Original Article

MicroRNA-23a regulates cell migration and invasion by target PTEN in gastric cancer

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Abstract: Objective: To investigate the biological significance of abundant miR-23a expression in gastric cancer (GC) and its correlation with PTEN in the pathogenesis of GC migration and invasion. Methods: The human gastric cancer cell lines SGC-7901, AGS, the immortalized cell line GES-1 derived from normal gastric mucosa. Cell transfection and selection of stable cell lines and the gene and protein levels of miR-23a and PTEN were examined to determine the molecular relationship between them in the pathogenesis of gastric cancer. Results: Inhibition of miR-23a effectively reduced migration and invasion of GC cell lines. Bioinformatics and luciferase reporter assay revealed that miR-23a specifically targeted the 3'-UTR of PTEN and regulated its expression. Down-regulation of PTEN enhanced migration and invasion of GC cell lines. Furthermore, in tumor tissues obtained from gastric cancer patients, the expression of miR-23a was negatively correlated with PTEN and the high expression of miR-23a combined with low expression of PTEN might serve as a risk factor for cancer patients. Besides, miR-23a-mediated suppression of PTEN led to activation of AKT/ERK pathways and epithelial-mesenchymal transition (EMT) in GC cells, and finally enhances the activity of GC cell proliferation and movement and promotes GC xenograft tumor growth in mouse models. Conclusion: Our study showed that miR-23a, by down-regulation PTEN, enhances migration and invasion in GC cells.

Keywords: Gastric cancer (GC), PTEN, epithelial-mesenchymal transition (EMT), invasion, migration, target therapy

Introduction

Gastric cancer (GC) is the fifth most frequently diagnosed cancer worldwide but the second most common cause of cancer death [1, 2]. Rapid malignant progression, dismal survival rate and high frequency of recurrence and metastasis remain the crux of GC treatment. Investigation of mechanisms involved in GC recurrence and metastasis might lead to development of novel therapeutic strategies.

miRNAs are a family of small highly conserved endogenous non-coding RNAs which inhibit translation of target genes by base pairing to the 3'-UTR of mRNAs. Recently, a series of miRNAs have been shown to play critical roles in the processes of EMT of cancer cells to promote the progression and metastasis of human malignancies, in which miR-23a is an important component involved in the cellular signaling pathways that regulates EMT processes [3, 4] and is always up-expressed in many kinds of malignancies. Recent studies showed that several tumor suppressors including PTEN, TPM1, PDCD4, maspin, and TIMP3 were targets of miR-23a [5-7], suggesting that miR-23a is an important oncogenic miRNA which is closely related to tumor growth and metastasis [8].

AGS is a GC cell line established from primary tumor and recurrent tumor respectively of the same patient [9]. They possess significantly different proliferative and invasive ability. Therefore, we analyzed the differential expression of miRNAs in AGS using microRNA microarray [10] and discovered extraordinarily high expression levels of miR-23a in AGS. Interestingly, qRT-PCR showed that expression levels of miR-23a were even higher than the internal reference gene U6. What is the biological significance of such abundant miR-23a expression in GC? Here, we
showed that microRNA-23a enhances migration and invasion through PTEN.

Materials and methods

Cell culture

The human gastric cancer cell lines AGS, SGC-7901 and the normal gastric epithelium cell line GES-1 were grown in RPMI 1640 medium supplemented with 10% FBS (Hyclone). The cell cultures were incubated in room air at 37°C in a humidified atmosphere of 5% CO₂.

Reverse transcription and real-time polymerase chain reaction to quantify mature miR-23a

Total RNA was extracted with TRizol (Invitrogen). For mature miRNA expression analysis, cDNA was synthesized with the Taqman MiRNA Reverse Transcription kit (Applied Biosystems) and 100 ng of total RNA (100 ng/μL), along with 1 μL of miR-23a (Applied Biosystems) specific primers that were supplied with the miRNA Taqman MicroRNA Assay, according to the manufacturer’s instructions. Quantitative real-time polymerase chain reaction (PCR) analyses were performed in triplicate on a 7900HT Real-Time PCR System (Applied Biosystems), and the data was normalized to RNU6B (Applied Biosystems) for each reaction. The thermal cycling profile used was as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Quantification was performed according to the standard ΔΔCT method.

