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Long non-coding RNA PLAC2 suppresses the survival of gastric cancer cells through down-regulating C-Myc

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Abstract. – OBJECTIVE: The aim of this study was to explore the effects of long non-coding ribonucleic acid (lncRNA) placenta-specific protein 2 (PLAC2) on the biological behaviors of gastric cancer (GC) cells by regulating the expression of c-Myc gene and its mechanism.

PATIENTS AND METHODS: The expression of PLAC2 in GC tissues and different GC cell lines was detected *via* quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). The effects of PLAC2 on apoptosis and cycle, migration, and invasion of GC cells were detected using flow cytometry, wound healing assay, and transwell assay, respectively. After interference in PLAC2 expression, the changes in c-Myc expression were determined through qRT-PCR and Western blotting.

RESULTS: The expression level of PLAC2 was downregulated in 38 out of 45 cases of GC tissues compared with that in normal gastric tissues, and it also declined in GC cells. The results of flow cytometry showed that after overexpression of PLAC2, the cell cycle was arrested in the G1/G0 phase, and the apoptosis rate was increased. The results of wound healing assay and transwell assay revealed that both migration and invasion of GC cells were inhibited. After overexpression of PLAC2, the mRNA and protein expression levels of c-Myc declined.

CONCLUSIONS: LncRNA PLAC2 affects the biological behaviors of GC cells by regulating the expression of c-Myc gene.

Key Words:
LncRNA PLAC2, Gastric cancer, Biological behaviors, c-Myc.

Introduction

Gastric cancer (GC) is the most common highly-invasive malignant tumor in the digestive system, whose morbidity and mortality rates are increasing year by year[1]. According to statistics, there are 35,000 cases of GC deaths in China every year, mostly due to distant metastasis of advanced GC[2]. Currently, the specific pathogeny of GC remains unknown, and the median survival time of GC patients undergoing conventional drug therapy is less than 15 months[3]. To improve the existing diagnosis and treatment of GC, studying the mechanism of GC occurrence and metastasis may have a substantial influence on the therapeutic strategies in the future, and provide an effective therapeutic method for GC patients.

Long non-coding ribonucleic acids (lncRNAs) affect important biological processes of a variety of cells, such as invasion and metastasis of tumor cells[4,5]. HOX tran antisense RNA (HOTAIR) is highly expressed in breast tumors, which can target specific genomic loci through chromatin repressor proteins, thus promoting metastasis of breast cancer[6]. Fang et al[7] showed that lncRNA-p21 associated with hnRNP-K is involved in regulating the transcriptional reaction as a p53-dependent repressor. LncRNA MALAT1 is involved in regulating the cell growth and tumor metastasis, there are many downstream target genes, and different activation pathways participate in the regulation of different behaviors[8]. The above findings suggest that lncRNAs may serve

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as important regulators of tumorigenesis, but the regulatory effect of lncRNAs on specific human tumors and their association with tumorigenesis have not been fully clarified.

The expression of lncRNA placenta-specific protein 2 (PLAC2) is dysregulated in various tumors and it exerts important biological functions[9,10]. Zheng et al[11] found that PLAC2 has a downregulated expression in non-small cell lung cancer, which suppresses the tumor growth by adsorbing microRNA-21 (miR-21). Moreover, PLAC2 induces apoptosis of hepatocellular carcinoma *via* upregulating the p53 expression[12]. However, the expression and function of PLAC2 in GC have not been reported yet. In the present study, it was discovered using *in vitro* experiments that PLAC2 was downregulated in GC and inhibited the formation of malignant phenotype.

Patients and Methods

Tissue Specimens

Tissue specimens were taken from patients undergoing radical gastrectomy in Xinghua People's Hospital from January 2016 to December 2017, and the patients were definitely diagnosed with gastric adenocarcinoma by more than two pathologists and they received no radiotherapy, targeted therapy, and immunotherapy before surgery. After excision, tissue specimens were immediately stored in liquid nitrogen at -180°C for subsequent experiments. This study was approved by the Ethics Committee of Xinghua People's Hospital and patients signed a written informed consent.

