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LncRNA RPPH1 predicts poor prognosis and regulates cell proliferation and migration by repressing P21 expression in gastric cancer

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Abstract. – OBJECTIVE: The purpose of this study was to explore the expression and biological functions of long non-coding ribonucleic acid (lncRNA) ribonuclease P RNA component H1 (RPPH1) in gastric cancer (GC), and to analyze the correlations of lncRNA expression with the clinical features and prognosis of GC patients.

PATIENTS AND METHODS: The relative expression of RPPH1 in tissue specimens from 60 GC patients was measured *via* quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR), and the correlations of RPPH1 expression with tumor-node-metastasis (TNM) stage, lymph node metastasis, etc. in GC patients were analyzed. Then, qRT-PCR was performed to detect the relative expression level of RPPH1 in GC cells. Moreover, colony formation assay, 5-Ethynyl-2'-deoxyuridine (EdU) staining, wound-healing assay, and transwell assay were employed to investigate the influence of RPPH1 on GC cell functions. After interfering in the expression of RPPH1, the changes in p21 (CDKN1A, cyclin dependent kinase inhibitor 1A) expression were determined through qRT-PCR and Western blotting.

RESULTS: It was shown in qRT-PCR assay results that the expression of RPPH1 was upregulated in 60 cases of GC tissues. Statistical analysis revealed that RPPH1 expression was positively correlated with the TNM stage, lymph node metastasis, and infiltration depth in GC patients. Besides, highly expressed lncRNA RPPH1 suggested poor prognosis of GC patients. Based on the results of qRT-PCR assay, the expression of RPPH1 in GC cells was upregulated. After interfering in RPPH1 expression, both colony formation assay and EdU staining indicated that the proliferative capacity of GC cells was repressed. Furthermore, it was manifested in the results of wound-healing and transwell assays that the migratory and invasive abilities of GC cells were weakened. Finally, the qRT-PCR and Western blotting assay results demonstrated that p21 expression was upregulated after interfering in the expression of RPPH1 in GC cells.

CONCLUSIONS: The expression of lncRNA RPPH1 is upregulated in GC, suggesting that the prognosis of the patients is poor. Highly expressed RPPH1 promotes the proliferation and metastasis of GC cells by regulating p21 expression.

Key Words:
Gastric cancer, LncRNA RPPH1, Proliferation, Metastasis, p21.

Introduction

Gastric cancer (GC) is a common malignant tumor of the digestive system in clinic, whose etiology and pathogenesis have not been fully understood yet[1]. Radical resection is the only means capable of curing GC at present, but most patients have been in the middle and advanced stage when diagnosed due to inconspicuous early symptoms of GC. Therefore, the patients cannot receive radical surgery, and they are prone to relapse and metastasis even if surgical resection is available, presenting relatively poor prognosis[2]. Hence, it is urgently necessary to elaborate the molecular mechanism of GC onset, so as to find ideal early diagnostic markers and highly-efficient specific therapeutic targets.

Long non-coding ribonucleic acids (lncRNAs), a category of non-coding RNAs with over 200 nucleotides in length, cannot encode proteins but can regulate gene expressions at the transcriptional and post-transcriptional levels[3-5]. They are also implicated in the biological process of tumor cells, including proliferation, migration, and apoptosis. The expression of lncRNAs is dysregulated in GC, and lncRNAs can act as cancer promoters or suppressors to modulate the occurrence and development of the disease. Shi et al[6] found that lncRNA

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GACAT1 (gastric cancer associated transcript 1) regulates the expressions of SP1 (Sp1 transcription factor) and ZBTB2 (zinc finger and BTB domain containing 2) by adsorbing micro RNA (miR)-149, thus facilitating the proliferation and metastasis of GC. Ma et al[7] reported that lncRNA GCAWKR (the gastric-cancer-associated WDR5 and KAT2A binding lncRNA) promotes the progression of GC by binding to chromatin modifiers WDR5 (WD repeat domain 5) and KAT2A (lysine acetyltransferase 2A), while lncRNA AB007962 expression is downregulated in GC tissues and cells and associated with the prognosis of GC patients[8]. However, there are no reports about the expression and function of lncRNA ribonuclease P RNA component H1 (RPPH1) in GC.

