miR-384 targets metadherin gene to suppress growth, migration, and invasion of gastric cancer cells

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Abstract
Objective: MicroRNA-384 (miR-384) has been reported to function as a tumor suppressor in multiple cancers; however, its role in gastric cancer (GC) remains unclear.
Methods: We measured expression levels of miR-384 in GC cell lines and in a normal gastric cell line (GES-1). The association between miR-384 and the metadherin gene (MTDH) was assessed by luciferase reporter assay and western blot. The effects of the miR-384/MTDH axis on GC cell behaviors were measured by CCK-8, wound-healing, and transwell invasion assays.
Results: miR-384 was significantly downregulated in GC cell lines compared with normal gastric cells. MTDH was identified as a direct target of miR-384 by bioinformatics analysis, luciferase assay, and western blot. Functional assays demonstrated that miR-384 inhibited GC cell proliferation, migration, and invasion through targeting MTDH.
Conclusion: These results reveal that miR-384 acts as a tumor suppressor in GC and suggest that the miR-384/MTDH axis may be a potential therapeutic target for GC.

Keywords
miR-384, metadherin, gastric cancer, tumor suppressor, microRNA, cell invasion, cell proliferation, cell migration

Date received: 2 August 2018; accepted: 13 November 2018

Introduction
Gastric cancer (GC) ranks as the second most diagnosed cancer and second leading cause of cancer-related deaths in China. Although the incidence and mortality of...
GC have decreased significantly in recent years, the number of cases diagnosed annually continues to increase as a result of population growth. Notably, the survival rate of patients with GC remains poor due to a lack of suitable screening and treatment biomarkers. There is thus an urgent need to investigate the mechanisms underlying GC initiation and progression.

MicroRNAs (miRNAs) are endogenous RNAs that can inversely regulate gene expression via binding to the complementary 3'-untranslated region (3'-UTR). Over 1,000 miRNAs have been identified in the human genome to date, and are responsible for regulating approximately 30% of all human genes. miRNAs have been reported to function as either tumor suppressor genes or oncogenes. For example, miR-100 was downregulated in GC and regulated GC cell proliferation by targeting chemokine (CXC motif) receptor 7, and was also identified as a prognostic indicator for GC. In contrast, miR-3129 was overexpressed in GC and functioned as oncogene by regulating retinoblastoma protein, suggesting that this miRNA might be a therapeutic target for GC. These studies have collectively revealed the importance of miRNAs in regulating GC progression.

miR-384 is located at chromosome Xq21.1. The expression of miR-384 has been reported to be downregulated in multiple human cancers, including breast cancer, osteosarcoma, glioma, and non-small-cell lung cancer, indicating a tumor suppressive role in these cancers. These studies also identified multiple target genes of miR-384, thus establishing its importance in cancers. However, the role of miR-384 in GC has not been investigated.

In the present study, we determined the expression levels of miR-384 in GC cell lines. We further transfected GC cell lines with synthetic miRNAs to investigate the biological roles of miR-384 in GC cell behaviors. We also investigated the direct target of miR-384 by dual-luciferase reporter and western blot assays, and carried out functional assays to explore the mechanism whereby miR-384 regulates GC cell behaviors.

Materials and methods

Cell lines and culture

Three GC cell lines (BGC-823, SGC-7901, HGC-27) and one normal gastric cell line (GES-1) were purchased from the American Type Culture Collection (Manassas, VA, USA). The cell lines were incubated in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (Thermo Fisher Scientific) at 37°C in a humidified atmosphere containing 5% CO2.

Target gene identification

Target genes of miR-384 were predicted using TargetScan algorithm (http://www.targetscan.org/vert_72/). Among the predicted miR-384 targets, metadherin (MTDH) was selected for further investigation.

