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LncRNA HOTTIP promotes proliferation and inhibits apoptosis of gastric carcinoma cells *via* adsorbing miR-615-3p

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Abstract. – OBJECTIVE: To explore the role of long-noncoding ribonucleic acid HOXA transcript at the distal tip (lncRNA HOTTIP) in the proliferation and apoptosis of gastric carcinoma cells.

MATERIALS AND METHODS: The expressions of lncRNA HOTTIP in gastric carcinoma cell lines MGC-803, HGC-27, SNU-1, and SGC-7901 and normal gastric mucosa cell line RGM-1 were detected by real-time fluorescence quantitative polymerase chain reaction (PCR) and compared. The effects of lncRNA HOTTIP on proliferation and apoptosis of gastric carcinoma cells were detected by cell counting kit-8 (CCK-8), colony formation assay, and flow cytometry, respectively. StarBase v2.0 website was adopted to predict the relationship between lncRNA HOTTIP and target miRNAs. Dual-Luciferase reporter assay was performed to verify the sponge effect of lncRNA HOTTIP on miR-615-3p. CCK-8 experiment was conducted to detect its effect on proliferation of gastric carcinoma cells after co-silencing lncRNA HOTTIP and miR-615-3p.

RESULTS: LncRNA HOTTIP was highly expressed in gastric carcinoma cell lines MGC-803, HGC-27, SNU-1, and SGC-7901 than in normal gastric mucosa cell line RGM-1. After knockdown of lncRNA HOTTIP, the proliferation function of gastric carcinoma cells was markedly weakened, and the proportion of apoptotic cells increased. LncRNA HOTTIP was able to adsorb miR-615-3p *via* a sponge effect. Notably, knockdown of miR-615-3p restored the effect of silenced lncRNA HOTTIP on the proliferation function of gastric carcinoma cells.

CONCLUSIONS: LncRNA HOTTIP is highly expressed in gastric carcinoma cells. It affects cell proliferation and apoptosis in gastric carcinoma by adsorbing miR-615-3p *via* a sponge effect.

Key Words:

Gastric carcinoma, LncRNA HOTTIP, MiR-615-3p, Proliferation, Apoptosis.

Introduction

Gastric carcinoma is a malignant tumor with a high incidence worldwide. The canceration of gastric mucosa epithelial cells leads to the tumorigenesis of gastric carcinoma[1,2]. Smoking, eating habits, and irregular lifestyle make the higher incidence rate of gastric cancer in China higher than the worldwide average level[3]. Besides, genetic characteristics such as single nucleotide polymorphisms, gene choroidal neovascularization (CNV) changes, and chromosome variations are also important causes for gastric carcinoma[4]. Early-stage gastric carcinoma, like pinpoint carcinoma, is likely to be ignored or not found in routine physical examination due to the atypical symptoms. Middle stage and advanced gastric carcinoma are difficult to be treated and have a high mortality rate[5]. Early diagnosis of gastric carcinoma and early surgical resection or chemotherapy can evidently help to enhance the survival rate and improve the prognosis of patients. Influencing factors on malignant phenotypes of cell functions can provide new treatment ideas for patients with gastric carcinoma.

Long-noncoding ribonucleic acids (lncRNAs) are a class of important molecules of noncoding regulatory RNAs, which have attracted much attention in recent years[6]. Because of the evident difference of spatio-temporal expression, lncRNAs can dynamically regulate gene expressions and play a significant regulatory role in cell epigenetics[7]. As characterized by gene expression disorder, lncRNAs are abnormally expressed in cancer cells, such as pancreatic cancer[8], colorectal cancer[9], liver cancer[10], etc. LncRNA HO XA transcript at the distal tip (lncRNA HOTTIP), a new noncoding regulatory RNA discovered in recent years, may exert a marked influence on the cell functions of gastric carcinoma and affect its progress.

