Original Article
MiR-762 regulates the activation of PI3K/AKT and Hippo pathways involved in the development of gastric cancer by targeting LZTS1

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Received December 24, 2021; Accepted June 2, 2022; Epub July 15, 2022; Published July 30, 2022

Abstract: Objective: MiR-762 has been confirmed as a tumor promoter in multiple tumors, while few reports illustrate its role in gastric cancer (GC). Thus, this research aimed to investigate whether miR-762 is involved in GC development. Methods: MiR-762 expression in the tumor tissues from GC patients and GC cell lines was analyzed by qRT-PCR. The assays including CCK-8, transwell, and flow cytometry were performed to reveal the functions of miR-762 in GC. The target genes of miR-762 were searched by online databases, and then were verified by dual-luciferase reporter assay. Western blot was performed to investigate the activation of PI3K/AKT and Hippo pathways in GC. Results: MiR-762 was aberrantly upregulated in the tumor tissues and cell lines, and miR-762 silencing could effectively reduce the viability and promote apoptosis of GC cell lines. The study identified LZTS1 as a target gene of miR-762. It was also found that the effects of miR-762 on GC cells could be reversed by LZTS1, and miR-762 could upregulate the activation of the PI3K/AKT pathway but inhibit the Hippo pathway by targeting LZTS1. Conclusion: MiR-762 activates PI3K/AKT and suppresses Hippo pathways to boost GC proliferation and invasion by targeting LZTS1.

Keywords: Gastric cancer, miR-762, LZTS1, PI3K/AKT, Hippo

Introduction
Gastric cancer (GC) is characterized by high malignancy and morbidity. At present, how to overcome GC has become a medical challenge [1, 2]. Moreover, more than 60% of the patients have progressed to a late stage when they are diagnosed with GC, causing a challenge to public health [3]. At present, therapeutic strategies such as chemotherapy, radiotherapy, and surgical intervention have been used for gastric cancer treatment [4]. However, the long-term survival rates of GC patients remain unsatisfactory. Multiple pathogenesis and tumor development mechanisms make GC one of the most intractable problems in clinical practice [5]. In recent years, many studies have shown that some short non-coding RNAs may play pivotal roles in the progression of GC. miRNAs contain 18-25 nucleotides [6, 7]. The functions of miRNAs on cellular activity have been shown to reduce effective abundance of the related proteins [9]. Moreover, given the effects of miRNAs on cells, controlling the cellular abundance of miRNAs is increasingly accepted as a feasible therapeutic strategy for treatment of diseases ranging from inflammation to cancer [10]. The effect of miRNAs on the progression of GC has been widely investigated as a possible treatment target [11]. Multiple studies have indicated an aberrant abundance of miRNAs involved in the malignant behavior of GC cells [12]. The study has identified miR-762, as a biomarker that is increased in the serum of patients with various cancers. However, the functional mechanism of miR-762 in GC remains unknown [13]. This study focused on investigating the abundance of miR-762 in GCs and revealed the functional mechanism of miR-762 in GC.

Material and methods
Pathological sample
This study was approved by the ethics committee of Yantaishan Hospital (No: 20200901). 15
pairs of cancer and corresponding para-cancerous tissues from patients with GC treated at Yantaishan Hospital from April 2018 to May 2019 were collected to examine the abundance of miR-762. All patients signed an informed consent.

Cell culture

The gastric cancer cell lines (MGC-803, BGC-823 and SGC-7901) and normal cell line (GES-1) were obtained from Yaji Co., Ltd, Shanghai, China. All cells were cultured in RPMI-1640 medium (Procell Life Science & Technology Co., Ltd, Wuhan, China) containing 10% fetal bovine serum (Thermo Fisher, Massachusetts, USA), at 37°C in a humidified incubator with 5% CO₂.

