miR-199a Regulates the Tumor Suppressor Mitogen-Activated Protein Kinase Kinase Kinase 11 in Gastric Cancer

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Small noncoding microRNAs (miRNAs) have been shown to play an important role in tumor proliferation and metastasis. However, their function and mechanism in the proliferation and metastasis of gastric cancer has not yet been elucidated. Here, we investigated the relationship between miRNA-199a and gastric cancer proliferation and metastasis. Using real-time reverse-transcriptase (RT)-polymerase chain reaction, we found that miR-199a is highly expressed in gastric cancer compared to normal gastric tissues and in metastatic, compared to non-metastatic gastric cancer tissues. MiR-199a positively regulated gastric cancer cell proliferation, migration and invasion. Further studies showed that miR-199a promoted proliferation and metastasis of gastric cancer cells through a regulatory pathway in gastric cancer that has yet to be described. miR-199a may be useful as a new potential therapeutic target for gastric cancer.

Key words miR-199a; mitogen-activated protein kinase kinase kinase 11; proliferation; metastasis; gastric cancer

Gastric cancer is one of the most common types of cancer worldwide. 1) Proliferation and metastasis of cancer cells remains the main cause of gastric cancer-related death. 2) Better knowledge of changes in gene expression during proliferation and metastasis may lead to improvements in the treatment of advanced gastric cancer. MicroRNAs (miRNAs) are a class of endogenous noncoding, double-stranded small RNA molecules that are synthesized as primary miRNA hairpins, and cleaved by two RNAase III enzymes, Drosha and Dicer. The mature miRNAs negatively regulate gene expression by targeting mRNAs for translational repression or promotion of RNA degradation. In this way, miRNAs are important in the regulation of most central cellular processes, including cellular proliferation and apoptosis. 3–7)

Recently, some miRNAs have been demonstrated to have crucial functions in cancer proliferation and metastasis. 8–13) To date, more than 800 miRNAs have been identified in humans, although most of the miRNAs involved in tumor proliferation and metastasis, especially in gastric cancer, are not well defined. Recently, Uedo et al. 14) investigated the relationship between miRNA expression, and progression and prognosis of gastric cancer. By miRNA expression analysis, they found that 22 microRNAs were upregulated, and 13 were downregulated in gastric cancer, including miR-199a-1/2. Here, we investigated the role and mechanism of miR-199a-1/2 in proliferation and metastasis of gastric cancer. We find a possible connection between miR-199a and the putative tumor suppressor mitogen-activated protein kinase kinase kinase kinase 11 (MAP3K11), previously associated with metastatic properties of gastric cancer cells. 15)

MATERIALS AND METHODS

Human Cell Lines and Clinical Samples The human gastric cancer cell lines BGC823 and AGS were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 2 mM L-glutamine (Invitrogen, Carlsbad, CA, U.S.A.), and grown at 37°C in 10% CO2. Tissues from 8 patients with metastatic, from 8 patients with non-metastatic cancer, from 8 patients with normal gastric mucosa and from 5 adjacent noncancerous counterparts were snap-frozen and stored in liquid nitrogen after collection. Formalin-fixed and paraffin-embedded samples were obtained from non-metastatic gastric cancer tissue, and metastatic gastric cancer tissue, from 80 patients who underwent surgery at the first affiliated hospital of Harbin Medical University, Harbin, China. All patients provided written informed consent for the use of their tissues. All cases of gastric cancer were clinically and pathologically proven (data not shown). Study protocols were approved by the hospital’s Protection of Human Subjects Committee.

RNA Extraction and Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) RNA extraction and quantitative reverse transcriptase (qRT) real-time PCR were performed as previously described. 16) Total RNA was isolated from formalin-fixed paraffin-embedded specimens as 15 sections of 10 microns, and miRNAs were extracted from tissues or cells using Trizol reagent (Invitrogen, Carlsbad, U.S.A.) and processed and enriched with an miRNeasy mini-column (miRNeasy Mini Kit, Qiagen, Hilden, Germany). In brief, tissue samples were treated with Trizol and then chloroform. The mixture was centrifuged at 12000×g for 15 min at 4°C. A 1.5-fold volume of 100% ethanol was added to the aqueous layer. The mixture was then applied to an miRNeasy mini column (Qiagen) and processed according to the manufacturer’s recommendations. qRT-PCR was performed with a mirVana qRT-PCR miRNA detection kit (Ambion, U.S.A.) using hsa-miR199a primers (Ambion), according to the manufacturer’s instructions. U6 snRNA primers (Ambion) were used as a control.

