour tissues. MiR-103a was down-regulated in 64 cases (64/80, 80%) (Fig. 1c). In general, expression of miR-103a in these GC tissues was significantly lower than that in the adjacent non-tumour tissues (Fig. 1d, $P < 0.001$). In addition, expression of miR-103a in metastatic GC tissues was significantly lower than in non-metastatic tissues ($P < 0.01$,

Figure 1. miR-103a was down-regulated in human GC cell lines and tissues. (a) Pathological examination of one human gastric cancer (cancer) and adjacent non-neoplastic tissue (Normal) using haematoxylin & eosin (H&E) staining (original magnification, $\times 100$). (b) Relative expression of miR-103a in four cell lines derived from gastric cancers and one non-malignant gastric cell line (GES) was determined by qRT-PCR. (c) Relative expression of miR-103a in 80 primary gastric cancers compared to their pair-matched non-tumour tissues. Data shown as log₁₀ of relative ratio change of gastric cancer tissues relative to normal tissues. Quantification of miR-103a was measured by qRT-PCR with specific primers for miR-103a and snRNA U6. (d) Relative miR-103a expression levels in GC tissues and adjacent normal regions; (e) and (f) Statistical analysis of association between miRNA level, pTNM stage (I, II, III and IV) and pM stage (No metastasis and Metastasis, respectively); ** $P < 0.01$.

independent-samples *t*-test). Moreover, low levels of miR-103a expression were associated with pTNM stage ($P < 0.01$, stage I versus stage III, $P < 0.01$, stage II versus stage IV, $P < 0.01$, stage III versus stage IV) (Fig. 1e).

miR-103a inhibited GC cell proliferation

To explore the role of miR-103a in GC, MGC-803 was transfected with miR-103a mimics or inhibitor, with high level of transfection efficiency (Fig. 2a). CCK-8 proliferation assays indicated that cell population growth was significantly lower in miR-103a mimic-transfected MGC-803 cells compared to scramble MiRNA-transfected cells, or untreated cells. Conversely, miR-103a inhibitor significantly increased proliferation of MGC-803 cells (Fig. 2b).

miR-103a inhibited cell migration and invasion

We used *in vitro* wound healing and transwell assays to analyze the role of miR-103a in cell migration and invasion. Cells treated with miR-103a mimics had less migratory ability than scrambled control or untreated cells, at 24 and 48 h after scratching. In contrast, miR-103a inhibitor significantly accelerated migration of MGC-803 cells (Fig. 2c). We also conducted Matrigel invasion assays to measure directional invasion ability of the cells.

Invasiveness of cells transfected with miR-103a mimics was significantly lower than scrambled control and untreated cells. However, miR-103a inhibitor significantly increased invasion of MGC-803 cells (Fig. 2d).

miR-103a targeted c-Myb in GC cells

MiRNAs exert biological functions through negatively regulating their target genes. It has been previously reported that c-Myb plays an important role in GC. As predicted by PicTar, there was a complementary sequence between has-miR-103a and c-Myb 3'UTR (23) (Fig. 3a). The effect of miR-103a on translation of c-Myb mRNA was assessed by luciferase reporter assay (Fig. 3b). More importantly, miR-103a overexpression remarkably reduced luciferase activity of wild type reporter genes, but not mutant c-Myb3'UTR, indicating that miR-103a directly targeted c-Myb3'UTR. Overexpression of miR-103a reduced protein levels but not mRNA levels of c-Myb (Fig. 3c,d).

Overexpression of c-Myb impaired miR-103a-induced inhibition of proliferation and invasion

We then performed rescue experiments to further validate whether c-Myb was involved in anti-tumour

Figure 2. Overexpression of miR-103a inhibited proliferation, migration and invasion of gastric cancer cells. (a) Expression levels of miR-103a were examined by qRT-PCR after transfection of 20 nmol/l of miR-103a mimics, inhibitors or scramble or no transfection. (b) Cells treated with miR-103a mimics, inhibitors, scramble or no transfection, were measured by CCK8 assay after different time periods. (c) Wound healing assays of MGC-803 cells after treatment with miRNA mimics, inhibitors or scramble or no transfection; relative ratio of wound closure per field is shown. (d) Transwell analysis of MGC-803 cells after treatment with miRNA mimics, inhibitors or scramble or no transfection; relative ratio of invasive cells per field is shown below, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

properties of miR-103a in GC cells. C-Myb expression vector, pcDNA3.0-c-Myb was used to restore c-Myb expression (Fig. 4a). miR-103a suppressed tumour promoting functions of c-Myb (Fig. 4b,c) and c-Myb was up-regulated in GC tissues. c-Myb protein level was analyzed in eight miR-103a down-regulated GC tissues; it was up-regulated in them compared to their pair-matched

adjacent non-tumour tissues (Fig. 5). Expression of miR-103a in eight GC tissues was shown in Fig. S1.