Cell transfection

The miR-762 inhibitors and related negative control (inhibitor-NC) were purchased from Xi’an Xiyue Biotechnology Co., Ltd (Xi’an, China). Si-LZTS1 and control vectors were also designed and purified by Xi’an Xiyue Biotechnology Co., Ltd (Xi’an, China). The SGC7901 cells were seed in 6-well plates. Cell transfection was performed when the cell confluence reached 70%. 4 μg of DNA or 100 pmol RNA and 10 μL Lipofectamine 2,000 were incubated with 250 μL of medium without FBS for 5 min, respectively. Subsequently, the diluted DNA or RNA were mingled with diluted Lipofectamine 2,000 (1:1) and then incubated for 25 min at room temperature, respectively. Finally, the cells were cultured with 500 μL of the mixture for 24 hours.

qRT-PCR

The RNA in tissues and cells was extracted with Trizol reagent. Subsequently, the RNAs were reverse transcribed into cDNA by using a kit (Shanghai Yihui Biotechnology Co., Ltd, Shanghai, China). The primers were designed by Geneseed Biotech Co., Ltd (Guangzhou, China). Reaction systems (10 μL) of qRT-PCR were prepared for PCR reaction. Notably, all operations used the guidelines of the KAPA qRT-PCR kit (Sigma-Aldrich, Missouri, USA). The primer sequences of U6 and miR-762 are shown in Table 1.

Western blot

The RIPA buffer was used to extract the total proteins of the tissues or cells. The concentrations of the proteins in the extracts were quantified by BCA protein assay kits (Shanghai Meilian Scientific Research and Biotechnology Co., Ltd, Shanghai, China). The total proteins were separated with polyacrylamide gel. Wet transfer method was performed for transferring the proteins to PVDF membranes. Subsequently, the PVDF membranes were immersed in 5% fat-free milk for 1 hour. After that, the diluted primary antibodies were used to incubate the membranes at 4°C overnight. Next day, after being washed with Tris Buffered saline Tween solution, the membranes were incubated with secondary antibodies at 25°C for 2 hours. Finally, the abundance of interest proteins was evaluated by a chemiluminescence detection system. Antibodies: anti-LZTS1 (1:1000, ab2643656, ThermoFisher, Massachusetts, USA); anti-p-PI3K (1:1000, ab2816326, ThermoFisher, Massachusetts, USA); anti-p-AKT (1:1000, ab2533699, ThermoFisher, Massachusetts, USA); anti-YAP1 (1:1000, ab2219137, ThermoFisher, Massachusetts, USA).

Transwell assay

To observe the cell migration, 2×10⁵ cells and 100 μL serum-free RPMI 1640 medium were injected to the upper chamber, and 600 μL medium including 10% FBS was injected to the lower chamber. SGC7901 cells were cultured in an incubator with 5% CO₂ at 37°C for 24 hours. The migrated cells on the lower surface of the upper chamber were fixed in methanol for 10 min. Cell staining was performed with 0.1% w/v crystal violet (Bohubio, Shanghai, China) for 30 min. After that, the excess dye in the chambers was removed. The migrated cells were observed and photographed with a light microscope (Leica DMI8, WETZLAR, Germany).

For cell invasion, the upper chamber was coated with matrigel. Subsequently, 5×10⁵ cells were incubated in the upper chamber of each well for 24 h. Then, 4% paraformaldehyde

### Table 1. Primer sequence

<table>
<thead>
<tr>
<th>Name of primer</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>miR-762-F</td>
<td>5’-ACACGGGGCGUGCAGGCGAGCCGAGCCTC-3’</td>
</tr>
<tr>
<td>miR-762-R</td>
<td>5’-CTCAGGGCGUGGGCCGAGCCGAGCCGAGCAGA-3’</td>
</tr>
<tr>
<td>U6-F</td>
<td>5’-CTCGCTTCGACGACA-3’</td>
</tr>
<tr>
<td>U6-R</td>
<td>5’-AACGCTTCAGATTTTGCGT-3’</td>
</tr>
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was used for cellular fixation, and 0.4% trypan blue was used for cellular staining. After that, the cells invaded into the lower chamber were counted using a microscope (Leica DMi8, WETZLAR, Germany).