Cell Culture

Human GC SGC-7901, AGS, MKN45, and BGC823 cell lines, and normal human gastric mucosal epithelial cell line GES-l were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in the Dulbecco's Modified Eagle's Medium (DMEM) or Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), streptomycin (100 μg/mL), and penicillin (100 U/mL) in an incubator with 5% CO_2 and humidity at 37°C.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

The total RNA was isolated from GC tissue and para-carcinoma normal tissues using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), the RNA concentration, and purity were measured using a NanoDrop 2000 spectrophotometer, and RNA was reversely transcribed into complementary deoxyribonucleic acid (cDNA) (TaKaRa, Otsu, Shiga, Japan), followed by cDNA amplification under the following conditions: pre-denaturation at 95°C for 10 min, denaturation at 95°C for 15 s, and annealing at 60°C for 32 s for a total of 50 cycles. Then, the dissolution curve was detected, the Ct value of each sample was automatically analyzed using the computer system, and the relative expression level of lncRNA was calculated using $2^{-\Delta\Delta Ct}$ method. The experiment was repeated for 3 times. Primers used were shown below: lncRNA PLAC2 F 5'-CCATTCCATTCATTTCTCTTTCCTA-3', R 5'-GGCGTAGGCGATTGGGGATCG-3', c-MYC F 5'-ATTTAAGGAGCGGATTTAGC-3' R 5'-TTTTCGAGTCGAAACACACT-3', glyceraldehyde 3-phosphate dehydrogenase (GAPDH) R 5'-AGCGAGCATCCCCCAAAGTT-3' F 5'-GGGCACGAAGGCTCATCATT-3'.

Plasmid Construction and Transfection

The PLAC2 overexpression plasmids were commercial products synthesized and identified by Shanghai Genechem Co., Ltd. (Shanghai, China), in which PLAC2 sequences were subcloned into pcDNA vectors (pcDNA-PLAC2), with pcDNA3.1 empty vectors as negative controls. GC cell lines were inoculated into a 6-well plate (5×10^5 cells/well), and they were transfected with pcDNA-PLAC2 and empty vectors using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) when 70% of them were fused. The medium was replaced after 6 h, and the cells were collected for qRT-PCR after 48 h.

Wound Healing Assay

The cells were inoculated into a 24-well plate (1×10^4 cells/well). When about 70% of cells were fused, they were scratched uniformly along the diameter of plate using a 20 μL pipette tip perpendicular to the bottom of plate. The floating cells shed were washed away with phosphate-buffered saline (PBS), and fresh medium containing 2% FBS was added. Then, the width of the wound was observed and measured at 0 and 36 h, and the cell migration ability was determined. The assay was repeated for 3 times.

Transwell Invasion Assay

The transfected cells were cultured till the logarithmic growth phase, resuspended in the

serum-free medium, and added into the upper chamber (1×10^4 cells/well) containing growth medium supplemented by 10% fetal bovine serum and growth factor at a normal concentration at the bottom. After incubation for 24 h, the cells on the upper side of membrane were removed with Matrigel and the top of membrane was wiped with a cotton swab. Then, the cells on the lower side of membrane were fixed and stained with 1% crystal violet dye, followed by photography and observation under an optical microscope.

Detection of Apoptosis Via Flow Cytometry

The transfected cells were collected, washed twice with cold PBS, and resuspended in binding buffer, followed by staining using the Annexin V- fluorescein isothiocyanate (FITC) apoptosis assay kit (BD Bioscience, Franklin Lakes, NJ, USA) at room temperature in the dark for 30 min. The apoptosis rate was measured using a flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) within 1 h.

Detection of Cell Cycle Distribution Via Flow Cytometry

The transfected cells in the logarithmic growth phase were digested with trypsin, washed twice with PBS, and fixed with 75% ethanol at 4°C overnight. On the next day, the cells were incubated with RNase at 37°C for 30 min, and stained with propidium iodide (PI) for 30 min, followed by detection of cell cycle (the percentage of cells in the G0/G1, S, and G2/M phases) using the flow cytometer.

Western Blotting

The total cell lysates were extracted using radioimmunoprecipitation assay (RIPA) buffer, and the protein was separated through 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), sealed with 5% skim milk powder, and incubated with c-MYC and GAPDH antibodies (1:5000) at 4°C overnight. Then, the protein was incubated again with the secondary antibodies for 2 hour, and detected using the enhanced chemiluminescence (ECL) substrate kit. The experiment was repeated for 3 times.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. The data were expressed as ($\bar{\chi}\pm s$), and statistical analysis was performed using Student's *t*-test. The association between the expression levels of PLAC2 and c-Myc was detected *via* Pearson correlation analysis. $p<0.05$ suggested the statistically significant difference.