Transfection Cells were transfected for 24 h with 60 nm of miR-199a-2 precursor or inhibitor (Ambion) with Lipo-fectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.) in antibiotic-free medium, at 30 to 50% confluency, in 60-mm dishes. Transfection with small interfering (si)RNA against MAP3K11 (Dharmacon, Lafayette, CO, U.S.A.), or non-
targeting siRNA controls at 100 nm, was in 6-well culture plates, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.). Cells were collected at 0 and 24 h. Gene silencing was verified by real-time PCR.

miRNA Target Predictions The computer-based miRNA target detection programs, TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org/microrna/home.do) were used to predict miR-199a binding sites in potential target miRNAs.

Dual-Luciferase Activity Assay Dual-luciferase activity assays were performed as previously described. Luciferase reporter constructs had miRNA target sites subeloned into the XhoI and NotI sites of the pMIR-REPORT vector (Ambion). The synthetic sequences were 5′-tccagGAGGACUCACAGCAUACUGAGAUGACACAGCAUA-CACUGGAgc-3′ and 5′-ggccgcTCCAGGCTGTCTGTTGACTCTCCTTCCAGTATGCTGTGACTTCC-3′. The pMIR-RB-3′ UTR-mut reporter construct with point mutations in the seed sequence was synthesized using a site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). AGS cells were transfected for 24 h in 24-well plates with 0.5 μg of pMIR constructs with or without 40 nM of miR-199a-2 precursor or inhibitor. Luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega), 24 h after transfection. Relative luciferase activity was normalized to Renilla luciferase activity. Each sample was assayed in triplicate.

3′-4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium Bromide (MTT) Cell Growth Assay 4000 cells were transfected with 60 nM of miRNA precursor or inhibitor for 4000 cells were assayed in triplicate. Relative luciferase activity was normalized to Renilla luciferase activity. Each sample was assayed in triplicate.

Detection of Apoptosis and Cell Death Apoptosis and cell death assay were performed as previously described. The Annexin V FITC apoptosis detection kit (BD Pharmingen, San Jose, CA, U.S.A.) was used to detect cell apoptosis. 105 cells were seeded in a 6-well plate, then were transfected with or without the miRNA precursors or inhibitors and incubated for 48 h. Cells were then washed twice with phosphate buffered saline (PBS) and resuspended in Annexin V-binding buffer. Cell suspension was incubated with 5 μl of Annexin V fluorescein isothiocyanate (FITC) and 5 μl of propidium iodide in the dark for 15 min at room temperature. The samples were then analysed on the FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, U.S.A.). The results were counted. Each experiment was performed in triplicate.

Wound-Healing Assay 1×105 cells were plated in a 12-well plate. Two parallel wounds of 1 mm were made using a pipette tip at 100% confluence. Wound size after 24 h was measured using Zeiss LSM Image Browser software, version 3.1, in three independent experiments.

In Vitro Invasion Assay In Vitro invasion assays were performed as described using Boyden chamber assay. Matrigel was added to a polycarbonate filter and air-dried. Filters were placed into 24-well plates and 700 μl of DMEM containing 10% bovine serum was added into the lower compartment. Cells were suspended at 2×103/ml in DMEM containing 1% bovine serum, and preincubated for 10 min with or without miR-199a-2 precursor or inhibitor. Cells incubated for 48 h that had invaded the bottom surface of the filter were fixed with methanol and stained with hematoxylin. The number of penetrating cells was counted with a microscope at 200× magnification, in 10 random fields per well. Each experiment was performed in triplicate.

In Vitro Migration Assay In vitro migration assays were performed as previously described using transwells [8-μm pore size, Corning Costar Corp., U.S.A.] without Matrigel. Cells were suspended at 1×105/ml in DMEM containing 1% bovine serum and preincubated for 10 min with or without miR-199a-2 precursor or inhibitor. After incubation for 24 h, cells that had migrated into the bottom surface of the filter were fixed with methanol and stained with hematoxylin. Penetrating cells were counted as described above. Each experiment was performed in triplicate.