CCK-8 assay

SGC7901 cells were cultured in 96-well plates. After 48 h, CCK-8 reagent (Amyjet, Wuhan, China) was added to each well. After incubation for 4 hours, the absorbance value was detected at 450 nm by a microplate reader (Molecular Devices, Shanghai, China).

Dual-luciferase reporter gene assay

The sequence of LZTS1 was named as LZTS1-wild type (LZTS1-wt), and the sequence of LZTS1 with mutant 3’-UTR was named as (LZTS1-mut). Subsequently, LZTS1-wt or LZTS1-mut were respectively inserted into the pmirGLO luciferase reporter vectors. After that, the vectors transfected with LZTS1-mut and LZTS1-wt were respectively transfected into HEK-293T cells along with miR-762 mimics or miR-NC, and then HEK-293T cells were incubated for 48 hours. Finally, the luciferase activity of HEK-293T was observed by a dual-luciferase reporter assay system.

Flow cytometry assay

The trypsinase without EDTA (0.25%, EDTA-free) was used for cell digestion. Subsequently, the cells were washed with ice phosphate-buffered saline (PBS). After that, 2×10^3 cells were suspended in 5 µL of ice Annexin V-FITC binding buffer (10 µg/mL) and 10 µL of propidium iodide (PI 20 µg/mL), and then the cells were incubated in dark for 10 min. Finally, cellular apoptosis was observed by flow cytometry equipment (BD Biosciences, New Jersey, USA).

Immunohistochemistry (IHC)

The tissues were embedded in paraffin and then cut into 5 µm sections. Then, the sections were immersed in citrate buffer (0.01 M, PH 6.0) and heated in a microwave for deparaffinization, rehydration, and antigen repair. The sections were incubated in blocking buffer (AmyjetScientific Co., Ltd, Wuhan, China) for 1 hour. LZTS1 IgG antibody (5 µg/ml) was used for incubation of the sections at 4°C for 12 hours. The sections were incubated with secondary antibody. After that, the peroxidase reaction was observed with 3’-diaminobenzidine. Finally, the sections were stained with hematoxylin - eosin stain.

Statistical analysis

The data analysis was performed with SPSS 20.0. Chi-square test or ANOVA with Tukey’s post hoc-test was performed to calculate the difference among groups. P<0.05 was considered a significant difference.

Results

MiR-762 was aberrantly upregulated in GC tissues and cell lines

To observe the relationship between gastric cancer and abundance of miR-762, we detected the relative expression of miR-762 using qRT-PCR. The results showed that miR-762 was upregulated in cancer tissues compared to para-cancerous tissues (Figure 1A, P<0.01).
Furthermore, increased miR-762 was also found in MGC-803, BGC-823, and SGC-7901 cells, as compared to GES-1 cells. Those findings suggest that miR-762 overexpression is a key event of gastric cancer (Figure 1B, P<0.01).

**MiR-762 silencing suppressed the growth and invasion of GC cells**

To observe the functions of miR-762 in the development of GC, the miR-762 inhibitors were transfected into the SGC-7901, and CCK-8, transwell assay and flow cytometry were performed to illustrate the changes in biologic behavior of GC cells. The CCK-8 assay proved that the viability of the SGC-7901 cells treated with miR-762 inhibitors decreased significantly compared to the cells transfected with negative control (Figure 2A, P<0.01). Furthermore, the Transwell assay reflected that miR-762 inhibitors significantly reduced the numbers of the invaded and migrated cells (Figure 2D-F, P<0.01). Flow cytometry results proved that miR-762 inhibition significantly increased the apoptosis level of SGC-7901 cells. These observations suggest that miR-762 is involved in the development of cancer cells (Figure 2B, 2C, P<0.01).