Results

Expression of LncRNA PLAC2 Declined in GC

The relative expression level of PLAC2 in 45 cases of GC tissues was detected *via* qRT-PCR. The results revealed that the expression of PLAC2 was downregulated in 38 cases compared with that in para-carcinoma tissues (Figure 1A). To study the biological function of PLAC2 in GC cells, its relative expression level in GC cells was detected *via* qRT-PCR. The results showed that the expression of PLAC2 was downregulated (Figure 1B). Then, PLAC2 overexpression vectors were synthesized and transiently transfected into GC cells, and the overexpression efficiency was determined using qRT-PCR (Figure 1C and D).

PLAC2 Promoted Apoptosis and Inhibited Proliferation of GC Cells

The PLAC2 overexpression plasmids and control plasmids were transfected into GC cells. After 48 h, the cells in experimental group and control group were collected and detected. The results of flow cytometry manifested that the apoptosis rate of GC cells rose (Figure 2A and 2B). After treatment of cells in the same way, it was found *via* flow cytometry that the GC cell cycle was distributed in the G1/G0 phase (Figure 2C and 2D).

PLAC2 Inhibited Migration and Invasion of GC Cells

Subsequently, the effects of PLAC2 on migration and invasion of GC cells were studied. After treatment as described above, the changes in cell migration ability were determined using wound healing assay. It was observed that compared with that in control group, the cell migration ability in the experimental group was inhibited (Figure 3A and 3B). The results of the transwell assay discovered that after overexpression of PLAC2, both cell migration and invasion were suppressed (Figure 3C and 3D).

PLAC2 Regulated c-Myc Expression

The results of qRT-PCR indicated that the messenger RNA (mRNA) expression of c-Myc

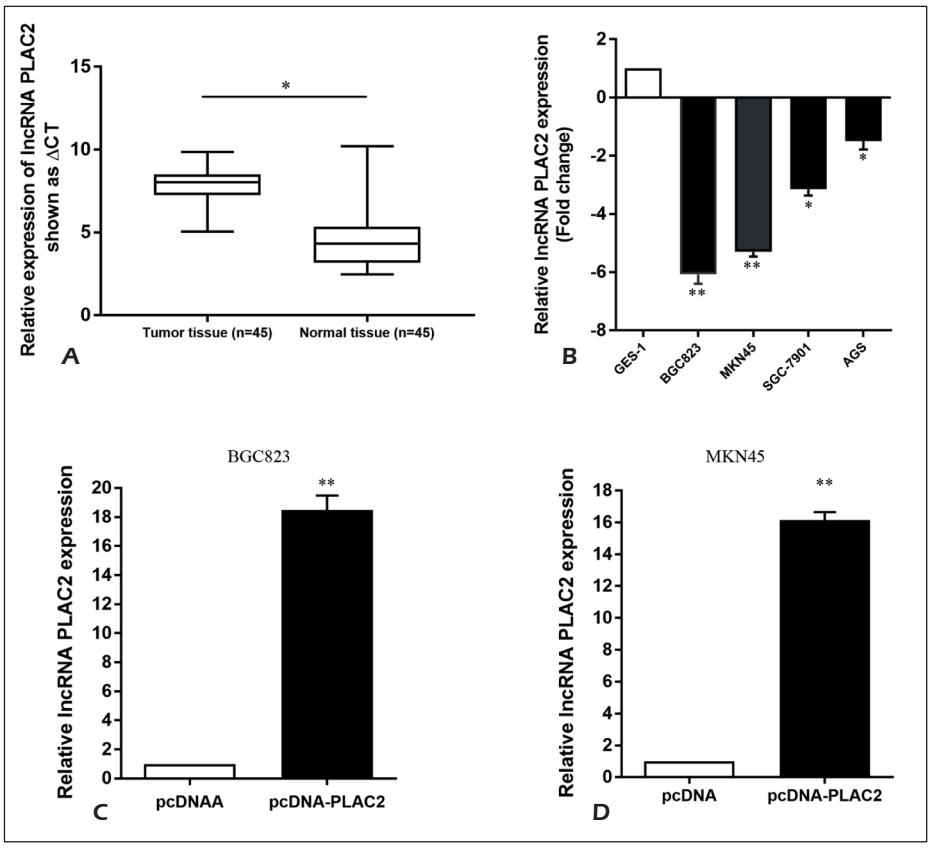

Figure 1. Expression of lncRNA PLAC2 declines in GC. **A**, The relative expression level of PLAC2 in 45 cases of GC tissues is detected via qRT-PCR. The results reveal that the expression of PLAC2 is down-regulated in 38 cases compared with that in para-carcinoma tissues. **B**, The expression level of PLAC2 in GC cells is detected via qRT-PCR, and the results show that the expression of PLAC2 is down-regulated. **C**, and **D**, The overexpression efficiency is determined using qRT-PCR.