Transfection of the miR-23a precursor

Cells were seeded 24 h prior to transfection into 24-well or 6-well plates or 6 cm dishes. Hsa-miR-23a (Applied Biosystems) or a miRNA mimic control (Applied Biosystems) was transfected with Lipofectamine 2000 (Invitrogen) at a final concentration of 50 nmol/L. The sequences of the mature miR-23a used in this study were GGUGCAGUGCUGCAUCUCUGGU and UGAGAUGAAGCACUGUAGCUC. The cells were harvested at 24 h (for RNA extraction), 48 h (for protein extraction) or 72 h (for apoptosis assays).

Cell viability assays

An Alamar blue assay was used to measure cell proliferation. This assay is based on the quantitative metabolic conversion of blue, non-fluorescent resazurin to pink, fluorescent resorufin by living cells. After 72 h of incubation, an Alamar blue (Invitrogen) stock solution was aseptically added to the wells to equal to 10% of the total incubation volume. The resazurin reduction in the cultures was determined after a 2-6 h incubation with Alamar blue by measuring the absorbances at 530-nm and 590-nm wavelengths on a Synergy HT Multi-Mode Microplate Reader (Bio-tek Instruments).

Western blot

Cells were harvested, washed twice in PBS, and lysed in lysis buffer (protease inhibitors were added immediately before use) for 30 min on ice. Lysate was centrifuged at 10000 rpm and the supernatants were collected and stored at -70 in aliquots. All procedures were carried out on ice. Protein concentration was determined using BCA assay kit (Tianlai Biotech).

miRNA real-time PCR

$1 \times 10^2$–$1 \times 10^7$ cells were harvested, washed in PBS once, and stored on ice; complete cell lysate was prepared by addition of 600 μl lysis binding buffer and vertex; 60 μl miRNA aomogenet additive was added to the cell lysate and mixed thoroughly by inverting several times; sample was stored on ice for 10 min, followed by addition of equal volume (600 μl) of phenol: chloroform (1:1) solution; sample was mixed by inverting for 30-60 sec, and then centrifuged at 12000 g for 5 min; the supernatant was transferred to a new tube and the volume was estimated; 1/3 volume of 100% ethanol was added and mixed; the mixture was loaded to the column at room temperature and centrifuged at 10000 g for 15 sec; The flow-through was collected and the volume was then estimated; 2/3 volume of 00% ethanol was added and mixed; The mixture was loaded to column at room temperature and centrifuged at 10000 g for 15 sec; The flow-through was discarded; 700 μl miRNA wash solution was added to the column, followed by centrifugation at 10000 for 10 sec; the flow-through was discarded; 500 μl miRNA wash solution was added to the column, followed by centrifugation at 10000 for 10 sec; the flow-through was discarded; the column was transferred to a new tube and 100 μl preheated elution solution (95 degree) was
added at room temperature; RNA was collected by centrifugation at 12000 g for 30 sec.

Luciferase assay

Forty-eight hours after transfection, the cells were rinsed in PBS. Experiments for each treatment were performed in triplicate. Luciferase activity was assessed using the dual-luciferase reporter assay system (Promega, USA) with a luminometer (Promega, USA). The luciferase activity after cell lysis was measured and then normalized to the activity of renilla luciferase driven by the constitutional promoter in the pRL vector. Basal promoter activity was measured relative to the activity observed with the pGL3 vector alone.

Invasion assays

After adjusting the cell density to 1×10⁶ cells/ml in 0.1% BSA-RPMI, 100,000 cells in 100 μl were added to the top chamber of a 24-transwell apparatus (6.5-mm diameter, 8-μm pore size; Costar 3422, Corning Inc., Corning, NY) in the absence or presence of fibronectin (25 μg) in the lower chamber. After overnight incubation at 37°C in an atmosphere containing 5% CO₂, living cells (with diameters ranging from 7 to 14 μm) that passed through the membrane were collected from the lower well and counted in a cell Coulter Counter channelizer 256. Results from triplicate wells were expressed as mean ± S.D.

Wound healing assay

The gastric cells transiently transfected with miR-23a precursor or negative control miRNA precursor were seeded in a 100-mm Petri dish. A wound was made by scratching on the Petri dish bottom, followed by another 48 hours growth.

Immunoblotting

For immunoblotting, the human gastric cancer cells were collected 72 hours after transfection with miR-23a precursor or negative control miRNA precursor. Cells were lysed using 1×RIPA buffer (Upstate Biotechnology, Lake Placid, NY) containing a protease inhibitor cocktail (Sigma, St. Louis, MO). After cell lysis, 45 mg of protein was loaded on a 10% SDS gel followed by transfer to PVDF membrane. Antibodies against SGPP2 (Abcam, Cambridge, MA), Smad4 (Cell Signaling Technologies, Danvers, MA), and Actin (Sigma, St. Louis, MO) were used. Secondary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The detected signals were visualized by an enhanced chemiluminescence kit (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China), as recommended by the manufacturer.

