Silencing Ribosomal Protein L22 Promotes Proliferation and Migration, and Inhibits Apoptosis of Gastric Cancer Cells by Regulating the Murine Double Minute 2-Protein 53 (MDM2-p53) Signaling Pathway

ABCE 1 Zhenqing Sun*
ABCE 1 Zhigang Qiu*
DE 1 Zhengkun Wang
DE 2 Honghui Chi
CE 3 Peipei Shan

* Zhenqing Sun and Zhigang Qiu contributed equally to this paper

Corresponding Author: Peipei Shan, e-mail: shanpeipei@qdu.edu.cn

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Background: The aim of this study was to investigate the effect of ribosomal protein L22 (RPL22) on gastric cancer (GC) cell proliferation, migration, and apoptosis, and its correlation with the murine double minute 2-protein 53 (MDM2-p53) signaling pathway.

Material/Methods: The RPL22 expression in GC tissues and cells was detected by quantitative reverse transcription-polymerase chain reaction and western blotting. RPL22 was overexpressed in the MKN-45 cells by the transfection of a vector, pcDNA3.1 (pcDNA)-RPL22, whereas it was silenced in the MGC-803 cells by the transfection of short interfering (si) RNA (si-RPL22). Flow cytometric analysis, cell viability assays, wound healing assays, and transwell assays were utilized to explore the influences of RPL22 on the apoptosis, proliferation, migration, and invasion. Nutlin-3 (an MDM2-p53 inhibitor) was used to inhibit MDM2-p53 signaling.

Results: The RPL22 expression was downregulated in GC tissues and cells. It was significantly lower in the advanced GC tissues than in the early GC tissues, and was significantly lower in the lymphatic metastatic tissues than in the non-lymphatic metastatic tissues. The transfection of si-RPL22 accelerated the ability of GC cells to proliferate and metastasize, whereas apoptosis was dampened. The transfection of pcDNA-RPL22 exerted the opposite effect on the GC cells; MDM2 expression was upregulated in RPL22-silenced GC cells, while the expression of p53 was downregulated. In vitro, treatment with nutlin-3 reversed the promoting effects of si-RPL22 on GC progression.

Conclusions: In vitro, the silencing of RPL22 aggravates GC by regulating the MDM2-p53 signaling pathway.

Keywords: Apoptosis Inducing Factor • Cell Migration Assays • Cell Proliferation • Stomach Neoplasms

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We investigated the expression of RPL22 in GC cells and tissues, and the possible functions of RPL22 in GC tumorigenesis, in vitro. The interactions between RPL22 and MDM2-p53 signaling in the GC cells were evaluated. These findings have potential applications for the clinical treatment of GC.

### Material and Methods

#### Tissue Collection

GC tissues and normal tissues were collected for the detection of RPL22 expression. From September 2016 to November 2018, 40 paired GC tissues and normal tissues were obtained from 40 patients with GC (22 men and 18 women, aged 35 years to 55 years). The inclusion criteria were patients with a first-time diagnosis, no history of radiotherapy, chemotherapy, or other adjuvant therapy. The exclusion criteria were the presence of other malignant tumors, and patients who had received GC treatment before admission.

#### Cell Transfection

RPL22 was silenced and overexpressed in the MKN-45 and MGC-803 cells by the transfection of RPL22 short interfering (si) RNA (si-RPL22) and a vector (pcDNA) pcDNA3.1-RPL22 (pcDNA-RPL22), respectively (Table 1). pcDNA-RPL22, pcDNA negative control (pcDNA-NC), si-RPL22, and siRNA negative control (si-NC) were purchased from Han Biotech (Shanghai, China). Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was used to perform the transfection for transfection was approximately 48 h, followed by the collection of transfected cells for the experiments. In addition, nutlin-3 (a MDM2-p53 inhibitor, ab120646, Abcam, Cambridge, UK) was used to treat the transfected cells for 60 min.

#### Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

A qRT-PCR was used to detect the expression of RPL22 in the GC tissues and cells. Total RNA was initially extracted from the cells and tissues using TRizol Reagent (Promega, Madison, WI, USA). A qRT-PCR was used to detect the expression of RPL22 in the GC tissues and cells. Total RNA was initially extracted from the cells and tissues using TRizol Reagent (Promega, Madison, WI, USA).