Cell transfection

The cells were divided into three groups and transfected with miR-384 mimic, miR-384 inhibitor, or negative control (NC) miRNA (RiboBio Inc., Guangzhou, China), respectively. Constructs overexpressing MTDH and NC vector were purchased from GenScript (Nanjing, China). Cell transfection was conducted using Lipofectamine 2000 (Thermo Fisher Scientific) following the manufacturer’s instructions. Cells were collected for subsequent experiments after transfection for 48 hours.
Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the transfected cells using TRIzol reagent (Beyotime, Haimen, Jiangsu, China), according to the manufacturer’s protocols. The extracted RNA was reverse transcribed to cDNA using a BeyoRT™ cDNA synthesis kit (Beyotime), according to the manufacturer’s protocol. qRT-PCR was conducted using BeyoFast™ SYBR Green qPCR Mix (Beyotime) with an ABI 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific) according to the following protocol: initial step at 95°C for 60 seconds, followed by 95°C denaturation for 10 seconds, and 60°C annealing for 30 seconds (40 cycles). Expression levels of miR-384 were analyzed using the 2^(-ΔΔCt) method with U6 snRNA as an internal control.14 The primers used in this study are shown in Table 1.

Western blot assay

Total protein was isolated from transfected cells using radioimmunoprecipitation assay lysis buffer (Beyotime), according to the manufacturer’s recommendations. The protein concentration was determined using a bicinchoninic acid kit (Beyotime). Samples with equal amounts of protein (30 μg) were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane (Beyotime). The membranes were incubated with the following primary antibodies: rabbit anti-MTDH (ab124789), rabbit anti-matrix metalloproteinase (MMP)-9 (ab73714), and rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (ab181602) (all Abcam, Cambridge, MA, USA) after blocking with 5% non-fat milk. The membranes were subsequently treated with horseradish peroxidase-linked goat anti-rabbit secondary antibody (ab205718; Abcam) after washing with TBST. Band signals were detected using enhanced chemiluminescence reagents (Beyotime) and analyzed using Image J 1.42 software (NIH, Bethesda, MD, USA).

Luciferase activity assay

Wild-type (wt) or mutant (mut) sequences containing the putative binding site of miR-384 in the MTDH 3’-UTR were cloned into pmirGLO vector and designated as MTDH-wt or MTDH-mut, respectively. Cells were co-transfected with MTDH-wt or MTDH-mut and miR-384 mimic or NC using Lipofectamine 2000 (Thermo Fisher Scientific), according to the manufacturer’s instructions. After 48 hours of co-transfection, the cells were collected for luciferase activity determination using a dual-luciferase reporter assay system (Promega, Madison, WI, USA) following the manufacturer’s instructions.

Cell counting kit-8 (CCK-8) assay

Proliferation of the transfected cells was measured by CCK-8 assay (Beyotime). Transfected cells were seeded at 5,000 cells/well onto 96-well plates and incubated for 0, 24, 48, or 72 hours. CCK-8 reagent was added to the plates at the above indicated points and incubated for a further 2 hours. The optical density at 450 nm was

| Table 1. Primer sequences used in this study |
|-----------------|-----------------|
| Gene      | Sequence                                   |
| miR-384   | Forward: 5’-TGTTAAATCAGG AATTAAA-3’  |
|           | Reverse: 5’-TGTTACAGGCAT TATGAA-3’   |
| U6 snRNA | Forward: 5’-CGCTTCGGCCAGC ACATATAAC-3’ |
|           | Reverse: 5’-TTCACGAATTTC GTGTCAT-3’   |
then determined using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Experiments were conducted in triplicate.

**Wound-healing assay**

Migration of the transfected cells was measured by wound-healing assay. Transfected cells were seeded at 50,000 cells/well onto 6-well plates and incubated to approximately 90% confluence. A wound was created at the cell surface using a pipette tip and cell debris was removed by rinsing with TBST. Wound width was calculated under a microscope (Olympus, Tokyo, Japan) at 0 and 24 hours after scratching. Experiments were conducted in triplicate.

**Transwell invasion assay**

Invasion of the transfected cells was measured by Transwell invasion assay. Transfected cells were seeded onto the upper chamber (8 μm; Corning Inc., Corning, NY, USA) coated with Matrigel. RPMI-1640 without fetal bovine serum was added to the upper chamber, while the lower chamber was supplemented with RPMI-1640 and 10% fetal bovine serum. Non-invading cells at the upper surface were removed using a cotton swab after incubation for 24 hours and the invaded cells were stained with 0.5% (w/v) crystal violet and counted under a microscope (Olympus).