Tumor-bearing (Xenografts) study

1.5×10⁵ murine gastric adenocarcinoma cells re-suspended in 150 μl PBS were injected subcutaneously into the flank of the normal C57B/16 mice at age about 8 weeks (5 mice per group). Both gastric cell line and the mice were in C57BL/6 background and no rejection occurred. The animals were maintained in a pathogen-free barrier facility and closely monitored by animal facility staff. The grown tumors (xenografts) were measured every 3 days starting 23 days post inoculation of cells using caliper as length × width × width/2 (mm³). 6.26 μg of miR-23a precursor or negative miRNA (GenePharma, Shanghai, China) mixed with 1.6 μl transfection reagent Lipofectamine 2000 (Invitrogen) in 50 μl PBS was injected into the tumors every 3 days, for total of 3 times. 32 days after inoculation, the animals were sacrificed and the xenografts were isolated, the weight (gram) and volume (cm³) of the xenografts were determined. All procedures were conducted according to the Animal Care and Use guideline approved by Xinxiang Medical University Animal Care Committee.

Results

miR-23a expression is up-regulated in highly metastatic cancer cells

miR-23a is widely over-expressed in tumorigenesis including GC cell lines. Furthermore, miR-23a was closely connected with motility, migration, and invasion of human cancers. To explore the expression of miR-23a in cancer cell lines, different cancer cell lines varied in their metastatic potentials were selected and measured the relative expression of miR-23a by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR), compared with normal cell lines. The relative expression of miR-23a was significantly up-regulated in AGS and SGC-7901 cell lines (6.73 ± 1.01 in AGS, 4.35 ± 0.53 in SGC-7901; P < 0.01 versus normal cell line).
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To confirm the metastatic potential of miR-23a high expression cells (AGS) and miR-23a low expression cells (GES-1), the harvested cells were injected into the abdominal cavity of BALB/c (nu/nu) mice at $2.5\times10^6$ cells per mouse. Two weeks later, mice were dissected under body vision microscope and the cancer nodules were monitored and counted. The results showed that AGS cells formed more
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Cancer nodules with larger size than GES-1 cells (Figure 1B).

To investigate the effect of miR-23a on cellular motility, GC cells with different expression status of miR-23a, including GES-1 and AGS cell lines, were measured by wound healing, transwell migration and invasion assays after transfected miR-23a expression vector or treated with miR-23a inhibitor. Transfection of miR-23a expression vector significantly enhanced the capacity of wound healing in GES-1 cells, as compared with the parental control cells without transfection (Figure 1C). However, the capacity of wound healing in AGS cells was significantly attenuated after treated with miR-23a inhibitor, as compared to the parental control cells without treatment (Figure 1D).

Meanwhile, after transfected with miR-23a expression vector, the relative migrated and invaded cell number of GES-1 cells was significantly more than that of the parental control cells (Figure 1E), and the relative migrated and invaded cell number was significantly decreased in the miR-23a inhibitor-treated AGS cells, as compared with the parental control cells (Figure 1F), suggesting that miR-23a plays an important role in the regulation of GC cellular motility, including the cancer cell invasive and metastatic capacity.

Over-expression of miR-23a induced EMT by regulating the activity of PTEN-mediated AKT and ERK signaling in GC cells

To further confirm the role of miR-23a in regulating cancer cell EMT, we constructed an
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expression vector of GFP-tagged miR-23a and transfected into GES-1 cells. Totally 5×10⁵ GES-1 cells were transfected with miR-23a vectors at a concentration of 2 μg/well, the transfection efficiency was (65.10 ± 2.00)% 24 h after transfection. Followed by G418 selection, the transfection efficiency was (92.32 ± 15.22)%. In the miR-23a-transfected cells, miR-23a expression was increased to more than 18-fold, as compared with the parental cells without transfection (Figure 2A), indicating that transfection of miR-23a expression vector could increase the relative expression of miR-23a. To examine whether the forced re-expression of miR-23a could induce EMT, the protein expression of EMT biomarkers, the phosphorylation levels of EMT-associated AKT and ERK signaling and the expression of their upstream signal regulatory factors, PTEN was measured by Western blotting assay. Compared with the negative vector control group, over-expression of miR-23a increased the expression levels of β-catenin and vimentin, while decreased the expression of E-cadherin (Figure 2B), suggesting that over-expression of miR-23a could induce EMT in GES-1 cells. Meanwhile, over-expression of miR-23a decreased the expression of PTEN and increased the expression of p-AKT and p-ERK in GES-1 cells (Figure 2C), suggesting that over-expression of miR-23a could activate AKT and ERK pathways. These results supported that miR-23a could regulate EMT by modifying the activity of PTEN- and mediated AKT/ERK pathways.