### Background

Gastric cancer (GC), a gastrointestinal malignant tumor, has serious consequences on a patient’s life and health [1]. GC is asymptomatic in the early stages, and diagnosed at an advanced stage in the majority of patients [2]. It has high mortality and successful therapeutic strategies are limited [3]. At present, surgery is considered the most effective and radical treatment for GC [4], and despite improvements in the surgical techniques and other treatments, the prognosis for advanced GC remains poor [5]. Therefore, exploring the mechanisms underlying GC tumorigenesis can make a valuable contribution in the clinical treatment of GC.

Ribosomal proteins (RPs) are a major component of ribosomes and play fundamental roles in ribosome biogenesis [6]. Increasing evidence has shown RPs to be closely associated with cell growth and tumorigenesis in various cancers, including GC [7]. The expression of ribosomal protein L34 (RPL34) is upregulated in GC cells, whereas a knockdown of RPL34 significantly suppresses proliferation and facilitates apoptosis in GC cells [8]. The expression of ribosomal protein L15 (RPL15) is upregulated in GC cells, and RPL15 silencing can restrain the growth of GC cells [9]. Ribosomal protein S13 (RPS13) and ribosomal protein L23 (RPL23) are upregulated in GC cells, and promote multidrug resistance in GC cells by suppressing apoptosis [10]. Ribosomal protein L22 (RPL22) is a part of the 60S large ribosomal subunit [11]. Yang et al [12] showed that there was minimal expression of RPL22 in non-small cell lung cancer (NSCLC). Ferreira et al reported that RPL22 played a crucial role in microsatellite instability in the carcinogenesis of colorectal and endometrial tumors [13]. Rao et al showed that the loss of RPL22 promoted lymphoma progression through accelerating angiogenesis [14]. However, the possible role and function of RPL22 in GC tumorigenesis is still unclear.

Protein 53 (p53) is acknowledged as a tumor suppressor, which modulates cellular processes, including the cell cycle, senescence, and apoptosis [15]. The ubiquitination and degradation of the p53 proteasome are controlled by human murine double minute 2 (MDM2), an oncogenic E3 ligase [16]. MDM2 exerts its tumor-suppressing function by inhibiting p53 activity and stability [17]. Increasing evidence suggests that p53 and MDM2 play vital roles in many cancers, including breast cancer [18], lung cancer [19], hepatocellular carcinoma [20], and GC [21]. Previous research has shown that several RPs play a critical role in MDM2-p53 signaling. For example, RPL5 and RPL11 bind to MDM2 and activate p53 [22,23]. RPL23 [24] and RPS7 [25] are MDM2-binding partners. A previous study proved that RPL22, a p53 activator, inhibited the colony formation of osteosarcoma cells through the regulation of MDM2-p53 signaling [26]. However, the regulatory mechanisms between RPL22 and MDM2-p53 signaling on GC progression are relatively unknown.
USA), followed by synthesizing complementary DNA (cDNA) using the PrimeScript RT reagent kit (Takara, Kyoto, Japan), and performing a qRT-PCR with synergy brands green (SYBR Green) FAST Mastermix (Qiagen, Dusseldorf, Germany). The relative expression level was assessed by the 2^-ΔΔCT method (27). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the expression of RPL22. The primer sequences are listed in Table 1.

### Western Blotting

The protein levels of RPL22, MDM2, p53, and GAPDH were determined by a western blot. The MGC-803 and MKN-45 cells were primarily lysed with a radioimmunoprecipitation assay (RIPA) buffer to collect the total protein. This was followed by separation of the protein product with a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which was then transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% bovine serum albumin for 1 h, followed by an overnight incubation at 4°C with the diluted primary antibodies anti-RPL22 (1: 1000, ab77720; Abcam, Cambridge, UK), anti-MDM2 (1: 1000, #86934; Cell Signaling Technology, Danvers, MA, USA), anti-p53 (1: 1000, #2527; Cell Signaling Technology, Danvers, MA, USA), and anti-GAPDH antibodies (1: 1000, #5174; Cell Signaling Technology, Danvers, MA, USA). After washing 3 times, the horseradish peroxidase (HRP)-conjugated secondary antibody (anti-rabbit; #14708; Cell Signaling Technology, Danvers, MA, USA) was added. Following incubation for 1 h at room temperature, the immunoblotting was visualized using an electrochemiluminescence exposure solution under a gel imaging system (Ultra-Violet Products, Upland, CA, USA).