This research group took the lead in discovering that RPPH1 expression is upregulated in GC, highly expressed RPPH1 has positive correlations tumor-node-metastasis (TNM) stage, and other clinical features, and interfering in RPPH1 expression represses the proliferation and metastasis of GC cells. The present study provided biomarkers for prognosis factors of GC patients and offered theoretical bases for clinical seeking of targeted therapy for GC.

Patients and Methods

Clinical Data

A total of 60 GC patients admitted to and treated in The Affiliated Hospital of Qingdao University from January 2012 to December 2015 were selected. All the patients were confirmed by postoperative histopathological examination. The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). Inclusion criteria: 1) patients meeting the diagnostic criteria for GC, 2) those who were diagnosed for the first time, 3) those undergoing radical or palliative resection, without any anti-tumor therapy before operation, and 4) those with complete clinical, pathological and follow-up data. Exclusion criteria: 1) patients complicated with malignant tumors at other sites, systemic infectious disease or serious disease in vital organs, such as liver and kidney, 2) those who received any preoperative anti-tumor therapy, or 3) those with incomplete clinical, pathological or follow-up data. This investigation was approved by the Ethics Committee of The Affiliated Hospital of Qingdao University, and all the patients or their families signed the informed consent.

Extraction and Reverse Transcription of RNA

The total RNA was extracted from cancer and para-carcinoma tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as per the instructions. The concentration of the total RNA was measured, and then, the total RNA was reversely transcribed into cDNA by means of Prime-Script™ quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) kit (TaKaRa, Otsu, Shiga, Japan).

QRT-PCR

SYBR® Green dye method (TaKaRa, Otsu, Shiga, Japan) was adopted for quantitative reverse transcriptase PCR according to the following conditions: pre-denaturation at 95°C for 10 min, denaturation at 95°C for 10 s, annealing at 57°C for 20 s, and extension at 72°C for 15 s. 3 parallel samples were set up for each sample to be detected. Later, qRT-PCR was performed using ABI7500 system (Applied Biosystems, Foster City, CA, USA) and SYBR Green PCR Master Mix (TaKaRa, Otsu, Shiga, Japan). Finally, the expression level of lncRNA RPPH1 in GC tissues and corresponding para-carcinoma tissues was analyzed through $2^{-\Delta\Delta Ct}$ method. The primer sequences are as follows: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) R: 5'-ATTTCCTTGAGGCTGGCACA-3' and F: 5'-AATTGGATGCGTGTCCCTCC-3', lncRNA RPPH1 R: 5'-TGGAAGAAGTCTCCTCATGACC-3' and F: 5'-AATAGGCCAGA GGCTTCAC-3', and p21

Cell Culture

Human GC cell line (SGC7901, MKN45, MKN28, BGC823, MGC803) and normal human gastric mucosal epithelial cell (GE-1) were purchased from Shanghai Institutes for Biological Sciences (Shanghai, China). Then, the cells were cultured in a mixture medium of Roswell Park Memorial Institute-1640 (RPMI-1640) medium or Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), penicillin (100 U/mL) and streptomycin (100 μg/mL, Beyotime Biotechnology, Shanghai, China) and placed in a constant-temperature incubator with 5% CO_2 at 37°C.

Cell Transfection

The cells were inoculated overnight and then transfected with 10 nM small interfering RNA (siRNA) and siRNA-free blank control (Invitrogen, Carlsbad, CA, USA) in Opti-MEM® (Invitrogen, Carlsbad, CA, USA) using Lipofectamine Reagent

(Invitrogen, Carlsbad, CA, USA). After cell growth for 10-12 h, the transfection experiment was conducted, with a final concentration of 10 nmol. 48 h later, the cells were collected, and the interference efficiency was determined *via* qRT-PCR.