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In this investigation, by comparing the expression differences of lncRNA HOTTIP in gastric carcinoma cell lines MGC-803, HGC-27, SNU-1, SGC-7901, and normal gastric mucosa cell line RGM-1, the effects of lncRNA HOTTIP on the proliferation and apoptosis of gastric carcinoma cells were detected. The sponge adsorption effect between lncRNA HOTTIP and miR-615-3p was predicted by the online database and verified by Dual-Luciferase reporter assay. The role of lncRNA HOTTIP in the proliferation and apoptosis of gastric carcinoma cells was further explored by a series of functional experiments.

Materials and Methods

Materials

Gastric carcinoma cell lines MGC-803, HGC-27, SNU-1, SGC-7901, and normal gastric mucosa cell line RGM-1 were all purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Dulbecco Modified Eagle's Medium (DMEM; HyClone, South Logan, UT, USA), fetal bovine serum (FBS; Gibco, Rockville, MD, USA), trypsin (Gibco, Rockville, MD, USA), 1% penicillin-streptomycin solution (100×) (Beyotime, Shanghai, China), reverse transcription kit and SYBR Green quantitative Polymerase Chain Reaction (qPCR) kit (TaKaRa, Otsu, Shiga, Japan), TRIzol reagent and Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA), Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan), fluorescein isothiocyanate (FITC)- apoptosis detection kit, small interfering RNA (siRNA; si-1#, Si-2#, and si-3#) with targeted and specific knockdown of lncRNA HOTTIP, negative control (si-NC), miR-615-3p mimetic and its negative control (miR-NC) designed and chemically synthesized by Shanghai Bioengineering Co., Ltd. (Shanghai, China), Dual-Luciferase reporter gene detection kit (Solarbio, Beijing, China) and pmirGLO empty plasmid (Promega, Madison, WI, USA), microplate reader, and qPCR meter (BIO-RAD, Hercules, CA, USA), flow cytometer (Beckman, Franklin Lakes, NJ, USA), cell incubator and biosafety cabinet were prepared.

Cell Culture and Transfection

Gastric carcinoma cell lines MGC-803, HGC-27, SNU-1, and SGC-7901 and normal gastric mucosa cell line RGM-1 were resuscitated in 37°C water bath and centrifuged for 5 min (1000 rpm/min). After suspended in complete medium, they were seeded in a T25 culture flask for culture. The complete medium was prepared by DMEM containing 10% FBS and 1% penicillin-streptomycin solution. The culture flask was placed in a cell incubator containing 5% CO_2 and the cell growth status was daily observed. Cells in good growth state were plated in six-well plates with appropriate density and transfected according to the steps of Lipofectamine 2000 operation instruction after cell adherence.

RNA Extraction and quantitative Real Time-Polymerase Chain Reaction (RT-qPCR) Detection

The total RNA of each cell line was extracted by TRIzol method. The expressions of lncRNA HOTTIP and miR-615-3p were detected by fluorescence qRT-PCR. Each gene primer was designed by Primer Premier 5.0, and synthesized and verified by Shanghai Bioengineering Co., Ltd. (Shanghai, China). LncRNA HOTTIP forward primer (5'→3') 'CACTGCGTGACCTTGGTTCA', reverse primer (5'→3') 'CTCCCACATCCTGTAAAAGGAC'. MiR-615-3p forward primer (5'→3') 'GAGCTTGGCCGGACTGAAC', reverse primer (5'→3') 'TGTCATAGATTCCAAAGCGTAGC'. GAPDH forward primer (5'→3') 'TGACATAGACAACAAGACGCC', reverse primer (5'→3') 'TCAAGGGGAGAGTCAGTACCT'. U6 forward primer (5'→3') 'CTCGCTTCGGCAGCACATAT', reverse primer (5'→3') 'TTGCGTGTCATCCTTGCG'. The total PCR system was 25 μL, including 1 μL of each primer, 0.5 μL of template complementary deoxyribonucleic acid (cDNA), 12.5 μL of SYBR Premix Taq, and 10 μL of dH_2O. The qRT-PCR conditions were as follows: 95°C for 2 min, followed by 35 cycles of 95°C for 35 s, 58°C for 45 s and 72°C for 30 s, and finally 72°C for 5 min.