Western Blots Cells were washed twice with Hank’s balanced salt solution and lysed directly in radio immunoprecipitation assay (RIPA) buffer. Cell or tissue lysates (60 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) minigel and transferred to nitrocellulose membranes (Millipore, Billerica, MA, U.S.A.), which were incubated with a primary antibody against MAP3K11 (Santa Cruz Biotechnology, CA, U.S.A.) or a mouse monoclonal anti-β-actin (diluted 1: 5000; Sigma Chemical Co.) at room temperature for 2 h, washed extensively with 0.1% Tween-20 in phosphate-buffered saline and incubated with secondary antibody conjugated with horse-radish peroxidase at 1: 3000 dilution (Santa Cruz Biotechnology). Signals were visualized using enhanced chemiluminescence (Amersham Life Science Inc., Buckinghamshire, U.K.).

Statistical Analysis Data are expressed as the mean± standard error of the mean (S.E.M.) from at least three independent experiments. Bands from Western blots were quantified by Quantity One software (BioRad). The difference between groups was performed with ANOVA, and a post-hoc test. Luciferase reporter assays were analyzed by two-tailed Student’s t-test. Analysis, including Kaplan–Meier survival curves, used the Statistical SPSS software package (SPSS Inc., Chicago, U.S.A.). Differences were considered significant for p-values less than 0.05.

RESULTS

Increased Expression of miR-199a in Metastatic Gastric Cancer Correlated with Prognosis We investigated the expression of miR-199a in 8 metastatic and 8 nonmetastatic snap-frozen gastric cancer tissues and 8 normal gastric tissues. Our results showed that miR-199a levels were significantly higher in metastatic gastric cancer than in nonmetastatic gastric cancer in all 8 cases, in which miR-199a levels were significantly higher than in normal gastric tissues (Fig. 1A). These results suggest that miR-199a expression was significantly overexpressed in human gastric cancer. We then examined the expression of miR-199a in formalin-fixed and paraffin-embedded paired samples of metastatic and nonmetastatic gastric cancer tissues from 80 patients. Our re-
sults showed that miR-199a was highly expressed in most of the 40 metastatic gastric cancer tissues compared to the 40 nonmetastatic gastric cancer tissues. Furthermore, clinical relevance was confirmed by the observation that miR-199a expression correlated with prognosis (Figs. 1B, C).

**Effect of miR-199a on Proliferation, Invasion and Migration of Gastric Cancer Cells in Vitro** Firstly, we investigated the effect of miR-199a on proliferation of gastric cancer cell AGS and BGC823 using *in vitro* MTT assay. As shown in Fig. 2A, both knockdown and forced expression of miR-199a were effective. We found that increased miR-199a expression by transfection with a miR-199a-2 precursor significantly promoted the proliferative ability of AGS and BGC823 with an average promotion rate of 38.5% for AGS cells, and 42.2% for BGC823 cells. Knockdown of miR-199a by transfection with a miR-199a inhibitor significantly suppressed the proliferative ability of these cells, with an average inhibition rate of 62.5% for AGS cells, and 55.1% for BGC823 cells (Fig. 2D). Similar significant effects were observed in *in vitro* migration assays (Fig. 2E). These results provide strong evidence that miR-199a plays a role in promoting proliferation and metastasis of gastric cancer cells.

**MiR-199a Targets MAP3K11 Expression** MiRNAs exert their functions by targeting the 3′ untranslated region (UTR) of target genes to induce mRNA degradation or translational repression. Predicting the target genes of miR-199a using a combination of the TargetScan and miRanda miRNA-target-prediction programs, we identified the 3′UTR of MAP3K11 as one of the potential targets of miR-199a. To confirm whether the predicted miR-199a target site in the 3′UTR of MAP3K11 mRNA was responsible for its regulation, we performed luciferase reporter assays. These results showed that miR-199a significantly decreased the relative luciferase activity in AGS cells (Fig. 3A). We investigated if miR-199a affected the MAP3K11 protein levels by Western blot, or mRNA levels by qRT real-time PCR. We found that miR-199a decreased the MAP3K11 protein level but did not affect the mRNA level in the AGS and BGC823 cell lines (Fig. 3B). Taken together, our results strongly support that MAP3K11 is a direct target of miR-199a.