Inhibition of miR-23a could reverse EMT by inactivating AKT and ERK signal activity through up-regulation of PTEN

AGS is a high-metastatic GC cell line and has a high expression level of miR-23a and low expression of PTEN. To further investigate the effect of miR-23a on EMT process of GC cells, the inhibitor of miR-23a was transfected into AGS cells and the expression of miR-23a was measured by real time qRT-PCR. The expression of miR-23a was decreased to more than 6-fold after treated with miR-23a inhibitor, as compared to the parental cell control (Figure 2D). The relative expression levels of EMT biomarkers were measured by Western blotting analysis. The results demonstrated that inhibition of miR-23a increased the protein expression of E-cadherin and decreased the expression of β-catenin and vimentin (Figure 2E). Meanwhile, the relative expression of p-AKT and p-ERK were decreased accompanied by an increase of PTEN and hSulf-1 (Figure 2F), indicating that inhibition of miR-23a could reverse EMT by inactivating AKT and ERK pathways through up-regulation of PTEN.

PTEN regulated gastric cancer cell invasion

Transwell invasion (n = 4) assays showed that GES-1 cells that were transfected with the PTEN siRNA (200 nM) had greater invasive potentials than the control (siRNA control). Meanwhile, Transwell invasion (n = 4) assays showed that AGS cells that were transfected with the PTEN siRNA (200 nM) had greater inva-
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Sive and migratory potentials than the control (siRNA control). All these data suggested that cell invasion ability was significantly enhanced after PTEN downregulation compared with the control (Figure 3A, 3B).

**miR-23a directly inhibited PTEN expression via its 3’UTR**

To understand the mechanisms by which inhibition of miR-23a reduced migration and invasion, we used bioinformatics analysis to identify miR-23a targets. There was a conserved binding site of miR-23a in the PTEN 3’UTR (Figure 4A). To test whether PTEN is a target of miR-23a, we conducted a standard luciferase reporter assay in 293 T cells after cotransfection with (i) a plasmid containing a fragment of PTEN 3’UTR that included either the wild type or mutant predicted miR-23a binding site and (ii) the miR-23a mimic or the mimic control. Data represents the mean ± SD of at least three independent experiments. *P < 0.05. C. Western blot assay showed increased PTEN expression in GES-1 cells after transfection with the miR-23a inhibitor (800 nM). D. Real time PCR showed that miR-23a did not affect PTEN mRNA expression.

**Figure 4. PTEN was a direct target of miR-23a.** A. Putative binding site for miR-23a in the 3’UTR of PTEN was revealed by TargetScan. B. The miR-23a binding site on PTEN 3’UTR was confirmed by luciferase assay in 293 T cells after cotransfection with (i) a plasmid containing a fragment of PTEN 3’UTR that included either the wild type or mutant predicted miR-23a binding site and (ii) the miR-23a mimic or the mimic control. Data represents the mean ± SD of at least three independent experiments. *P < 0.05. C. Western blot assay showed increased PTEN expression in GES-1 cells after transfection with the miR-23a inhibitor (800 nM). D. Real time PCR showed that miR-23a did not affect PTEN mRNA expression.

MiR-23a promotes the growth of GC cell xenografts in nude mice

The parental, pGL3-miR21-EGFP-transfected and pGL3-Ctrl-EGFP-transfected GES-1 cells were used to establish GC xenograft models in nude mice. Three weeks after cell transplantation, tumors in the miR-21-transfected group grew more quickly compared with the parental and negative control groups, and the difference was further intensified on day 35 (Figure 5A). Tumor weight in the miR-21-transfected group was higher than that in the other two groups.
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(Figure 5B). In the miR-23a-transfected tumor cells, the expression of miR-23a was positively increased (Figure 5C), the expression of PTEN was down-regulated, and the expression of p-AKT, p-ERK and Ki67 were up-regulated, compared with the other two groups.