### Cell Viability Assay

A CCK-8 assay was used to measure the viability of the GC cells. The MGC-803 and MKN-45 cells were planted in 96-well plates (2×10^4 cells/well), and cultured for 24, 48, 72, and 96 h, respectively. Next, approximately 10 μL of CCK-8 solution (BD Biosciences, San Jose, CA, USA) was added and the incubation continued for another 2 h at 37°C. Cell viability was determined using a microplate reader (BioTek Instruments Inc., Winooski, VT, USA) at an optical density of 450 nm (OD450).

### Flow Cytometric Analysis

The cell apoptosis was determined using flow cytometric analysis. The cells (1×10^6 cells/mL) were cultured for 24 h in 96-well plates, followed by staining with fluorescein isothiocyanate (FITC) (5 μL) and propidium iodide (PI) (10 μL), using the Annexin V-FITC apoptosis detection kit (Thermo Fisher Scientific, Waltham, MA, USA) at 25°C for 20 min in the dark. The apoptotic cells (FITC+, PI±) were measured using a flow cytometer (BD Biosciences, San Jose, CA, USA). The Annexin V measurements were used as the horizontal axis and those of PI were used as the vertical axis. The cells represented in the lower left quadrant were viable cells, the cells in the upper left quadrant were necrotic cells, the cells in the lower right quadrant were early apoptotic cells, and those in the upper right quadrant were late apoptotic cells. The total apoptotic cells were calculated as the cells in the upper right quadrant plus cells in the lower right quadrant.

### Wound Healing Assay

The migration of the GC cells was determined by a wound healing assay. After transfection, the MGC-803 and MKN-45 cells were plated on 6-well plates (5×10^4 cells/well), and a vertical scratch was created at the center of the well using a 1 mL aseptic liquid remover. The migration of the GC cells was assessed using Image-Pro Plus Analysis software (Media Cybemetics, Silver Spring, MD, USA). The relative migration rate was calculated as (1–scratch area at 48 h/scratch area at 0 h)×100%.

### Transwell Assay

A transwell assay was used to assess the invasion abilities of the GC cells. The cells (2×10^5 cells/well) cultured in a serum-free medium were seeded into the Matrigel precoated (BD Biosciences, San Jose, CA, USA) upper chambers. A medium (600 μL) containing 100 ng/mL of chemokine stromal cell-derived factor-1 (Sino Biological, Beijing, China) was added to the lower chambers. After 24 h of culturing, the invasion cells were fixed in methanol and stained with 0.1% crystal violet for 30 min. Cells from 4 random fields were photographed and counted.

| Name of primer | Sequences (5’-3’) |
|----------------|-------------------|
| RPL22-F        | CATGCCACCTAGGCTATGACT |
| RPL22-R        | TGGTAGGCCCCCTCAGTGTCTA |
| GAPDH-F        | GGGCGGAGTTTCCCTCCCAAT |
| GAPDH-R        | GGGCGGAGTTTCCCTCCCAAT |
| si-RPL22-1-F   | GCAUUGAAAUUUCAGGGUGU |
| si-RPL22-1-R   | UAGGAGGUGCAAUCUACU |
| si-RPL22-2-F   | GCACUAAAAGCGGGUUU |
| si-RPL22-2-R   | AUUAAUCUUUUGAUCGCCG |
| si-NC-F        | UUUUCCGAACUGUCAGU |
| si-NC-R        | ACCUGACACGUUCCAGAAGA |

F – forward; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; NC – negative control; R – reverse; RPL22 – ribosomal protein L22; si – short interfering RNA.

Table 1. Primer sequences used in the quantitative real-time polymerase chain reaction.
Statistical Analysis
Each assay was performed at least 3 times. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS version 22.0) (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 7.0 (Graphpad Software, San Diego, CA, USA). Data were presented as mean±standard deviation. Comparisons between the 2 groups were performed using the t test, and comparisons between >2 groups were carried out by one-way analysis of variance, followed by Fisher’s Least Significant Difference test. P<0.05 was considered statistically significant.

Each participant gave written informed consent. All the procedures followed in this study were approved by the Ethics Committee of our hospital (approval ID: QYFY WZLL25915).