**MiR-199a Promotes Proliferation and Metastasis of Gastric Cancer by Regulating MAP3K11** Although we confirmed MAP3K11 is a target of miR-199a, we did not know if miR-199a regulated MAP3K11 to promote proliferation and metastasis of gastric cancer cells. Thus, we investigated the hypothesis that miR-199a regulates cellular proliferation and metastasis through MAP3K11. Firstly, we found MAP3K11 expression levels in 5 gastric cancer tissues were all lower than that in normal gastric tissues and adjacent noncancerous tissues (Fig. 4A). Then, we examined the effect of MAP3K11 on the proliferative and metastatic abilities of gastric cancer cells. We found that specific siRNA that decreased the MAP3K11 protein level (Fig. 4B), significantly increased the proliferative, invasive and migratory abilities of AGS and BGC823 cells (Figs. 4C—E). As observed above, a decrease in proliferative and metastatic characteristics was induced by anti-miR-199a, which downregulated miR-199a, thereby upregulating MAP3K11. This effect was counteracted by co-transfection with MAP3K11 siRNA (Figs. 4C—E). This fur-
ther demonstrated that miR-199a was associated with the post-transcriptional downregulation of MAP3K11 in gastric cancer cells.

DISCUSSION

Uncontrolled proliferation and metastasis are factors in the mortality of gastric cancer patients. Currently, however, the exact proliferative and metastatic mechanisms of gastric cancer are still not fully elucidated. miR-199a-1 and miR-199a-2 are located on different chromosomes but the sequence of mature microRNA is the same. In this study, we confirmed that miR-199a is upregulated in gastric cancer in vivo, consistent with the findings of Ueda et al. and found that expression correlated with prognosis.

MAP3K11 was identified as a possible target of miR-199a by two target-predicting programs. MiR-199a was shown to regulate a reporter with a putative binding sequence from MAP3K11, which was previously identified as altered in expression in several cancer types, including AGS cancer cells. Mitogen-activated protein kinase kinase kinase 11 (MAP3K11) is also named mixed lineage kinase 3 (MLK3), which was identified from human thymus. The gene encoding MLK3 has been mapped to human chromosome 11q13.1—13.3, a region frequently altered in human malignancies. This kinase contains a SH3 domain and a leucine zipper-basic motif, which can activate MAPK8/c-Jun N-terminal kinase (JNK) kinase, and function as a positive regulator of JNK signaling pathway. Mota et al. showed that overexpression of an active MLK3 induced activation of the JNK pathway and apoptosis in SCG neurons. Also, this kinase can directly phosphorylate, and activates inhibitory κB (IκB) kinase α and β, and is found to be involved in the transcription activity of nuclear factor κB (NFκB) mediated by Rho family GTPases and CDC42. Although some studies showed that MAP3K11 might act as activator on growth in some tumor cells, its exact effects on tumor cells are not elucidated. Here, our results demonstrated that knockdown of MAP3K11 could promote the in vitro proliferation and metastasis of gastric cancer cell AGS and BGC823. The re-
results presented here are consistent with a tumor suppressor function of MAP3K11 in gastric cancer,\textsuperscript{15}) in that proliferative and metastatic properties increased with knockdown of MAP3K11. This may be due to the different genetic background of different tumor type.

We hypothesized that miR-199a would control cancerous properties of gastric cancer cells through MAP3K11, and using the AGS and BGC823 gastric cancer cell lines, found that increasing or decreasing miR-199a expression inhibited in vitro proliferative, invasive and migratory characteristics of gastric cancer cells. Further, we found that miR-199a was high expressed in AGS cells. Also MAP3K11 was lowly expressed in AGS cells. Our results showed that miR-199a could bind to the 3’UTR of MAPK311 to inhibit translation of MAP3K11. Of course, it is reasonable that when we down-regulated the expression of miR-199a by introducing the anti-miR-199a, the inhibitory effect of miR-199a on MAP3K11 will be inversed. Then anti-miR-199a increased the MAP3K11 protein. This demonstrated that the mature miR-199a may play its function on inhibiting apoptosis and promoting proliferation and metastasis of gastric cancer cells least via MAP3K11. These properties increased when MAP3K11 was knocked down with siRNA. Consistent with the model that miR-199a has post-transcriptional effect on MAP3K11, treatment of MAP3K11 knockdown cells with anti-miR-199a did not alter the increase in proliferative and metastatic properties.

Taken together, our results revealed that MAP3K11 appears to be a post-transcriptional target of miR-199a. Altering the levels of miR-199a or MAP3K11 altered the proliferative, invasive and migratory characteristics of gastric cancer cells in vitro, consistent with a model in which MAP3K11 inhibits proliferation and metastasis, and miR-199a acts as an oncogenic miRNA to promote proliferation and metastasis. The level of miR-199a expression in gastric cancer samples significantly correlated with patient prognosis, suggesting that miR-199a may be useful as a therapeutic target or diagnostic marker for metastatic gastric cancer.
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