**Statistical analysis**

Data were presented as mean ± standard deviation and compared by Student’s t-test (two groups) or one-way analysis of variance with Tukey’s post hoc test (more than two groups). Data analysis was conducted using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). Differences were considered significant when \( P < 0.05 \).

**Results**

**Downregulation of miR-384 in GC cell lines**

The expression levels of miR-384 in three GC cell lines (BGC-823, SGC-7901, HGC-27) and one normal gastric cell line (GES-1) were analyzed by qRT-PCR. miR-384 expression levels were significantly reduced in all the GC cell lines compared with the normal cells (BGC-8923 and HGC-27, \( P < 0.01 \); SGC-7901, \( P < 0.001 \)) (Figure 1). miR-384 expression levels in the GC cell lines descended in the order BGC-823, HGC-27, and SGC-7901 (Figure 1). We therefore selected SGC-7901 cells for subsequent functional studies.

**Overexpression of miR-384 decreased GC cell proliferation, migration, and invasion**

We investigated the biological roles of miR-384 in GC by performing gain- and loss-of function assays. miR-384 mimic significantly increased miR-384 expression in SGC-7901 cells, while miR-384 inhibitor decreased miR-384 expression levels (both \( P < 0.001 \)) (Figure 2a). Overexpression of miR-384 significantly decreased cell proliferation, while miR-384 inhibitor significantly increased cell proliferation (both \( P < 0.001 \)).

![Figure 1. miR-384 expression was significantly downregulated in GC cell lines (BGC-823, SGC-7901, HGC-27) compared with normal gastric cells (GES-1). ***P < 0.001, **P < 0.01. GC: gastric cancer; miR-384: microRNA-384](image-url)
as demonstrated by CCK-8 assay (Figure 2b). Furthermore, ectopic expression of miR-384 significantly decreased cell migration (both $P < 0.01$), as demonstrated by wound-healing assay (Figure 2c). Similar trends were observed in the Transwell invasion assay, indicating that cell invasion was increased by miR-384 inhibitor and
decreased by miR-384 mimic (both $P < 0.01$) (Figure 2d). We further analyzed the expression of MMP-9, as a marker of invasion, in cells transfected with synthetic miRNAs and showed that miR-384 overexpression significantly decreased the expression of MMP-9 (Figure 2e), while the miR-384 inhibitor had the opposite effect (Figure 2e). These results demonstrated that miR-384 suppressed GC cell proliferation, migration, and invasion.

MTDH was a direct target of miR-384

The online prediction tool TargetScan was used to identify target genes containing the putative binding site for miR-384. MTDH was shown to contain a complementary binding sequence for miR-384 in its 3’-UTR (Figure 3a). Transfection with miR-384 mimic significantly decreased luciferase activity in cells transfected with MTDH-wt ($P < 0.001$) but not MTDH-mut, as shown by luciferase reporter assay (Figure 3b). Furthermore, we examined the effects of miR-384 on MTDH expression by western blot, and showed that MTDH protein levels were significantly inhibited by miR-384 mimic (Figure 3c) but elevated by miR-384 inhibitor compared with NC (Figure 3c) ($P < 0.001$). These results suggested that MTDH was a direct target of miR-384 in GC.