**LZTS1 is a downstream target of miR-762**

Given the effect of miR-762 on SGC-7901 cells, the interactions between miR-762 and 3’-UTRs of mRNA might contribute to the effect of miR-762 on the progression of gastric cancer. MiRDB, the miRNAs target database, was used to screen downstream genes of miR-762. The results showed that LZTS1 was one of the targets of miR-762. To further confirm the binding between miR-762 and LZTS1, Dual-luciferase reporter assay was carried out and confirmed that miR-762 could significantly decrease the luciferase activity of LZTS1-wt compared to LZTS1-mut (Figure 3A, P<0.01). WB and IHC showed that LZTS1 was significantly downregulated in the tissues and GC cell lines (including GES-1, MGC-803, BGC-823 and SGC-7901), and increased miR-762 could inhibit the expression level of LZTS1 (Figure 3B-D, P<0.01).

**LZTS1 silencing reversed the effects of miR-762 downregulation on GC cells**

To confirm whether LZTS1 is involved in the regulation of miR-762 on gastric cancer, the LZTS1 silencing vectors and miR-762 inhibitors were co-transfected into SGC-7901 cells to observe the changes in the biological behavior of SGC-7901 cells. Compared to the cells with miR-762 inhibitor only, the proliferation and viability of the cells co-transfected with miR-762 inhibitor and si-LZTS1 were significantly enhanced (Figure 4A, P<0.01). Flow cytometry assay showed that the apoptosis level of the cells co-transfected with miR-762 inhibitor and si-LZTS1 was significantly lower than that of cells transfected with miR-762 inhibitor alone (Figure 4B, 4C, P<0.01). It was also observed that the inhibited invasion and migration of SGC-7901 induced by miR-762 silencing was reversed by LZTS1 downregulation (Figure 4D-F, P<0.01). These findings suggest that LZTS1 silencing could reverse the effect of miR-762 inhibition on SGC-7901 cells.

**MiR-762 inhibited the PI3K/AKT pathway and activated the Hippo pathway of GC cells**

To explore the pathogenic mechanism of miR-762 in GC, the miR-762 inhibitors and si-LZTS1 were co-transfected into SGC-7901 cells. The results confirmed that the expression levels of p-AKT, YAP1, and p-PI3K were significantly downregulated in the cells with miR-762 inhibition (Figure 5, P<0.01). Increased LZTS1 was also observed in SGC-7901 cells with reduced miR-762 expression (Figure 5, P<0.01). In addition, the effects of miR-762 silencing on the levels of p-AKT, YAP1, and p-PI3K were reversed in cells co-transfected with si-LZTS1 (Figure 5, P<0.01).

**Discussion**

This study explored the role of miR-762 in GC, and revealed an influence of miR-762 level on the proliferation, viability and apoptosis of GCs, and illustrated the functional mechanism of miR-762 in the progression of GC.

Dysfunction of miRNAs is one of major causes of the development and formation of many diseases ranging from organ inflammation to multiple cancers [12]. In this study, increased miR-762 was observed in the pathological samples and cell lines of GC. According to recent studies, miR-762 is involved in the progression of multiple cancers [14, 15]. For instance, it has been confirmed that miR-762 is overexpressed in the serum of the patients with non-small cell lung cancer, and it can be used as a biomarker for evaluating the malignancy of the tumor [16].
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Figure 2. MiR-762 silencing inhibited the proliferation, invasion, and migration and promoted apoptosis of SGC-7901 cells. A: The effect of miR-762 on the viability of SGC-7901 cells was observed by CCK-8 assay; B: The effect of miR-762 on the apoptosis level of SGC-7901 cells was measured by flow cytometry assay; C: The effects of miR-762 on the invasion and migration of SGC-7901 cells were observed by transwell assay (Scale bar =50 μm, 400×). **P<0.01.