Results
Decreased Expression of RPL22 Observed in Gastric Cancer Tissues and Cell Lines
The expression of RPL22 was initially detected in the tissues using a qRT-PCR analysis. In comparison with normal tissues, a declined expression of RPL22 was observed in the GC tissues (P<0.001) (Figure 1A). We determined the expression of RPL22 in gastric cancer cell lines (AGS, MKN-45, and MGC-803) and the normal human gastric epithelial cell line (GES-1) was detected by a quantitative real-time-polymerase chain reaction. The expression of RPL22 in gastric cancer cell lines and the normal human gastric epithelial cell line (GES-1) was detected by a western blot.

(A) *** P<0.001 vs normal tissues; (B, C) * P<0.05, ** P<0.01, *** P<0.001 vs GES-1.

Figure 1. Ribosomal protein L22 (RPL22) is downregulated in gastric cancer tissues and cell lines. (A) The mRNA expression of RPL22 in gastric cancer tissues and normal tissues was detected by a quantitative real-time-polymerase chain reaction. (B) The mRNA expression of RPL22 in gastric cancer cell lines (AGS, MKN-45, and MGC-803) and the normal human gastric epithelial cell line (GES-1) was detected by a quantitative real-time-polymerase chain reaction. (C) The protein expression of RPL22 in gastric cancer cell lines and the normal human gastric epithelial cell line (GES-1) was detected by a western blot.
**RPL22 Affects the Proliferation and Apoptosis of Gastric Cancer Cells**

To identify the role of RPL22 on GC carcinogenicity, first, we detected the transfection efficiency after transfection of si-RPL22-1/-2/pcDNA-RPL22 into the GC cells. The results of the qRT-PCR and western blotting revealed that RPL22 expression was distinctly elevated by the transfection with pcDNA-RPL22 in the MKN-45 cells, while it was dampened by the transfection with si-RPL22 in the MGC803 cells ($P<0.01$) (Figure 2A, 2B). The si-RPL22-1 was selected for subsequent transfection experiments due to its higher silencing efficiency. The CCK-8 assay results showed that the si-RPL22-1 transfection significantly increased the OD450 values of the MGC803 cells at 48, 72, and 96 h post culture. However, the overexpression of RPL22 obtained the opposite result ($P<0.01$) (Figure 2C). The apoptosis rate of the si-RPL22-1-transfected MGC803 cells was remarkably decreased ($P<0.01$), while that of the pcDNA-RPL22-transfected MKN-45 cells increased substantially when compared with the blank group ($P<0.01$) (Figure 2D).

**RPL22 Affects the Metastasis of Gastric Cancer Cells**

The abilities of migration and invasion were visibly elevated by the transfection of si-RPL22-1 in MGC803 cells compared to the blank cells (Figure 3A, 3B); however, these abilities were significantly restrained by the transfection of pcDNA-RPL22 in the MKN-45 cells ($P<0.01$).

**Silencing RPL22 Activates the MDM2-p53 Signaling Pathway**

We determined the protein expression of MDM2 and p53 after the transfection of si-RPL22-1/pcDNA-RPL22. The results of western blotting demonstrated that expression of MDM2 increased, while p53 expression decreased in the MGC803 cells in the si-RPL22-1 group compared to the cells in the blank group ($P<0.01$) (Figure 4A). In contrast, the transfection with pcDNA-RPL22 downregulated the expression of MDM2, and upregulated the expression of p53 in the MKN-45 cells ($P<0.01$) (Figure 4B).