Correlation between miR-23a and PTEN expression in clinical patients

From 2010 to 2011, 91 patients with gastric cancer got surgery in Peking Union Medical College hospital and among them only 38 (stage I) had follow-up data for statistical analysis. Patients with high miR-23a expression had DFS of 32.53 m ± 5.69 m (21.37 m-41.30 m) while patients with low miR-23a expression had DFS of 44.11 m ± 4.61 m (35.07 m-53.15 m) (P = 0.143, Figure 5D). Patients who were PTEN positive had DFS of 48.36 m ± 4.42 m (39.69 m-57.02 m) while patients were PTEN negative had DFS of 28.35 m ± 3.76 m (20.97-35.72 m) (P = 0.039, Figure 5E). Since miR-23a and PTEN were correlated, we draw the conclusion that higher miR-23a expression combined with low PTEN expression could serve as a risk factor for stage1 gastric cancer patients. We didn’t analyze patients in stages 2 and 3 due to the limited patient number.

Discussion

Tumor metastasis is a complex series of steps that involve a number of influential factors. Additionally, due to tumor heterogeneity, the mechanisms underlying distal metastasis could be totally different even though primary tumors possess similar clinical manifestations and histological types [11, 12]. Thus, the advance of detecting biomarker indicative of high-risk tumor metastasis may immensely benefit the approach of personalized cancer treatment. Lately, miRNA has been regarded as an excellent biomarker owing to several unique features: an average 22 nucleotides in length, more stable expression compared with mRNA and more likely to be detected in samples with mRNA and protein degraded. Furthermore, the convenience of synthesizing an overexpressed or an interfering sequence also made miRNA a potential candidate for novel therapeutic strategies [13, 14].
According to the prediction of biological databases, PTEN might be a target gene of miR-23a. PTEN is a well-defined tumor suppressor gene that has been discovered recently. PTEN gene encodes a 442-amino acid protein which contains an extracellular domain, a transmembrane domain and a cytoplasmic domain. Extracellular domain of PTEN of 373 amino acids includes three Ig-like C2-type domain connected by disulfide bonds [15-17]. Therefore, PTEN is considered to be involved in cell-cell interactions. Expression of PTEN is lost or reduced in a variety of cancers, including non-small cell lung cancer (NSCLC) [18, 19], breast cancer [20], cervix cancer [21, 22], and GC [23, 24]. This reduction has been associated with enhanced metastasis potential and poor prognosis.

Our study reports miR-23a could regulate invasion of GC cell via targeting PTEN. We confirmed that miR-23a could promote invasion through PTEN in GC cell lines. Whether miR-23a and PTEN expression are correlated in tumor tissues is not investigated before. Here, using clinical samples from 38 patients of GC, we analyzed miR-23a and PTEN expression by RT-PCR and immunohistochemistry, respectively. In 38 patients who were classified as stage 1 according to TNM, those who were PTEN positive had long DFS while patients were PTEN negative had short DFS and the difference was statistically significant. Patients who had high miR-23a expression had short DFS while those with low miR-23a expression had long DFS, although the difference was not statistically significant.

During the invasion and metastasis of cancers, it is important for tumor cells to acquire a movement ability and anoikis-resistance behavior [25, 26]. EMT is a multistage process that contributes to cancer cells with dramatic changes in cellular morphology, loss and remodeling of cell-cell and cell-matrix adhesions, and gain of migratory and invasive capabilities [27]. In the past years, several signal pathways have been identified that are critical for EMT in cancer progression and metastasis [28, 29]. Activations of AKT and ERK signaling by growth factors notably triggers EMT and endow cancer cells with strong ability to survive or to initiate metastatic tumors [30]. PTEN is a dual protein/lipid phosphatase inside the cells, inactivation of PTEN in cancer cells leads to activated downstream signaling including AKT and/or ERK. Therefore, it is not difficult to understand that miR-23a-mediated decrease of PTEN expression can activate downstream AKT and ERK signaling, and enhances cancer cell proliferation and movement ability by inducing EMT in GC cells. Moreover, this study also found that miR-23a can suppress hSulf-1 and PTEN expression in GC xenograft models in nude mice and increase the phosphorylation of AKT and ERK. The nuclear proliferation antigen Ki67 was up-regulated in miR-23a-transfected GC xenografts, which demonstrated that miR-23a increased the proliferation activity of GC cells, and promote tumor growth. This new insight into miR-23a regulatory function on GC cells may help us to design strategies for cancer target biotherapy.

In conclusion, we showed that miR-23a is highly expressed in more metastatic GC cells and inhibition of miR-23a effectively reduced migration and invasion of GC cells by down-regulation PTEN. More importantly, we found high expression of miR-23a combined with low expression of PTEN might serve as a risk factor for gastric cancer patients. Here, we provide new insights into the metastasis enhancer functions of miR-23a in gastric carcinoma. Identifying small molecules targeting miR-23a might lead to vigorous therapeutic strategies for gastric carcinoma.

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Disclosure of conflict of interest

None.

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