CCK-8 Proliferation Assay

The proliferation activity of gastric carcinoma cells was detected by CCK-8 proliferation experiment. After 24 h of transfection, cells in good growth condition were plated on 96-well plates with 3000 cells per well and 5 replicants per sample. 10 μL of CCK-8 reagent was added to each well at 24 h, 48 h, 72 h, and 96 h, respectively, and incubated at 37°C for 3 h. The absorbance value at 450 nm was detected by the microplate reader, and the cell proliferation activity was obtained *via* analysis.

Colony Formation Assay

Cells transfected for 24 h were digested with trypsin, prepared in cell suspension and inoculated into 6-well plates. After 15-20 days, visible colonies in the six-well plates were washed with polybutylene succinate (PBS), fixed with 4% paraformaldehyde, stained with crystal violet for 10 min, and then photographed and counted.

Detection of Apoptosis

The proportion of apoptotic cells was measured by the apoptosis detection kit, and the operation steps were carried out in strict accordance with the instructions. After 72 h of transfection, the cells were digested with trypsin, carefully washed by PBS and re-suspended. They were incubated with AnnexinV-FITC and propidium iodide (PI) in the dark for 20 min, respectively. The proportion of apoptotic cells was detected by flow cytometry.

Dual-Luciferase Reporter Assay

According to the relationship between lncRNA HOTTIP and miRNA predicted by StarBase v2.0 website, a binding site between miR-615-3p and lncRNA HOTTIP was obtained, which was verified by Dual-Luciferase reporter gene experiment. The experimental steps were carried out according to the operation of the Dual-Luciferase reporter gene detection kit. LncRNA HOTTIP-wild type (WT) and lncRNA HOTTIP-mutant type (MUT) were constructed based on the predicted binding sequence of miR-615-3p. Cells were co-transfected with lncRNA HOTTIP-WT or lncRNA HOTTIP-MUT and miR-NC or miR-615-3p mimetic for 48 h, and Luciferase activity was assayed.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.0 software (La Jolla, CA, USA). The measurement data were tested by the *t*-test. $p<0.05$ was considered to represent a statistically significant difference.

Results

Expression of LncRNA HOTTIP in Gastric Carcinoma Cell Lines and Normal Gastric Mucosa Cell Line

The expression of lncRNA HOTTIP in gastric carcinoma cell lines and normal gastric mucosa cell line was shown in Figure 1. By detecting the expression of lncRNA HOTTIP in gastric carcinoma cell lines (MGC-803, HGC-27, SNU-1, and SGC-7901) and normal gastric mucosa cell line RGM-1, it was found that lncRNA HOTTIP was significantly overexpressed in gastric carcinoma cell lines ($p<0.05$). HGC-27 cells expressed the highest level of lncRNA HOTTIP in the tested gastric carcinoma cell lines. It is indicated that lncRNA HOTTIP may play a special role in gastric carcinoma cells.

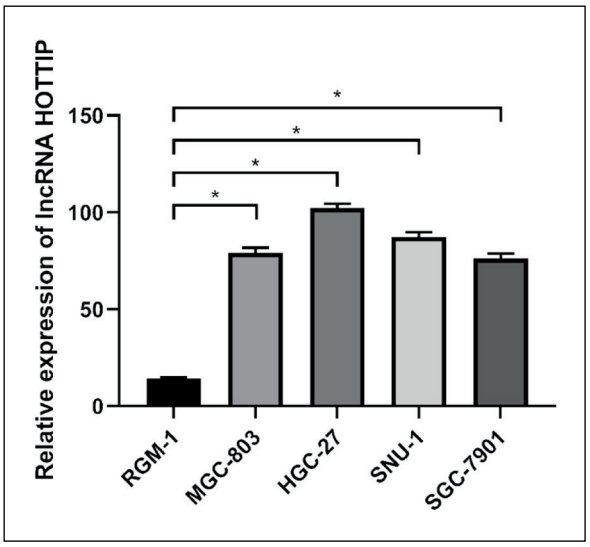

Figure 1. Relative expression of lncRNA HOTTIP in gastric carcinoma cell line (MGC-803, HGC-27, SNU-1, and SGC-7901) and normal gastric mucosa cell line RGM-1 (*$p<0.05$).