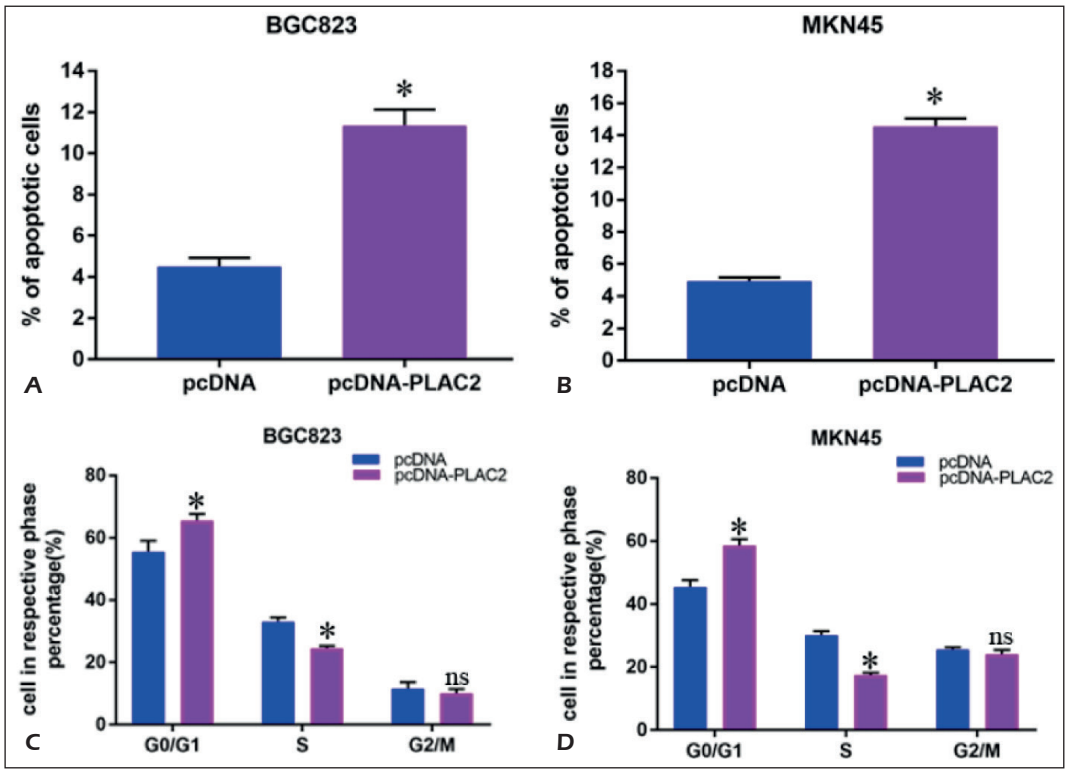

Figure 2. PLAC2 promotes apoptosis and inhibits proliferation of GC cells. **A**, and **B**, The results of flow cytometry manifest that the apoptosis rate of GC cells rises after overexpression of PLAC2. **C**, and **D**, It is found via flow cytometry that the GC cell cycle is distributed in the G1/G0 phase in experimental group compared with that in control group.

was downregulated after overexpression of PLAC2 (Figure 4A). Then, Western blotting was used to detect the protein expression of c-Myc, and it was observed that the protein expression of c-Myc declined after overexpression of PLAC2 (Figure 4B). Besides, their relative expressions in GC tissues were detected *via* qRT-PCR, and Pearson correlation analysis manifested that there was a regulatory relation between PLAC2 and c-Myc (Figure 4C).

Discussion

Currently, surgical resection, concurrent radiochemotherapy, and molecular targeted therapy for malignant tumors can reduce the pain as far as possible and prolong the life of patients, which are main treatment means for patients with malignant tumors, but the prognosis is relatively poor[13]. Therefore, it is urgently needed to find a new effective therapeutic method for tumor patients. The biomolecular treatment mode has become a research hotspot.