Colony Formation Assay

The cells were transfected with si-RPPH1 inhibitor, then, seeded into 6-well plates (1×10^3 cells/plate) using a medium containing 10% fetal bovine serum and preserved in the cell incubator at 37°C with 5% CO_2. Next, the transfected cells were placed in the 6-well plates and stored for 14 d, and the medium was replaced every 3 d. After that, the cells were washed with 1 mL of phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 15 min, and stained with 0.1% crystal violet for 20 min. Finally, the cells were washed with 1 ml of water for 3 times. Subsequently, the colonies were counted manually. Finally, two-tailed *t*-test was applied to evaluate the differences, and the experiment was repeated for each cell line for 3 times.

5-Ethynyl-2'-Deoxyuridine (EdU) Staining

The cells were paved on 24-well plates and transfected the next day. After 24 h, the EdU reagent A (Thermo Fisher Scientific, Waltham, MA, USA) was diluted at 1000:1 and added into the 24-well plates at 500 μL/well. 4-6 h later, the cells were washed with PBS, and the procedures were completed according to the instructions. Finally, the images were obtained through RFP and 4',6-diamidino-2-phenylindole (DAPI) channels separately under a fluorescence microscope and then subjected to Merge and statistical analysis.

Wound-Healing Assay

After transfection with si-RPPH1 or si-NC for 48 h, a wound was scratched on the adherent cells using the tip of a 200 μL sterile pipette. Next, the cells were washed in PBS for 3 times to remove any dissociative cells and fragments. Later, serum-free medium was added to culture the cells at 37°C with 5% CO_2. At 0 and 12 h after transfection, the wound-healing conditions were observed and photographed using a digital microscope. The experiment was repeated for 3 times.

Transwell Assay

The cells in experimental group and control group were prepared into cell suspension (1×10^6 cells/mL) using serum-free medium. Then, 100 μL of the cell suspension was seeded into the upper membrane of transwell chambers (for cell migration assay, the upper chambers were not coated with Matrigel. For cell invasion assay, the upper chambers were coated with Matrigel), and the lower membrane was added with 600 μL of medium containing 10% serum. After cell penetrating for 24 h, the chambers were taken out, the cells on the upper membrane were wiped out, and those on the lower membrane were stained with 0.1% crystal violet. Finally, the cells were photographed under an upright microscope and counted in 5 randomly selected fields of vision.

Western Blotting Assay

The cells in experimental group and control group were harvested to extract total proteins using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). After quantification, 20 μg of proteins were taken from every sample and subjected to denaturation at high temperature, followed by separation *via* sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Later, the PVDF membrane was sealed in 5% skim milk powder for 30 min and incubated with primary antibodies (diluted at 1:2000) overnight. The next day, the membrane was washed in Tris-buffered saline and Tween 20 (TBST), incubated with secondary antibodies for 1 h, and washed with TBST again, followed by color and image development *via* enhanced chemiluminescence (ECL) method.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. The measurement data were expressed by ($\bar{\chi}\pm s$), and independent-samples *t*-test was employed for comparison of results. Pearson correlation analysis was adopted for correlation analysis, and Kaplan-Meier method was used for survival analysis. $p<0.05$ suggested that the difference was statistically significant.

Results

LncRNA RPPH1 Expression Was Up-Regulated in GC and Suggested Poor Prognosis of Patients

The cancer and para-carcinoma tissue specimens were collected from 60 GC patients, from which the RNA was extracted, and the expression

