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Original article

Long intergenic noncoding RNA 01296 aggravates gastric cancer cells progress through miR-122/MMP-9

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ABSTRACT

Long non-coding RNAs (lncRNAs) play important roles on tumor development and progression in gastric cancer (GC). However, the biological function and regulatory mechanisms of LINC01296 in GC still remain unknown. The objective of this study is to investigate the clinical significance and pathological roles of LINC01296 in GC. Results showed that LINC01296 was up-regulated in GC tissue and correlated with poor prognosis. In vitro, LINC01296 knockdown was up-regulated in GC cells and LINC01296 knockdown suppressed GC cells proliferation, migration and invasion, and promoted apoptosis. In vivo xenograft assays, results showed LINC01296 knockdown significantly inhibited GC tumor growth. Bioinformatics analysis revealed that LINC01296 sponged miR-122, which was proved to target MMP-9. Western blot and RT-PCR showed that LINC01296 was positively correlated with MMP-9 expression, while miR-122 was negatively correlated to it. Overall, results indicate that LINC01296 acts as oncogenic lncRNA in GC carcinogenesis, suggesting the LINC01296/miR-122/MMP-9 regulatory pathway in GC tumorigenesis.

1. Introduction

Gastric cancer (GC) is one of most common malignancies of digestive tract carcinoma, especially in Asia [1]. All over the world, GC is the third leading cause of tumor death with extraordinarily high incidence rates and mortality rates [2]. With the development of early-stage diagnosis and late-stage comprehensive effective therapy, the incidence and fatality rate have been decreased [3]. Series of pathogenic factors contribute to the generation and development of GC, including heredity, inflammatory stimulation and virus infection [4]. However, there is still unclear to explicate the pathogenesis of GC. Taken together, it is necessary to discover the molecular mechanism underlying GC carcinogenesis and construct effective treatments [5].

Long noncoding RNAs (lncRNAs) are a type of non-coding RNAs (ncRNAs) with more than 200 nucleotides [6]. Increasing number of researches have revealed that lncRNAs take part in series of physiological and pathological process in tumors, included proliferation, invasion and metastasis [7]. LncRNAs are vital regulators to modulate transcriptional and post-transcriptional level regulation [8]. The aberrant expression of lncRNAs in tumor tissue is closely correlated with the tumorogenesis and progression [9]. For instance, lncRNA GHET1 is involved in the occurrence and development of gastric cancer and GHET1 knockdown inhibits GC cells activation [10]. RNA-seq and gene ontology annotations reveal DANCR promotes cell growth and tumorigenicity in gastric cancer [11]. Thus, studies about lncRNA suggest that lncRNAs could exert critical functions in tumor development and progression.

Microarray analysis reveals that LINC01296 is a novel intergenic lncRNA, which is located at chromosome 14q11.2. However, the role of LINC01296 in cancer is still unclear. In present study, we found LINC01296 was significantly up-regulated in GC. Our results reveal the role of lncRNA LINC01296 in GC cells and the regulation through miR-122/MMP-9, indicating the potential value of LINC01296 in GC therapy.

2. Materials and methods

2.1. Clinical tissue collection

A total of 60 cases of gastric cancer specimens and matched adjacent normal gastric tissue were obtained from the Tianjin First Center Hospital from Aug 2015 to Jun 2016. All tissues were verified by two independent clinical pathologists. None of the patients received any chemotherapy or radiotherapy treatment before the surgery. The tumors frozen in liquid nitrogen after resection and stored at −80 °C until use. This study was approved by the Ethics Committee of Tianjin First

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Center Hospital. Informed consents were obtained from all the patients for the research purposes.

2.2. Cell culture

The human GC cell lines (SGC-7901, BGC-823, MGC-803, and MKN-45) were obtained from the Chinese Institute of Biochemistry and Cell Biology (Shanghai, China). Normal gastric mucosa cell line GES-1 was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). All cells were cultured in RPMI 1640 Medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin in culture flasks at 37 ˚C with 5% CO_2.

2.3. RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from GC tissue samples and cell lines using TRIzol (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was synthesized from reverse transcription reaction system (20ul) using the SuperScript First-Stand Synthesis system (Invitrogen). RT-PCR was performed using SYBR Premix Taq (Applied Biosystems, US) at ABI PRISM 7900 thermocycler. GAPDH was used as an internal control. The primers for PCR were as follows: LINC01296, forward 5′-AGTTCCACCAAGTTTCTTCA-3′, reverse 5′-AGGTTTTGCCAGCATAGAC-3′; miR-122, forward, 5′-GCTCGAGATATGGAGGGCGCC-3′, reverse, 5′-GGAACGGCAGACACGTA TCC-3′; GAPDH, forward, 5′-GCACCGTCAAG GCTGAGAAC-3′, reverse, 5′-TGGTGAAGACGCCAGTGGA-3′. Relative levels of gene expression was expressed relative to GAPDH and calculated using the $2^{-\Delta\Delta Ct}$ method.