Discussion

Although miRNAs have been extensively studied in many types of cancer in recent years, their expression

Figure 3. miR-103a targets c-Myb in gastric cancer cells. (a) Sequences of miR-103a binding sites within human c-Myb 3'UTRs, and schematic reporter constructs, in this panel, c-Myb-WT represents reporter constructs containing the entire 3'UTR sequences of c-Myb. C-Myb-MUT represents reporter constructs containing mutated nucleotides. (b) Analysis of relative luciferase activities of c-Myb-WT, c-Myb-MUT in MGC-803 cells. Error bars are derived from triplicate experiments. (c) qRT-PCR analysis of c-Myb mRNA expression in MGC-803 cells after treatment with miRNA mimics or scramble or no transfection. Expression of c-Myb was normalized to GAPDH. (d) Western blot analysis of c-Myb expression in MGC-803 cells transfected with miR-103a mimics or scramble or no transfection. GAPDH was also detected as loading control.

Figure 4. Overexpression of c-Myb impaired miR-103a-induced inhibition of proliferation and invasion of GC cells. (a) Western blot analysis of c-Myb in MGC-803 cells co-transfected with either miR-103a mimic or scramble and 2.0 µg pCDNA-c-Myb or pCDNA empty vector. (b) Cell growth curves in MGC-803 cells transfected with different combinations. Relative ratio of invasive cells per field shown right. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

and potential roles in GC remained largely unknown (5,24). Here, we have shown that expression of miR-103a was significantly down-regulated in both GC tissues and cell lines. In addition, expression level of

miR-103a significantly correlated with pTNM stage. Furthermore, overexpression of miR-103a suppressed cell proliferation, migration and invasion in GC cells. *C-Myb*, an oncogene, was identified to be a novel and

Figure 5. c-Myb up-regulated in gastric cancer tissues. Western blot analysis of c-Myb expression in 8 miR-103a down-regulated GC tissues and pair-matched non-tumour tissues. GAPDH was also detected as a loading control.

direct target of miR-103a. Our findings suggest that miR-103a played a fundamental role in GC tumourigenesis by suppressing cancer cell proliferation, migration and invasion.

MiR-103 has previously been reported to be overexpressed in many neoplasms, such as colorectal cancer and endometrial cancer cell lines. MiR-103 correlated with extent of lymph node and distant metastasis in patients with colorectal cancer, serving as prognostic marker for metastasis and poor survival (17). MiR-103 has also been reported to stimulate population growth and invasion of endometrial cancer cell lines (18). Some debate still existed on whether miR-103 is up-regulated or down-regulated in GC (25). In our experiments, we have shown that expression of miR-103a was significantly lower in GC cell lines and tissues compared to normal gastric cells or tissues. Meanwhile, lower levels of miR-103a were associated with pTNM stage in clinical GC patient tissues. Although functioning as a tumour suppressor in GC cells, only a greater part (64/80, 80%), but not all, GC patients had down-regulation of miR-103a in their GC tissues. This may be because of distinct mechanisms of genesis of GC in different individuals. It is possible that there are other important genes or factors responsible for tumourigenesis in patients whose miR-103a was unchanged or up-regulated. Functional studies were also performed to analyze the role of miR-103a on behaviour of gastric cancer cells (MGC-803). We found that miR-103a dramatically suppressed their proliferation, migration and invasion. miR-103a inhibitor significantly accelerated proliferation, migration and invasion of MGC-803 cells and restoration of miR-103a repressed tumourigenic processes of gastric cancer, indicating that restoration of miR-103a might provide a survival advantage.

There are many proteins that seem, by using Target-Scan, to be regulated by MiR-103a among which we found c-Myb to be a target (23). This conclusion is supported by several reasons. The complementary sequence of miR-103a was identified in the 3'UTR of c-Myb mRNA. In addition, overexpression of miR-103a led to significant reduction in c-Myb protein level and restored miR-103a expression inhibited c-Myb 3'UTR luciferase

report activity. These effects were abolished by mutation of the miR-103a binding site. c-Myb is a well-acknowledged oncogene, overexpressed in a variety of human cancers. Overexpression of c-Myb contributes to malignant transformation by regulating genes participating in multiple aspects of tumourigenesis, such as cell population growth, angiogenesis and resistance to apoptosis (26–29). Recent studies have shown that c-Myb has played an important role in GC development and progression. Its high expression level correlates with advanced disease stage, lymph-node metastasis and poor survival rate (30–32). In our study, overexpression of c-Myb significantly increased proliferation and invasion of MGC-803 cells. Our data revealed that miR-103a inhibited gastric cancer development by targeting c-Myb, providing a potential mechanism of its post-transcriptional control.

In conclusion, data described here suggest that miR-103a functioned as a novel tumour suppressor in GC. It was dramatically down-regulated in both GC cell lines and clinical cancer specimens. In addition, *in vitro* experimentation demonstrated that overexpression of miR-103a inhibited cell population growth, cell invasion and migration of GC cells by suppressing c-Myb gene. These findings not only help us understand molecular mechanisms of GC carcinogenesis but also provide us with a strong rationale to further investigate the role of miR-103a as a diagnostic biomarker and therapeutic target for GC.

Acknowledgements

Funding: This work was supported by the Technological Innovation Special Foundation of Harbin Science and Technology Bureau (No. 2014RFQGJ188).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Expression of miR-103a in GC tissues. Relative expression of miR-103a in primary gastric cancer tissues compared to pair-matched non-tumour tissues. miRNA quantification data normalized to U6.

Table S1. Clinicopathologic characteristics of patients with GC

Table S2. Primer sequence