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MiR-199a-3p promotes gastric cancer progression by targeting ZHX1

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ABSTRACT

Accumulating evidence has indicated that microRNAs (miRNAs) act as critical epigenetic regulators in tumor carcinogenesis. Here, we report that miR-199a-3p was significantly upregulated in gastric cancer (GC) cell lines and tissues. Functional studies demonstrated that miR-199a-3p dramatically increased cell proliferation and suppressed cell apoptosis both in vitro and in vivo. Furthermore, the transcriptional regulator zinc fingers and homeoboxes 1 (*ZHX1*) was identified as one of the direct downstream targets of miR-199a-3p, miR-199a-3p bound to the *ZHX1* 3′ untranslated region (3′UTR) to regulate *ZHX1* protein expression. In addition, the expression of miR-199a-3p was inversely associated with that of *ZHX1* in GC cell lines. Overexpression of miR-199a-3p in SGC-7901 cells inhibited *ZHX1* expression, while reduction in miR-199a-3p by inhibitors in NCI-N87 cells enhanced *ZHX1* expression. Moreover, restoring *ZHX1* expression in SGC-7901/miR-199a-3p cells inhibited the cell proliferation induced by miR-199a-3p. Taken together, these findings suggest that miR-199a-3p may function as a novel tumor promoter in GC and its oncogenic activity may involve the direct targeting and inhibition of *ZHX1*.

1. Introduction

Gastric cancer (GC) is one of the most common cancers and the second lethal cancer worldwide [1]. Although mortality rates of GC have decreased significantly, the absolute number of GC cases and deaths is still a big burden [2]. Therefore, a better comprehending of the molecular pathways related to the carcinogenesis of GC is essential to reduce GC mortality. Evidence collected to date has indicated that the microRNAs (miRNAs) are newfangled regulators of tumor development and new targets for cancer treatments in GC [3].

MiRNAs are small non-coding, single-stranded endogenous RNA molecules that degrade mRNAs and suppress protein expression [4]. Compelling evidences had proved that miRNAs modulate most cellular processes involved in tumor biological behaviors such as cell proliferation, apoptosis, differentiation and metastasis [5]. Abnormal expression of miRNAs, which function as oncogenes or tumor suppressor genes, can exist in many malignant tumors and is closely related to the occurrence and development of tumors [6]. Although plenty of miRNAs in relation to GC have been studied, the underlying mechanism of these miRNAs in carcinogenesis of GC remains to be investigated.

MiR-199 was identified from mouse skin cells and it was cloned from the human osteoblast sarcoma cell [7]. The expression levels of miR-199a-3p vary in different tumor cells, and highly expressed miR-199a-3p can role as a potential oncogene in breast cancer [8], while suppressed miR-199a-3p can act as a possible tumor suppressor in hepatocellular carcinoma [9]. Our previous study found that the expression of miR-199a-3p in the plasma was substantially up-regulated in GC [10] and that miR-199a-3p in the plasma could serve as a potential diagnostic marker for early GC [11]. MiR-199a has been reported in recent studies on GC [12–14], while the role of miR-199a-3p in GC progression has not been explored.

In the present study, we demonstrated a general increase in the miR-199a-3p expression level in 52 GC tissues compared with the non-tumor tissues, and found that the miR-199a-3p expression levels are associated with tumor invasion and the TNM stage. In addition, we discovered that miR-199-3p could induce the growth and inhibit apoptosis of GC cells both in vitro and in vivo by directly targeting the transcription factor zinc fingers and homeobox 1 (*ZHX1*).

Abbreviations: GC, gastric cancer; MiRNAs, microRNAs; ZHX1, zinc fingers and homeoboxes 1; UTR, untranslated region; HCC, hepatocellular carcinoma

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2. Materials and methods

2.1. Cell culture and clinical specimens

Preparation of clinical samples, gastric cancer cell lines AGS, MKN-45, MKN-28, SGC-7901, NCI-N87, BGC-823, immortalized normal gastric mucosal epithelial cell line (GES-1) and human embryonic kidney cell line 293T (HEK 293T) was described in [15]. The six gastric cell lines and GES-1 cells were grown in RPMI-1640, while HEK 293T was grown in DMEM (Sigma, St. Louis, MO, USA) with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cell lines and clinical specimens using an mirVana™ miRNA Isolation Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The expression level of miR-199a-3p in cell lines and tissues was measured by qRT-PCR according to the Taqman® MicroRNA Assays protocol (Applied Biosystems) and normalized using U6 small nuclear RNA (RNU6B; Applied Biosystems) by the $2^{-\Delta CT}$ method. The relative expression ratio of miR-199a-3p in each paired tumor and non-tumor tissue was calculated by the $2^{-\Delta\Delta CT}$ method. The expression level of *ZHX1* mRNA was measured by qRT-PCR according to the SYBR Green real-time PCR Assay protocol (Applied Biosystems). The GAPDH mRNA level was used for normalization. The expression level of *ZHX1* mRNA relative to GAPDH mRNA was calculated using the $2^{-\Delta CT}$ method.