### Table 2. Correlation between the expression of RPL22 and clinicopathological features of gastric cancer patients.

| Parameter                        | Number (N=40) | RPL22 mRNA expression | $P$ value |
|----------------------------------|--------------|-----------------------|-----------|
| Age (years)                      |              |                       |           |
| <50                              | 7            | 0.6618±0.1622         | 0.9766    |
| ≥50                              | 33           | 0.6700±0.1226         |           |
| Gender                           |              |                       |           |
| Male                             | 22           | 0.7084±0.1370         | 0.6191    |
| Female                           | 18           | 0.6199±0.0994         |           |
| Stage                            |              |                       |           |
| Early stage                      | 29           | 0.7370±0.0692         | 0.0372*   |
| Advanced stage                   | 11           | 0.4883±0.0347         |           |
| Lymphatic metastasis             |              |                       |           |
| No                               | 23           | 0.7301±0.0450         | 0.0212*   |
| Yes                              | 17           | 0.5723±0.0501         |           |
| Tumor position                   |              |                       |           |
| Cardia                           | 6            | 0.4996±0.0089         |           |
| Gastric fundus                   | 5            | 0.6793±0.1779         | 0.7469    |
| Antrum                           | 22           | 0.7101±0.1096         |           |
| Gastric angle                    | 7            | 0.7046±0.0686         |           |
| Degree of histological type      |              |                       |           |
| Good                             | 9            | 0.6633±0.1669         |           |
| Moderate                         | 6            | 0.6688±0.0892         | 0.995    |
| Poor                             | 25           | 0.6704±0.1253         |           |

RPL22 – ribosomal proteins L22; * represented significantly different at $P<0.05$. 

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si-RPL22-1 Promotes the Proliferation, Migration, and Invasion of MKN-45 Cells by Modulating MDM2-p53 Signaling

To confirm the influence of RPL22 on MDM2-p53 signaling in the GC cells, MDM2-p53 signaling was blocked by nutlin-3 treatment. The treatment with 50 μM nutlin-3 dramatically elevated the p53 expression, as well as decreased the protein expression of MDM2 in the MKN-45 cells (P<0.01) (Figure 5A). Nutlin-3 not only reversed the promoting effects of si-RPL22 on proliferation, migration, and invasion, but also the inhibiting effects on the apoptosis of MKN-45 cells (P<0.01) (Figure 5B-5E).

Discussion

RPs are abundant RNA-binding proteins, and the expression of many RPs is altered during tumorgenesis [28]. Studies have shown the upregulation and downregulation of RPs in different types of cancers, including the upregulation of RPL7a...
Figure 3. Ribosomal protein L22 (RPL22) influences the migration and invasion of gastric cancer cells. (A) The relative migration of MKN-45 and MGC-803 cells was detected by a wound healing assay. (B) The relative invasion of MKN-45 and MGC-803 cells was detected by a transwell assay. BLANK – cells without transfection; pcDNA-NC – cells transfected with pcDNA negative control; pcDNA-RPL22 – cells transfected with pcDNA-RPL22; si-NC – cells transfected with siRNA negative control; si-RPL22-1 – cells transfected with siRNA-RPL22-1. (A, B) ** P < 0.01, *** P < 0.001 vs BLANK; && P < 0.01 vs BLANK.
[29], RPL19 [30], RPL15 [9], and RPL34 [28], in human colorectal carcinoma, breast cancer, and GC, respectively; and the downregulation of RPL22 in NSCLC [12]. Evidence suggests that the abnormal expression of RPs was closely associated with tumorigenesis. In the present study, the RPL22 expression was significantly reduced in GC cells (P<0.05) and tissues (P<0.001). We conjectured that RPL22 can play a crucial role in GC. Previous studies have demonstrated that the expression of RPs is correlated with clinicopathological features. The expression of RPS16 was significantly associated with the pathological tumor grade and clinical stage in prostate cancer [31]. The expression of RPL19 in patients with advanced colorectal cancer was higher than that in early-stage colorectal cancer and control group patients [32]. RPL13 has been associated with advanced stages of GC [33]. Therefore, RPs can serve as prognostic markers for human cancers. In the present study, we found that the expression of RPL22 was strongly correlated with the early tumor stage and non-lymphatic metastasis. These results reveal that RPL22 could be an underlying clinical prognostic factor for GC.