2.4. Cell transfection

The small interfering RNAs (siRNAs) targeting LINC01296 and miR-122 were synthesized by Shanghai GenePharma Co. Ltd. (Shanghai, China), including si-LINC01296 and miR-122 inhibitor. The transfection was performed using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. The subsequent experiments were carried out 48 h after transfection.

2.5. MTT assay and colony formation assay

Cell proliferation was measured using 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and colony formation. For the MTT assay, GC cells (SGC-7901, BGC-823, 2000 cells per well) were seeded into 96-well plates. After cultured for indicated time, the spectrophotometric absorbance was measured at 490 nm using a microplate reader absorbance test plate. For colony formation assay, SGC-7901 and BGC-823 cells were seeded at density of 500 cells/well in 6-well plates. After being cultured for 2 weeks, the colonies were fixed with 4% paraformaldehyde for 5 min and stained with 1% crystal violet for 10 min. Colonies were examined and counted under microscope. Plate efficiency = (colony numbers/inoculated cell numbers) × 100%. The assays were repeatedly performed in triplicate.

2.6. Dual-luciferase reporter gene assays

The sequences of LINC01296 containing the predicted miR-122 binding sites were amplified by PCR, and cloned into pGL3 Vector (Promega, Madison, WI, USA), named as pGL3-LINC01296-WT and pGL3-LINC01296-Mut. HEK 293T cells (5×10^3 cells per well) were co-transfected with miR-122 mimics or blank controls, and pGL3-LINC01296-WT or pGL3-LINC01296-Mut using Lipofectamine2000 (Invitrogen, California, USA), according to the manufacturer's instructions. Then, cells were seeded into in 96-well plates for 24 h culture. Then, luciferase activities were measured using Dual-Luciferase Reporter Assay System (Promega) on the Promega GloMax 20/20 luminometer according to manufacturer's protocol. The results were expressed relative to luciferase activity.

2.7. Wound healing assay

GC cells were seeded in 6-well plates and grew to about 90% confluence. Then, with culture solution was discarded, the monolayer was wounded by manually scraping with a 200 ul sterile pipette tip. The monolayers were washed with PBS and cultured for 10 h in basal medium. After scratching (0 h) and 24 h, the images were taken immediately using Olympus digital camera mounted on an Axiovert 25 microscope (Zeiss, Jena, Germany). The migration rate was quantified with measurements of the distance between cells.

2.8. Transwell invasion assay

Cell invasion assay was detected by Transwell chamber (Corning Life Sciences, Corning, NY, USA) with polycarbonic membrane (8 μm pore size) and Matrigel (BD, NJ, USA) according to manufacturer's instructions. At 48 h post-transfection and harvested, 3×10^4 cells (150 μL) were seeded in the upper chambers of Transwell chamber. 600 μL DMEM supplemented with 10% FBS was added into the lower chamber of Transwell chamber. After incubated about 12 h, the cells on the bottom surface of the transwell chambers were fixed with ice-cold methanol and stained with 0.1% crystal violet for 20 min. The invaded cells were counted in five randomly selected fields and images were acquired under an inverted light microscope (Leica DM IRB; Germany).

2.9. Flow cytometric analysis of apoptosis assay

Apoptosis assay was performed using flow cytometric analyses with Annexin V: FITC Apoptosis Detection Kits (BD Biosciences, USA), according to the manufacturer's instructions. Briefly, SGC-7901 and BGC-823 cells were treated with siRNAs for 48 h and washed three times in cold PBS. Then, cells were stained with Annexin V-FITC and PI in 500 μL of binding buffer, in the dark at room temperature for 15 min, and assessed by flow cytometry (FACScan; BD Biosciences, San Jose, CA, USA). All samples were assayed in triplicate.

2.10. Western blot analysis

Total protein was extracted using RIPA buffer (Beyotime Institute of Biotechnology) according to instructions. At 4 ˚C, the extracted liquid was centrifuged at 20,000*g* for 15 min. The protein concentration was determined using a BCA protein assay kit (Thermo, Waltham, MA, USA) with bovine serum albumin as a standard. Equal amounts of protein lysates were electrophoretically separated on SDS-PAGE gels and transferred to PVDF membranes. The membranes were blocked with 5% non-fat dried milk for 1 h at room temperature and then incubated with primary antibodies in 4 ˚C overnight. Primary antibodies contained: anti-MMP-9 (1:1000, Santa Cruz); anti-GAPDH (1:2000, Santa Cruz). After three washes, the blots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG for 1 h and subsequently visualized by enhanced chemiluminescence.

2.11. In vivo tumor xenografts formation assay

The in vivo animal experiment was approved by the Institutional Animal Care and Use Committee of Tianjin First Center Hospital. Ten male BALB/c nude mice (6 weeks old) were purchased for the experiments and randomly divided into two groups and maintained under pathogen-free conditions. Total 2×10^6 GC cells (SGC-7901, BGC-823) that stably transfected with scramble or sh-LINC01296 were suspended in 200 μL of serum-free RPMI 1640 medium and subcutaneously injected into one flank of each mouse. Tumor volumes were calculated