MTDH was a functional target of miR-384 during its inhibition on GC cells

We explored the role of MTDH in GC by co-transfecting miR-384 mimic and an MTDH construct into SGC-7901 cells. The MTDH construct significantly increased MTDH protein levels compared with the NC group, as shown by western
Figure 4. Overexpression of MTDH impaired miR-384-induced inhibition of GC cell proliferation, migration, and invasion. (a) MTDH and MMP-9 expression, (b) cell proliferation, (c) cell migration (×200), and (d) cell invasion in SGC-7901 cells transfected with MTDH construct or miR-384 mimic (crystal violet stain; ×200). **P < 0.01, ns: not significant. MTDH: metadherin; GC: gastric cancer; miR-384: microRNA-384; NC: negative control; OD: optical density; MMP-9: matrix metallopeptidase 9

blot (P < 0.001) (Figure 4a). Notably, the inhibitory effects of miR-384 mimic on MTDH levels could be reversed by transfection with the MTDH construct (Figure 4a). We also detected expression levels of MMP-9 after transfection with the MTDH construct and showed that MMP-9 expression was increased by MTDH overexpression (Figure 4a). Functional assays revealed that overexpression of MTDH significantly
increased GC cell proliferation, migration, and invasion (all $P < 0.01$), suggesting that the MTDH construct acted as an miR-384 inhibitor (Figure 4b-4d). Notably, transfection with the MTDH construct partly reversed the inhibitory effects of the miR-384 mimic on GC cell behaviors (Figure 4b-4d). These results suggested that MTDH was a functional target of miR-384 in GC.

**Discussion**

GC is prevalent in East Asia, especially in China. Various miRNAs have been suggested to play important tumor suppressor or oncogenic roles in GC, although the mechanisms whereby miRNAs impact the behaviors of GC cells remain unclear. Furthermore, miR-384 has been reported to function as a tumor suppressor in multiple human cancers, but its function in GC has not previously been examined.

In the present study, we showed that miR-384 expression was significantly downregulated in GC cell lines compared with normal gastric cells. Tumor cells are characterized by uncontrolled growth, accelerated invasion, and metastasis. We therefore investigated the effects of miR-384 on GC cell behaviors. miR-384 overexpression inhibited GC cell proliferation, migration, and invasion, while downregulation of miR-384 by miR-384 inhibitor had the opposite effect. Moreover, expression of the invasion biomarker MMP-9 was increased by miR-384 downregulation and decreased by miR-384 overexpression. These data provide the first evidence indicating that miR-384 functions as a tumor suppressor in the progression of GC.

Previous studies suggested that miRNAs act by regulating downstream tumor-associated genes. It is therefore necessary to investigate the targets of miR-384 in GC to determine the mechanisms underlying its role in the progression of GC.

Using mechanistic assays (luciferase reporter assay and western blot), we demonstrated that MTDH was a direct target of miR-384. MTDH is a highly conserved protein that was first identified in primary human fetal astrocytes, and which has subsequently been found to be overexpressed in multiple human tumor tissues and cell lines. Importantly, MTDH is considered as an oncogene in human cancers, and has been associated with tumor progression and metastasis. Recent studies showed that MTDH expression in human cancers was regulated by miRNAs. For example, miR-202 was revealed as a tumor suppressor in glioma and regulated cell proliferation, migration, and invasion through targeting MTDH, while miR-375 overexpression downregulated MTDH expression, and the miR-375/MTDH axis was revealed as a potential therapeutic target in esophageal squamous cell carcinoma. MTDH was recently found to be upregulated by miR-197 in GC, and therefore also has potential as a target for GC treatment. In the current study, overexpression of MTDH reversed the effects of miR-384 on GC cell behaviors, thus highlighting the importance of MTDH in GC. A previous study showed that miR-384 expression could regulate the expression of astrocyte elevated gene-1 in non-small cell lung cancer, thus affecting cell migration and invasion. The current results validated the role of miR-384 in cancer cell proliferation, migration, and invasion in another cancer type, GC.

The current study had some limitations. We only examined the functions of the miR-384/MTDH axis in GC in vitro, and further in vivo experiments should be performed to validate these results. Furthermore, the relationship between miR-384 and MTDH expression and overall survival of GC patients should also be investigated.

The results of this study further our knowledge regarding the biological roles of miR-384 and its target gene, MTDH,
in GC, and thus help to advance our understanding of the mechanisms underlying the progression of GC. These results also suggest that miR-384 may be a novel therapeutic target for GC management in the near future.

Declaration of conflicting interest
The authors declare that there is no conflict of interest.

Funding
This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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