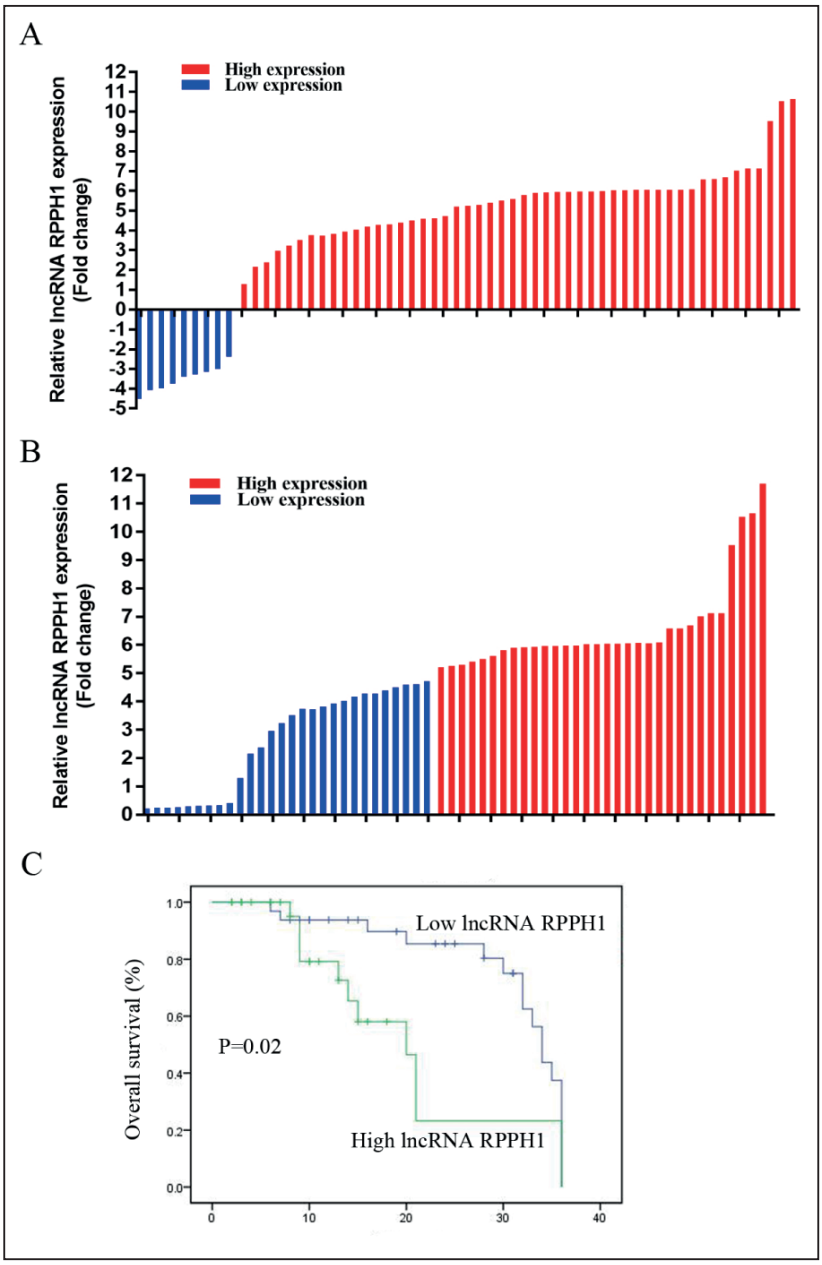

Figure 1. LncRNA RPPH1 expression is up-regulated in GC and suggests poor prognosis of patients. **A**, RPPH1 expression in 60 cases of GC tissues detected *via* qRT-PCR. The results show that the expression of RPPH1 is up-regulated in 51 cases of GC tissues. **B**, With the average value of the relative expression of RPPH1 as the cutoff point, the tissues are divided into RPPH1 high expression group (n=32) and RPPH1 low expression group (n=28). C, Kaplan-Meier survival analysis indicates that increased RPPH1 expression suggests unfavorable prognosis of the patients.

of RPPH1 in the tissues was measured *via* qRT-PCR assay. The results showed that compared with that in para-carcinoma tissues, RPPH1 expression was upregulated in 51 cases of GC tissues (Figure 1A). Next, with the average value of the relative expression of RPPH1 as the cutoff point, the tissues were divided into RPPH1 high-expression group (n=32) and RPPH1 low-expression group (n=28) (Figure 1B), and the correlations of RPPH1 expression level with the clinical features of GC patients were analyzed *via* chi-square test. It was manifested that the expression level of RPPH1 had positive correlations with the TNM stage (p=0.004), lymph node metastasis (p=0.005), and infiltration depth (p=0.014) (Table I). Subsequently, the Kaplan-Meier survival analysis revealed that the raised RPPH1 expression implied unfavorable prognosis of the patients (Figure 1C).