2.3. Transient transfection

MiR-199a-3p mimics (dsRNA oligonucleotides), negative control (NC), miR-199a-3p inhibitor, and inhibitor negative control were purchased from GenePharma (Shanghai, China). The pEGFP-*ZHX1* (1–873) plasmid was donated by Prof. Kazuya Yamada (Matsumoto University Graduate School of Health Science). Transfection of cells with oligonucleotides was performed using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) at a final concentration of 100 nM. Transfection efficiency was monitored by qRT-PCR.

2.4. Cell proliferation assay

At 24 h post transfection with miR-199a-3p mimics/inhibitors or control oligonucleotides, cells were seeded into 96-well plates ($1.5 \times 10\text{^}5$ cells/well) and cell proliferation was documented every 24 h for 4 days. Cell proliferation was assessed in triplicates by the water-soluble tetrazolium salt (WST) assay using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) following the manufacturer's instructions.

2.5. Soft agar colony formation assay

MiRNA mimics/inhibitors-transfected SGC-7901/NCI-N87 cells were resuspended with 0.3% soft agar (A9045, low gelling temperature, Sigma–Aldrich, St. Louis, MO, USA) in RPMI 1640 containing 10% FBS and layered on 0.6% solidified agar in RPMI 1640 containing 10% FBS in six-well plates ($1 \times 10\text{^}3$ cells/well) 24 h post transfection. The plates were incubated for 2 weeks. Colonies containing at least 50 cells were counted.

2.6. Flow cytometric analysis for apoptosis

One day before transfection, $2 \times 10\text{^}6$ cells were seeded into six-well culture plates without antibiotics. The cells were transfected with miR-199a-3p mimics/miR-199a-3p inhibitor or their respective controls. Forty-eight hours after transfection, cells were harvested and stained with the AnnexinV/PI double staining kit (BD Biosciences, USA) according to the manufacturer's protocol. Apoptotic cells were assessed in triplicates by flow cytometry on a FACScan (Beckman Instruments, Fullerton, CA, USA). Experiments were repeated three times.

2.7. Lentiviral transfection for stable expression clones

LV3-pGLV-H1-GFP+Puro plasmids with hsa-miR-199a-3p inhibitor or control oligonucleotides, namely LV-anti-miR-199a-3p and LV-anti-miR-NC, were purchased from GenePharma (Shanghai, China). Lentivirus transfections were performed according to the manufacturer's instructions to establish anti-miR-199a-3p-expressing stable clones in NCI-87 cells (NCI-N87/anti-miR). The control clones (NCI-N87/anti-NC) were produced by a similar method. The miR-199a-3p expression level was examined by qRT-PCR using U6 RNA as an endogenous control.

2.8. Tumour xenograft model, tumorigenicity assay and immunohistochemistry

All animal studies complied with protocols approved by the Committee on Animal Care in Shanghai Jiao Tong University School of Medicine. Cells (100 ml, $2 \times 10\text{^}6$ cells) from stable transfected lines LV-anti-miR-199a-3p and LV-anti-miR-NC were collected and inoculated subcutaneously into the right flank of 4-week-old male nude mice. Five mice were used for each group. Mice were checked weekly, and tumor nodules were measured with a caliper. After the mice were sacrificed, all tumor grafts were excised, weighed, harvested, fixed, embedded and examined histologically.

2.9. Cloning of 3′UTR of ZHX1 into pMIR-REPORT luciferase vector

The 3′UTRs of *ZHX1* was synthesized according to a ~59-bp genomic fragment containing the predicted miR-199a-3p binding sites. The mutant *ZHX1* 3′UTR construct was designed to mutate three intermittent nucleotides complementary to the miR-199a-3p seed-region. Both strands were annealed and cloned into the Hind III-MluI sites of the pMIR-REPORT miRNA expression reporter vector (Applied Biosystems).

2.10. Western blot analysis

ZHX1 protein levels were quantified by standard immunoblot procedures, using rabbit anti-human *ZHX1* antibody (1:2000, Abcam, USA). Monoclonal anti-GAPDH (1:5000, Abcam, USA) was used for loading control.

2.11. Statistical analysis

Student's *t* test or one-way ANOVA were used for statistical analysis when appropriate. All statistical analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). A two-tailed value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. The expression of miR-199a-3p is up-regulated in GC and correlates with TNM stage and tumor invasion

MiR-199a-3p expression was examined by quantitative real time RT-PCR (qRT-PCR) in GC cell lines and GES-1. As shown in Fig. 1 A, miR-199a-3p was generally upregulated in all GC cell lines