Effect of LncRNA HOTTIP on Proliferation Function of Gastric Carcinoma Cells

Since lncRNA HOTTIP was overexpressed in gastric carcinoma cell line, it was speculated that it has a marked carcinoma-promoting effect. Transfection efficacy of three lncRNA HOTTIP siRNAs (si-1#, si-2#, and si-3#) was tested in HGC-27 cells, and si-2# displayed the best efficacy ($p<0.05$). Therefore, lncRNA HOTTIP si-2# was selected to knock down lncRNA HOTTIP in subsequent experiments. Through CCK-8 experiment, it was found that the proliferation ability of HGC-27 cells with lncRNA HOTTIP knockdown was remarkably reduced at 96 h compared with NC group (Figure 2B). It is indicated that lncRNA HOTTIP can promote the proliferation of gastric carcinoma cells.

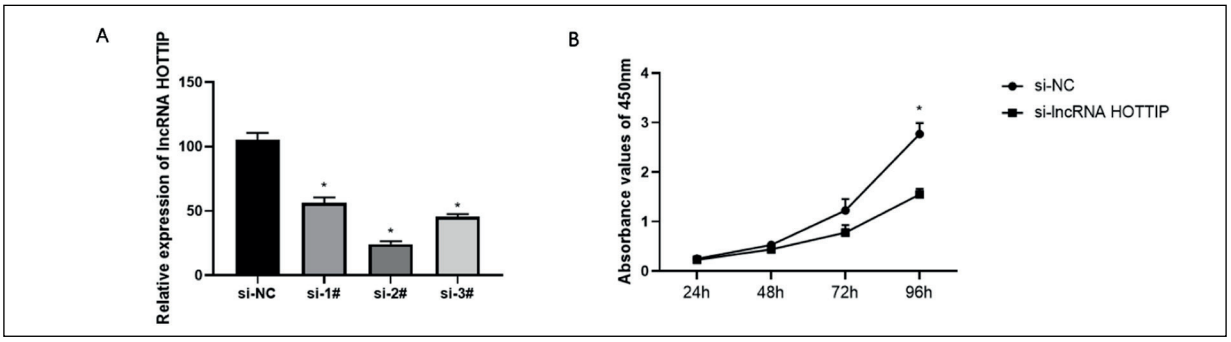

Figure 2. Effect of lncRNA HOTTIP on proliferation function of gastric carcinoma cells. **A,** Comparison of knockdown efficiency of lncRNA HOTTIP. **B,** Effect of silenced lncRNA HOTTIP on proliferation function of gastric carcinoma cells detected through CCK-8 experiment. (*p<0.05).

Effect of LncRNA HOTTIP on Colony Formation Function of Gastric Carcinoma Cells

Colony formation ability reflects cell proliferation function, and tumor cell colony formation ability is essential to tumor growth. Through colony formation experiments, it was found that knockdown of lncRNA HOTTIP inhibited the colony formation ability of gastric carcinoma cells, proving once again the effect of lncRNA HOTTIP on promoting the growth of gastric carcinoma cells (Figure 3).

Effect of LncRNA HOTTIP on Apoptosis of Gastric Carcinoma Cells

With the apoptosis detection kit, it was discovered that after knockdown of lncRNA HOTTIP, the portion of apoptotic cells increased evidently (p<0.05; Figure 4), suggesting that lncRNA HOTTIP can inhibit the apoptosis of gastric carcinoma cells and promote tumor development.