Si-RPPH1 Inhibited GC Cell Proliferation

In order to investigate the biological function of RPPH1 in GC cells, qRT-PCR assay was conducted to measure the expression level of RPPH1 in GC cells firstly. The results mani-

Table I. Association between lncRNA RPPH1 expression and clinicopathological features of the GC patients (n=60).

Characteristics	Cases (No.)	RPPH1		*p*-value
		Low level No. case	High level No. case	
Age (years)				
>60	26	14	12	0.435
≤60	34	14	20	
Sex				
Male	33	18	15	0.203
Female	27	10	17	
Tumour size (cm)				
>5	29	11	18	0.246
≤5	31	17	14	
Histological type				
Intestinal	30	13	17	0.816
Diffuse	30	15	15	
TNM staging				
I+II	35	22	13	0.004*
III+IV	25	6	19	
Lymph node metastasis				
No	29	19	10	0.005*
Yes	31	9	22	
Invasion degree				
T1	13	8	5	0.014*
T2	16	11	5	
T3	16	5	11	
T4	15	4	11	

fested that RPPH1 expression was upregulated in GC cells (Figure 2A). Next, the RPPH1-specific interference sequences were designed and synthesized to determine the interference efficiency 48 h later (Figure 2B). Moreover, it was shown in colony formation assay that the proliferative capacity of GC cells was repressed after interference in the RPPH1 expression in GC cells (Figure 2C, 2D). Finally, the results of EdU staining indicated that the cell proliferation rate declined after knockdown of RPPH1 expression (Figure 2E, 2F).

Si-RPPH1 Inhibited GC Cell Metastasis

Secondly, the wound-healing assay was employed to explore the impact of RPPH1 on the migratory ability of GC cells, and the results revealed that the migratory ability was decreased in experimental group in comparison with that in control group (Figure 3A, 3B). Besides, the transwell assay results demonstrated that the migratory and invasive abilities of GC cells was suppressed after interference in RPPH1 expression (Figure 3C, 3D).

LncRNA RPPH1 Regulated p21 Expression

For the purpose of studying the downstream molecular mechanism of RPPH1 in performing biological functions in GC cells, qRT-PCR assay was conducted, and it was found that the messenger RNA (mRNA) expression level of p21 was altered after interfering in RPPH1 expression (Figure 4A). Later, the results of Western blotting assay manifested that after interference in the expression of RPPH1, the protein expression level of p21 was changed (Figure 4B).

Discussion

The occurrence and development of GC is a complex process involving multiple factors and genes[9,10], whose etiology and pathogenesis have not been completely clarified yet. Due to the atypical symptoms of the disease in the early stage, the majority of GC patients have been in the middle and advanced stage when diagnosed with apparent symptoms, missing the best opportunity for radical operation. Besides, even though GC can