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The dysfunction of miRNAs can induce the phenotype alteration and malfunction of the cells. For instance, miR-133a-3p is significantly downregulated in esophageal squamous cell carcinoma cells, and overexpressed miR-133a-3p can impede the malignant behavior and advance the apoptosis of tumor cells [17]. This study found that the malignant behavior of GC cells was significantly suppressed after miR-762 downregulation. Several studies have confirmed that miR-762 plays a key role in regulating the biological behaviors of cancer cells. A study indicated that circ 0071662 can suppress the proliferation and invasion abilities of bladder cancers cells by sponging miR-762 [18]. GC development is a complex event which generally involves changes in several pathways. A study has indicated that miR-762 can activate the STAT3 and AKT pathway by inhibiting the expression of HPGD to promote the proliferation and invasion of cervical cancer cells [19]. In this study, it was found that miR-762 played a role in the activation of the Hippo and PI3K/AKT pathways. Therefore, we suggest that miR-762 is a key promoter in the development of GC. Considering the character of miRNAs in regulating the abundance of some proteins by combining with the 3’-UTR of the related mRNAs, we hypothesized that regulating the abundance of the downstream factors might be an important mechanism of miR-762 affecting the progression of GC [20, 21]. IRF7 is a down-

Figure 3. MiR-762 directly targets the 3’-UTR of LZTS1, and LZTS1 was significantly downregulated in the tissues and cells of gastric cancer. A: The binding effect of miR-762 on LZTS1 was observed by dual-luciferase reporter assay; B: The expression level of LZTS1 in the tissues was observed by immunohistochemistry (Scale bar =20 μm, 400×); C, D: The relative expression levels of LZTS1 in the cells of GES-1, MGC-803, BGC-823 and SGC-7901 were measured by western blot. *P<0.05, **P<0.01.
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stream factor of miR-762, and increased IRF7 induced by miR-762 downregulation can weaken the cell proliferation and invasion abilities of thyroid cancer [15]. This project confirmed that miR-762 could directly bind to the 3′-UTR of LZTS1, and aberrant abundance of LZTS1 was also measured in GCs when miR-762 was downregulated. LZTS1 is involved in multiple biological behaviors of the cells, and LZTS1 deficiency is related to a poor prognosis of multiple cancers [22, 23]. In this study, reduced LZTS1 was found in GC tissues, and LZTS1 downregulation could reverse the effects of miR-762 silencing on the malignant behaviors of GC cells. Studies showed that the expression level of LZTS1 was notably reduced in tumor tissues compared with the para-cancerous tissues, and increased LZTS1 could significantly inhibit the proliferation and invasion of GC cells. Recently, the anti-cancer properties of LZTS1 have been revealed by several studies showing that LZTS1 regulates the viability and apoptosis of the cells to impede the progression of multiple cancers. For instance, Guanen et al. indicated that miR-214 could downregulate the expression level of LZTS1 though the PI3K/AKT/mTOR pathway to promote the proliferation and inhibit the apoptosis of lung cancer cells [24]. Wan et al. found LZTS1 was a biomarker of uveal melanoma, and the low expression level of LZTS1 was closely associated with poor outcome of patients with uveal melanoma [25]. Moreover, we also found that LZTS1 upregulation could remarkably suppress the PI3K/AKT and boost Hippo pathways. One study has indicated that LZTS1 is associated with the changes in angiogenesis, cell proliferation, and autophagy of liver hepatocellular carcinoma cells, and LZTS1 upregulation can inactivate the PI3K/AKT pathway to suppress the

Figure 4. LZTS1 downregulation reversed the effects of miR-762 silencing on SGC-7901 cells. A: The effect of miR-762 silencing and LZTS1 downregulation on the viability of SGC-7901 cells was observed by CCK-8 assay; B, C: The effect of miR-762 silencing and LZTS1 downregulation on the apoptosis level of SGC-7901 cells was measured by flow cytometry assay; D-F: The effects of miR-762 silencing and LZTS1 downregulation on the invasion and migration of SGC-7901 cells were observed by transwell assay (Scale bar =50 μm, 400×). **P<0.01.
Progression of liver hepatocellular carcinoma [26]. The study of Li et al. has shown that LZTS1 is regulated by miR-135b, and the expression level of LZTS1 can significantly affect the activation of the Hippo pathway [15]. Therefore, we suggest that decreased LZTS1 controls the functional mechanism of miR-762 in GC.

**Conclusion**

MiR-762 can regulate the activities of PI3K/AKT and Hippo pathways to control the development of gastric cancer by targeting LZTS1.

**Disclosure of conflict of interest**

None.

**References**


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