Sponge Adsorption Effect Between LncRNA HOTTIP and MiR-615-3p

Based on the binding site of lncRNA HOTTIP and miR-615-3p predicted by StarBase v2.0 website, the sponge adsorption effect between lncRNA HOTTIP and miR-615-3p was speculated (Figure 5A). The lncRNA HOTTIP sequence containing the predicted miR-615-3p site or mutation site was cloned into empty plasmid, named lncRNA HOTTIP-WT and lncRNA HOTTIP-MUT, respectively. The Dual-Luciferase reporter gene system was used to detect the Luciferase activity changes in each group. It was found that transfection of miR-615-3p mimic remarkably inhibited Luciferase activity in lncRNA HOTTIP-WT group (p<0.05), but it had no evident effect on Lciferase activity in lncRNA HOTTIP-MUT group (Figure 5B). Besides, the regulatory effects of lncRNA HOTTIP on the expression level of miR-615-3p were examined in HGC-27 cells (Figure 5C, D). The above results revealed that there was a sponge effect between lncRNA HOTTIP and miR-615-3p in gastric carcinoma.

MiR-615-3p Was Responsible for LncRNA HOTTIP-Mediated Inhibition on Gastric Carcinoma Cell Proliferation

Changes in proliferation of HGC-27 cells with co-silencing lncRNA HOTTIP and miR-615-3p

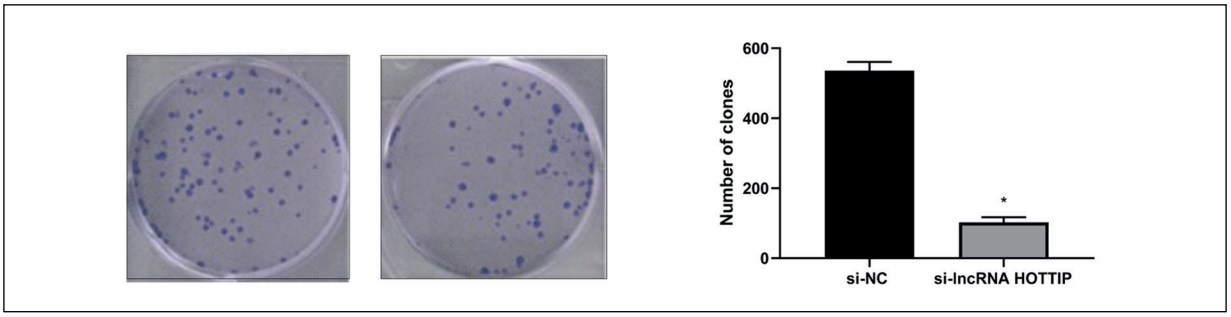

Figure 3. Effect of lncRNA HOTTIP on colony formation function of gastric carcinoma cells (magnification: 10×; *p<0.05).

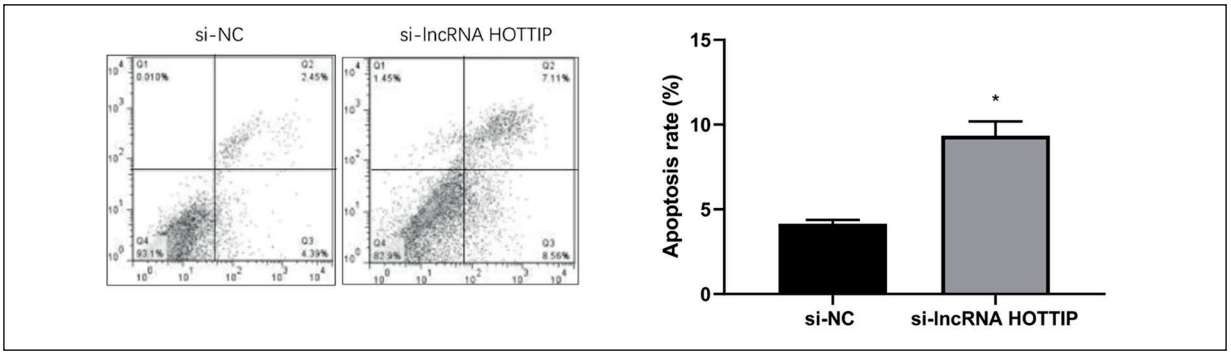

Figure 4. Effect of lncRNA HOTTIP on apoptosis cell ratio of gastric carcinoma cells (*p<0.05).

were detected *via* CCK8 experiments. As shown in Figure 6, knockdown of miR-615-3p reversed the inhibitory effect of silenced lncRNA HOTTIP on the proliferation of HGC-27 cells (p<0.05).