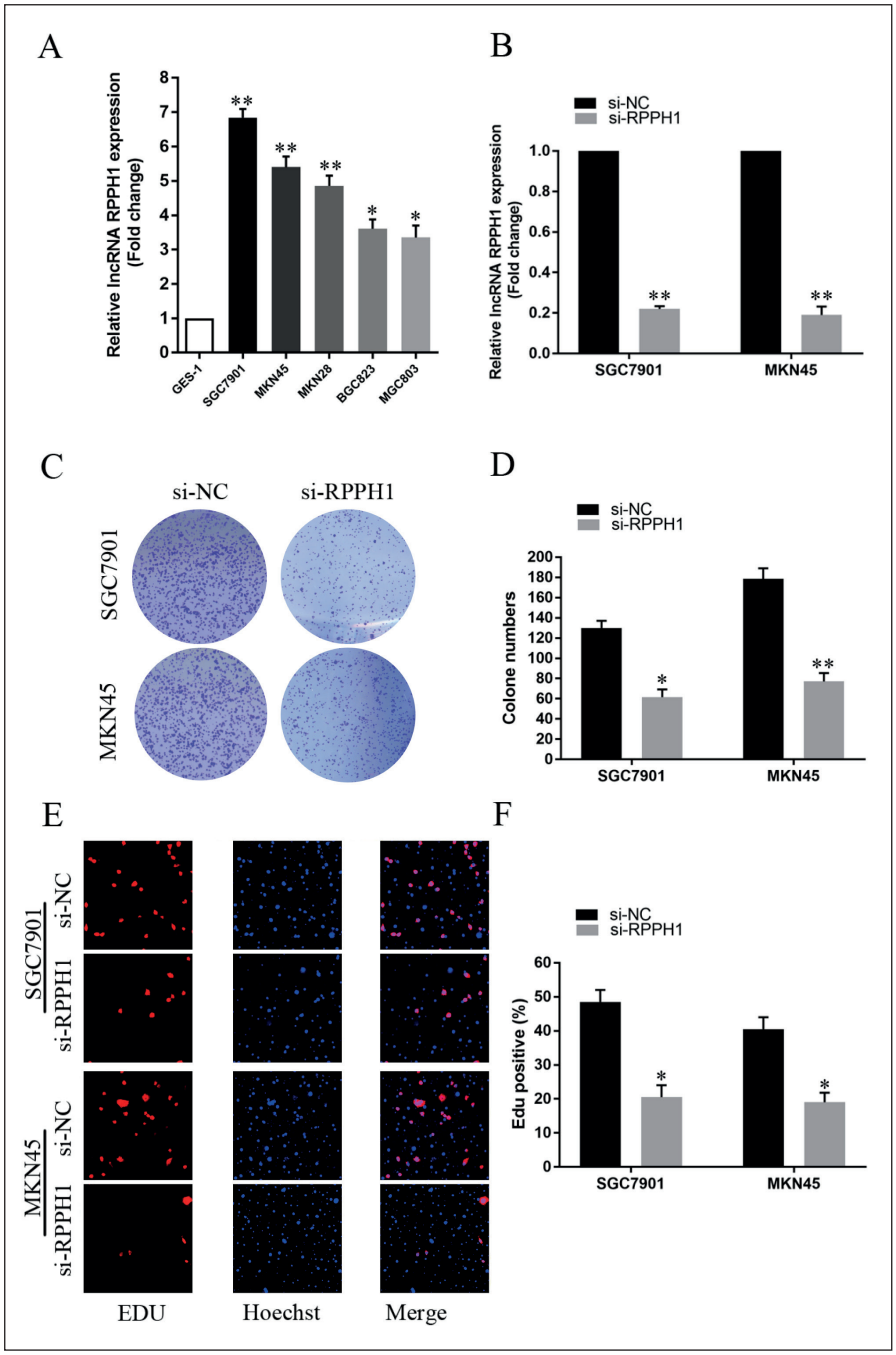

Figure 2. Si-RPPH1 inhibits GC cell proliferation. **A**, QRT-PCR assay manifests that RPPH1 expression is up-regulated in GC cells. **B**, RPP-specific interference sequences are designed to measure the interference efficiency *via* qRT-PCR. **C-D**, Colony formation assay results demonstrate that the proliferative capacity of GC cells is suppressed after interference in RPPH1 expression. (magnification: 40×) **E-F**, The results of EdU staining indicate that the knockdown of RPPH1 expression in GC cells reduces the cell proliferation rate (magnification: 400×).

be excised by operation, the patients are highly vulnerable to relapse and metastasis after operation, with relatively poor prognosis[11]. Hence, there is an urgent need to elucidate the molecular mechanism of GC onset, so as to seek for more ideal early diagnostic markers and highly-efficient specific therapeutic targets.

With the constant development of genome sequencing technology in recent years, a growing number of RNAs without protein-encoding function have been discovered, which play vital roles in the occurrence and development of tumors[12,13] Currently, lncRNAs are the most frequently researched ncRNAs in clinic, and they exert irreplaceable effects in modulating such biological processes as proliferation, apoptosis, invasion, and migration of tumor cells[14].