RPs are considered to be regulators in the tumorigenesis of different cancers. The knockdown of RPS15A can suppress cell growth and colony formation, as well as induce cell apoptosis in human glioblastomas [34]. In vitro, the knockdown of RPL34 significantly inhibited cell proliferation in esophageal cancer [35]. The knockdown of RPL13 was shown to attenuate cell growth and induce cell cycle arrest in gastrointestinal cancer [33]. We found that RPL22 silencing efficiently increased the OD450 values and decreased the apoptosis rate. Our findings suggest that RPL22 could be a participant in the progression and development of GC by modulating cell proliferation and apoptosis. Therefore, we inferred that RPL22 can inhibit the development of GC, and be a potential therapeutic strategy for GC patients. We further explored the mechanisms underlying the RPL22-mediated GC metastasis. Metastasis, which involves the transfer of malignant tumors from an organ to a distant organ, is a frequent cause of death in patients with cancer [36]. In our research, we found that RPL22 silencing efficiently elevated the number of migrating and invading cells. Our findings were similar to those of previous studies.
Figure 5. Ribosomal protein L22 (RPL22) inhibits the proliferation, migration, and invasion of MKN-45 cells through regulation of MDM2-p53 signaling. (A) The protein expression of MDM2 and p53 in MKN-45 cells was detected by a western blot. (B) The optical density (OD450) value of the MKN-45 cells was detected by a CCK-8 assay. (C) The apoptosis rate of the MKN-45 cells was detected by a flow cytometry assay. (D) The relative migration of the MKN-45 cells was detected by a wound healing assay. (E) The relative invasion of the MKN-45 cells was detected by a transwell assay. BLANK – cells without transfection; si-NC – cells transfected with siRNA negative control; si-RPL22-1 – cells transfected with siRNA-RPL22-1; si-RPL22-1+nutlin-3 – cells transfected with siRNA-RPL22-1 and nutlin-3. (A) ** P<0.01 vs control; (B-E) ** P<0.01 vs BLANK; * P<0.05, ** P<0.01 vs si-RPL22-1.
For example, the knockdown of RPS3 was shown to decrease the invasion and migration of osteosarcoma cells [37]. RPS24 silencing efficiently blocked the cell migration of colon cancer cells [38]. The silencing of RPS7 was shown to reduce cell invasion and migration, decrease the expression of β-catenin, MMP2 and MMP13 (genes), and increase the E-cadherin expression in RPS7-silenced cells [39]. Our results demonstrated that the silencing of RPL22 promoted the metastasis of GC cells. Therefore, our findings suggest that RPL22 could be involved in the progression of GC by the regulation of cell migration and invasion. Under clinical conditions, RPL22 can inhibit the metastasis of GC, thereby contributing to an improvement in the poor prognosis of GC.

p53 is a tumor suppressor, and its function is significantly inhibited by binding to the MDM2 oncoprotein [40]. Hence, blocking the MDM2-p53 interaction to reactivate the p53 function can be a potential therapeutic cancer strategy. In our study, p53 expression was significantly decreased in the si-RPL22-transfected GC cells (P<0.01), while MDM2 expression was significantly increased (P<0.01), indicating that RPL22 silencing activates MDM2-p53 signaling. Numerous studies reported that RP expression is correlated with MDM2-p53 signaling in tumor progression. S7 binds to MDM2 and activates p53, which induces apoptosis and inhibits cell proliferation in prostate cancer [41]. Furthermore, L23 inhibits MDM2-p53 interaction, stabilizes p53, and induces apoptosis in GC [22]. Based on the above findings and our results, we hypothesized that the anti-tumor effect of RPL22 is associated with the inhibition of MDM2-p53 signaling. To demonstrate the effect of RPL22 on MDM2-p53 signaling in GC cells, nutlin-3 was used to block MDM2-p53 signaling. We found that treatment with nutlin-3 significantly reversed the promoting effects of si-RPL22 on GC cell proliferation, migration, and invasion, as well as its inhibitory effect on cell apoptosis. Taken together, our results showed that the silencing of RPL22 expedited the proliferation, migration and invasion, and restrained the apoptosis of MKN-45 cells through activating MDM2-p53 signaling. Hence, the RPL22/MDM2-p53 can be an important regulatory axis in GC, which provides direction for the research of novel targeted drugs.

**Conclusions**

A low expression of RPL22 was found in the GC tissues and cells. RPL22 silencing expedited the GC cells' proliferation, migration, and invasion, as well as inhibited cell apoptosis by activating the MDM2-p53 signaling. Hence, we infer that RPL22 could be an underlying target for GC treatment. However, this study did not confirm the detailed mechanisms between RPL22 and MDM2-p53 signaling in vivo, which are a limitation of the present study. In the future, further experiments are needed to elucidate these issues.

**Department and Institution Where Work Was Done**

Department of Gastrointestinal Surgery, the Affiliated Hospital of Qingdao University, Qingdao, Shandong, China.

**Conflict of Interest**

None.
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