Discussion

Gastric carcinoma, one of the high incidence tumors of the digestive system, is a giant threat to people's health and even lives. In addition to genetic factors, the occurrence of gastric carcinoma may be related to epigenetic changes in tumor cells[11]. Oh et al[12] have shown that during the growth, invasion, metastasis, and epithelial-mesenchymal transformation of gastric carcinoma cells, changes of various genes may be an important promoter of phenotypic changes in gastric carcinoma cells. Moreover, changes in gene expressions of gastric carcinoma cells

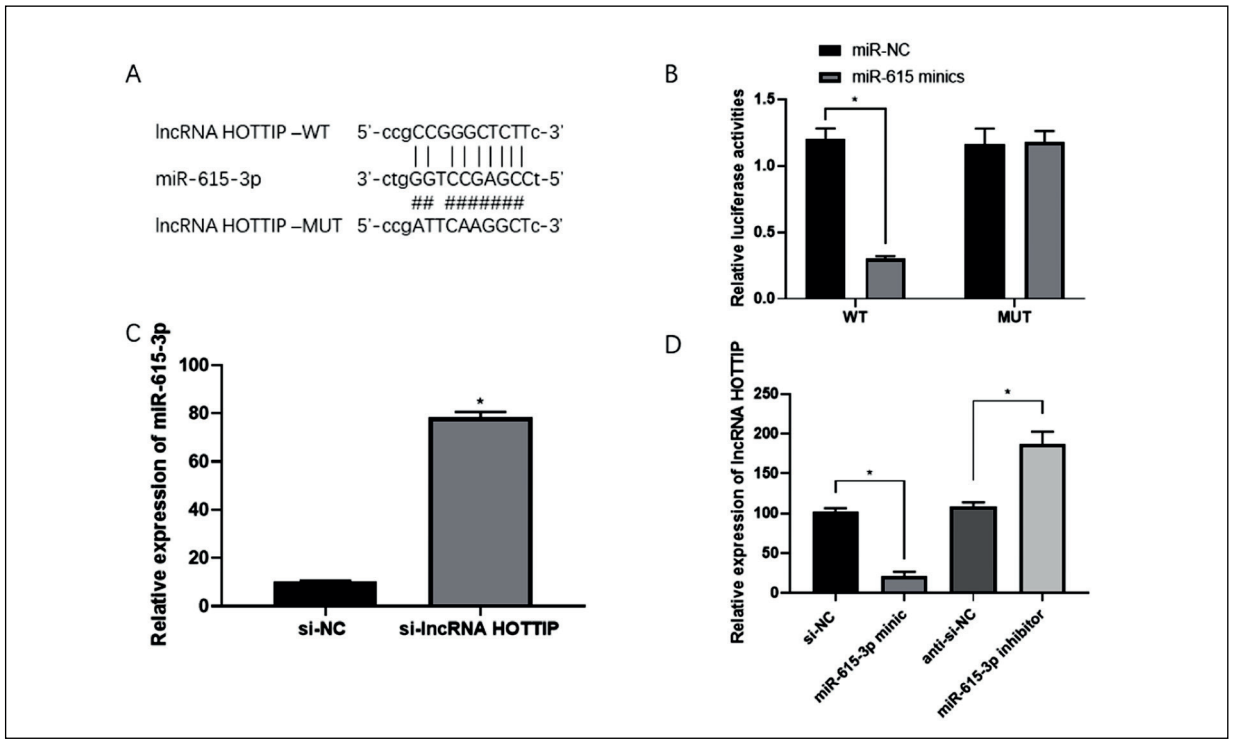

Figure 5. Sponge adsorption effect between lncRNA HOTTIP and miR-615-3p. **A,** StarBase v2.0 website is employed to predict the binding site of lncRNA HOTTIP and miR-615-3p. **B,** Dual-Luciferase reporter assay is performed to detect the interaction between lncRNA HOTTIP and miR-615-3p. **C-D,** QRT-PCR is adopted to detect regulatory effects between lncRNA HOTTIP and miR-615-3p (*p<0.05).