LncRNA RPPH1 is located on chromosome 14q11.2. It has been reported in literature that RPPH1 can accelerate the metastasis of colorectal

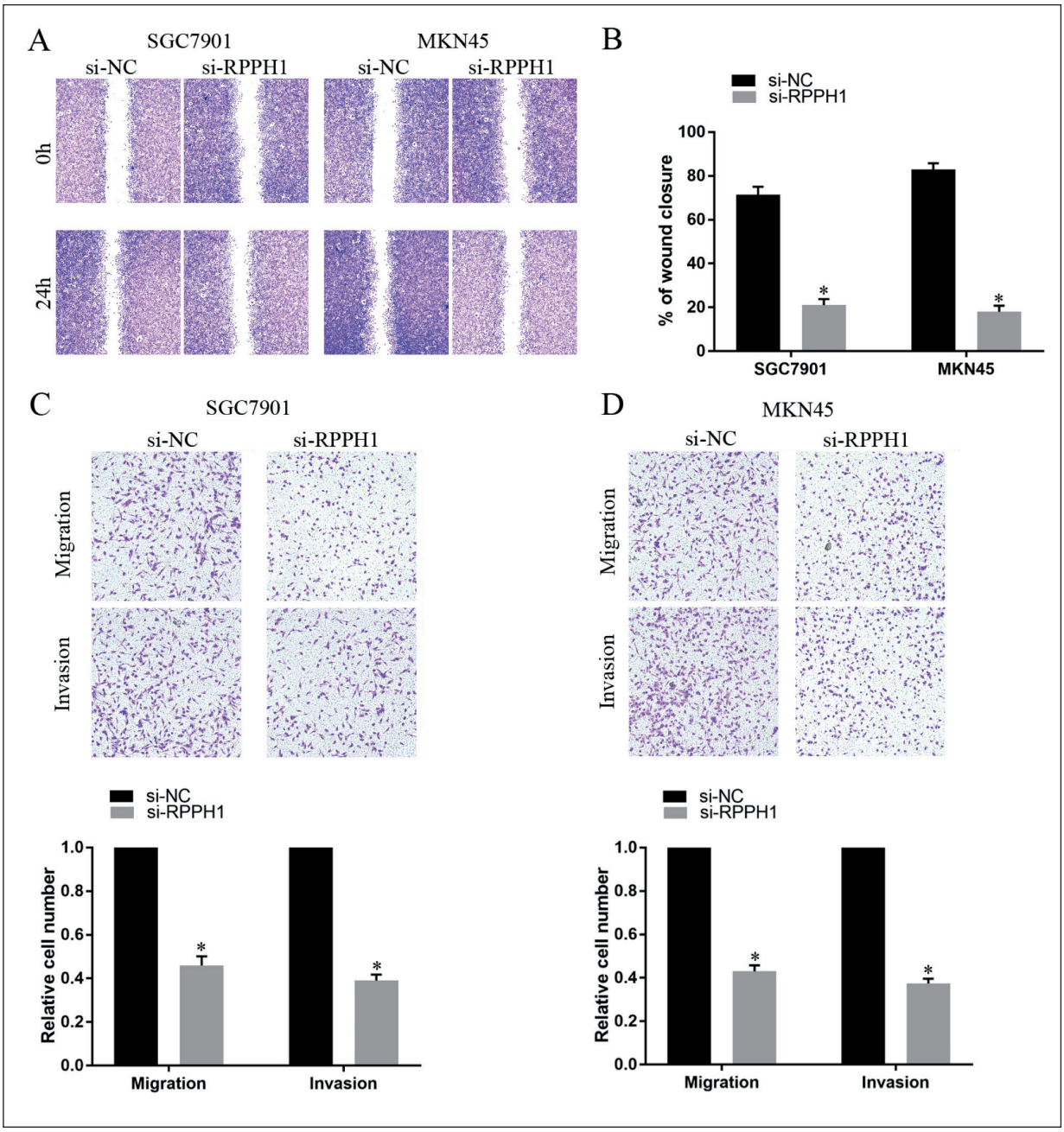

Figure 3. Si-RPPH1 inhibits GC cell metastasis. **A-B**, Wound-healing assay reveals that the migratory ability of GC cells is decreased after interfering RPPH1 expression. **C-D**, Transwell assay results exhibit that the migratory and invasive abilities of GC cells is suppressed after interference in RPPH1 expression (magnification: 40×).

cancer by regulating the expression of TUBB3 (tubulin, beta 3 class III)[15]. In the case of breast cancer, RPPH1 expression is upregulated, which promotes the proliferation of breast cancer cells by regulating miR-12[16]. It was discovered through *in-vitro* experiments in this research group that the expression of RPPH1 was increased in GC, and the proliferation and metastasis of GC cells was enhanced.

As a member of the cyclin-dependent kinase (CDK) inhibitory factor family, p21 is able to recognize cells with impaired DNA, arrest the cell cycle by disturbing the combination between CDK and cyclins, and prevent the further abnormal proliferation of cells[17,18]. Many lncRNAs regulate p21 expression by directly and indirectly binding to p21, thus affecting tumor progression. Tian et

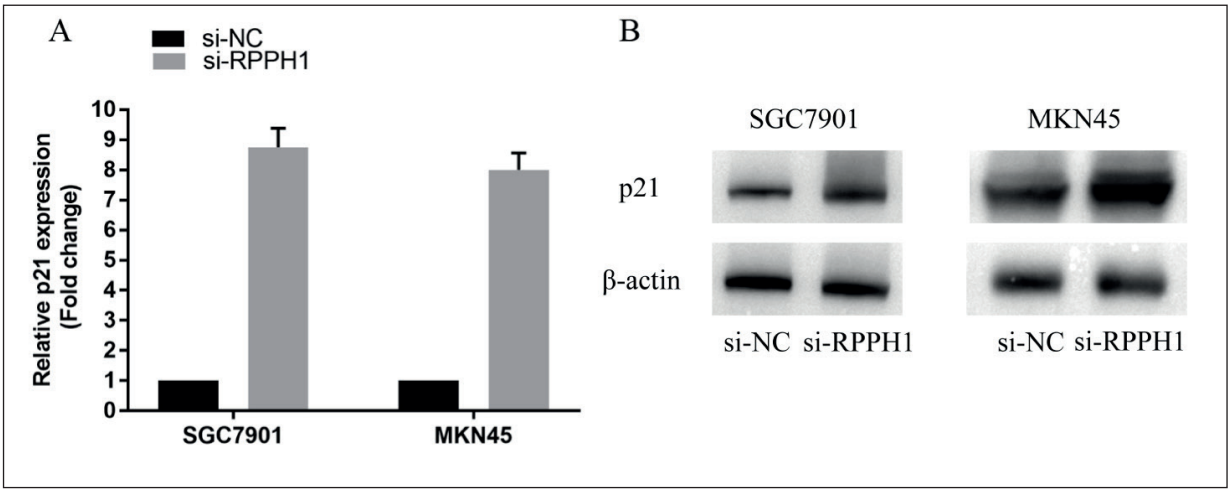

Figure 4. LncRNA RPPH1 regulates p21 expression. **A**, According to the results of qRT-PCR assay, the mRNA expression level of p21 is up-regulated after the expression of RPPH1 is interfered. **B**, Based on the Western blotting results, the protein expression level of p21 is up-regulated after interfering in RPPH1 expression.

al[19] has reported that linc00152 is highly expressed in GC, and it can summon PRC2 (polycomb-group proteins 2) to the promoter region of p21 and inhibit p21 expression *via* H3K27me3 (Trimethylation of lysine 27 of histone H3). Moreover, in gallbladder cancer, lncRNA UCA1 (urothelial cancer associated 1) can facilitate the growth and metastasis of gallbladder cancer cells by repressing the transcription of p21 and E-cadherin[20]. In the *in-vitro* experiments in this research, it was revealed that the mRNA and protein expression levels of p21 were upregulated after interference in RPPH1 expression in GC cells.

Conclusions

The results of this investigation indicate that expression of lncRNA RPPH1 is upregulated in GC, suggesting that the prognosis of the patients is poor. Highly expressed RPPH1 promotes the proliferation and metastasis of GC cells by regulating p21 expression.

Conflict of Interests

The authors declare that they have no conflict of interests.

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