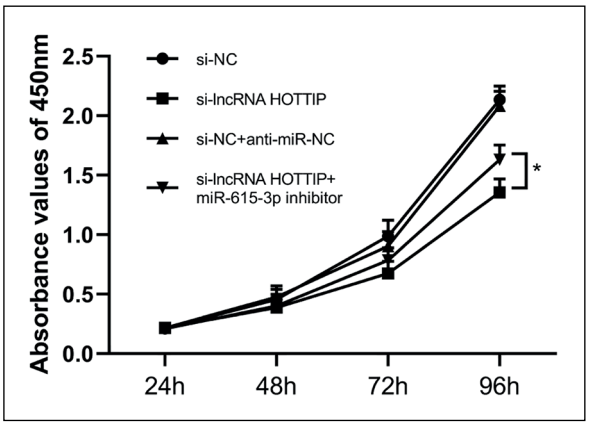

Figure 6. MiR-615-3p participates in lncRNA HOTTIP-mediated inhibition on gastric carcinoma cell proliferation (*p<0.05).

may be caused by many factors, such as changes in chromosome open region, DNA methylation, histone modification, and noncoding RNA regulation[13,14]. Among them, the regulation of gene expressions by noncoding RNAs, including cyclic RNAs and micro RNAs, is one of the critical reasons for the formation of cancer cell phenotypes[15].

LncRNAs, a kind of molecules well studied in recent years, have been proven to play a marked role in cell epigenetics regulation. They are also closely related to the development of gastric carcinoma. LncRNA HOXC-AS3 mediates gastric carcinoma by binding to YBX1[16]. LncRNA GMAN is highly expressed in gastric carcinoma tissues, and it can competitively bind to GMAN-AS, and thus influences metastasis of gastric carcinoma[17]. LncRNA FEZF1-AS1 can promote the proliferation of gastric carcinoma cells by inhibiting the expression of p21[18]. LncRNA HOTTIP is a kind of lncRNA closely related to tumor occurrence[19]. It's important clinical significance in early non-small cell lung cancer has been identified[20]. In addition, lncRNAs can promote the invasion and metastasis of breast cancer and epithelial-mesenchymal transformation through the Wnt signaling pathway[21]. By detecting the expression of lncRNA HOTTIP, this study found that lncRNA HOTTIP was highly expressed in gastric carcinoma cell lines MGC-803, HGC-27, SNU-1, and SGC-7901 than in normal gastric mucosa cell line RGM-1, indicating that the lncRNA may play an important role in the development of gastric carcinoma. Cell functional assay CCK-8 and colony formation experiment observed that lncRNA HOTTIP promoted proliferation and inhibited apoptosis of gastric carcinoma cells.

LncRNA may affect gene expressions by regulating transcription of upstream promoter region of coding gene, thus affecting protein activity or inhibiting transcription process of RNA polymerase. This research revealed that lncRNA HOTTIP can adsorb miR-615-3p in gastric carcinoma cells *via* a sponge effect, affecting expression changes of downstream genes. MiR-615-3p has been proven to promote the proliferation and migration of tumor cells and inhibit the apoptosis of carcinoma cells[22]. Through cell functional experiments and recovery tests, it was found that knockdown of miR-615-3p restored the effect of silenced lncRNA HOTTIP on the proliferation function of gastric carcinoma cells. These results indicated that lncRNA HOTTIP did affect the proliferation of gastric carcinoma cells through its sponge effect on miR-615-3p.

Conclusions

This study showed that lncRNA HOTTIP is upregulated in gastric carcinoma cell lines and tissues compared to controls. It is able to promote proliferation and inhibit apoptosis in gastric carcinoma cells through its sponge effect on miR-615-3p. Our findings provide a new direction in clinical treatment of gastric carcinoma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding

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