LncRNAs, important members of the non-coding RNA family, are composed of more than 200 nucleotides[14]. They are important regulators in tumors[15,16], whose abnormal expression plays a similar role to oncogenes or cancer suppressor genes in such tumors as GC. The molecular mechanism experiments showed that lncRNAs can regulate the gene expression at the epigenetic, transcriptional, and post-transcriptional levels, thereby participating in the occurrence and development of tumors[17]. In the present study, it was showed using *in vitro* experiments that PLAC2 was downregulated in GC and played a similar role to the cancer suppressor gene.

The c-Myc gene is a transcription factor located on chromosome 8, which encodes the intranuclear protein of p62 and participates in regulating the cell growth and proliferation[18]. The abnormal activation of c-Myc gene can be seen in most malignant tumor cells, such as breast cancer cells, gallbladder cancer cells, and nasopharyngeal car-

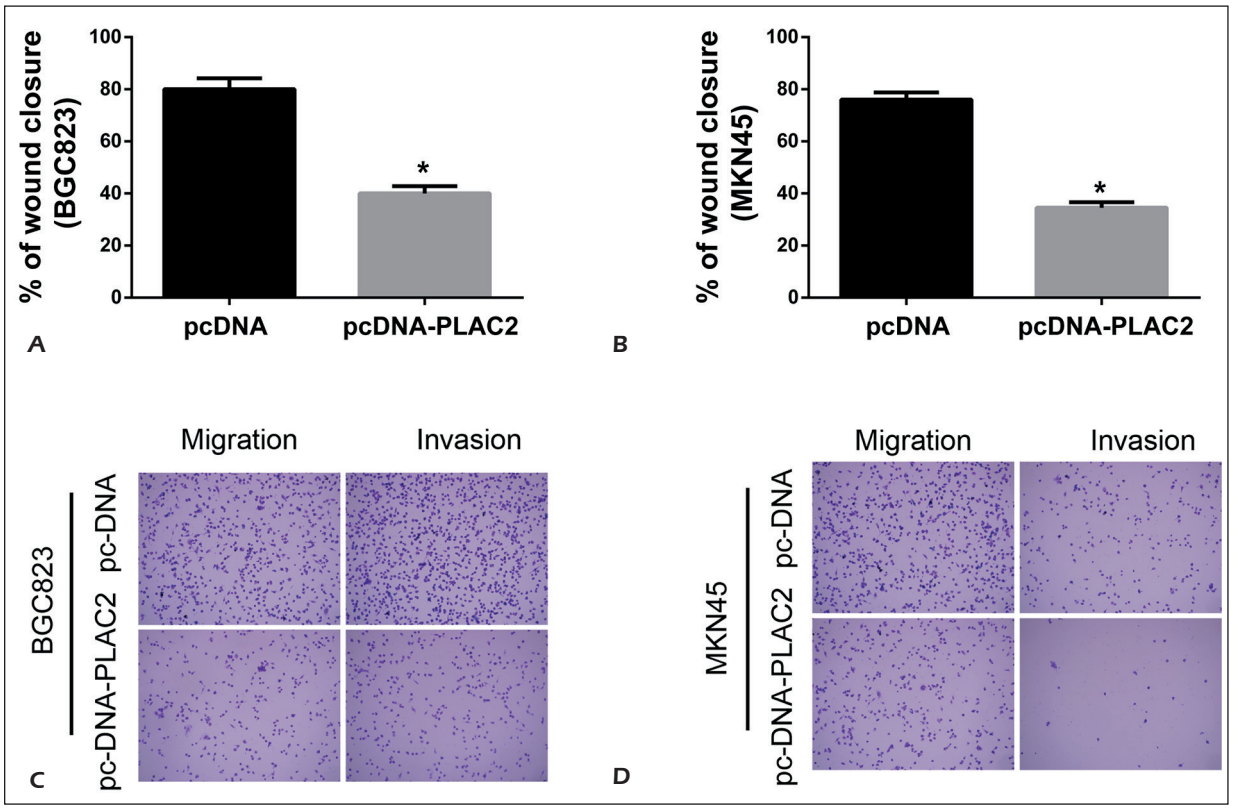

Figure 3. PLAC2 inhibits migration and invasion of GC cells. **A**, and **B**, The effect of PLAC2 on the migration ability of GC cells is studied via wound healing assay, and it is observed that compared with that in control group, the cell migration ability in experimental group is inhibited. **C**, and **D**, The results of transwell assay show that after overexpression of PLAC2, both cell migration and invasion are suppressed (magnification: 40×).

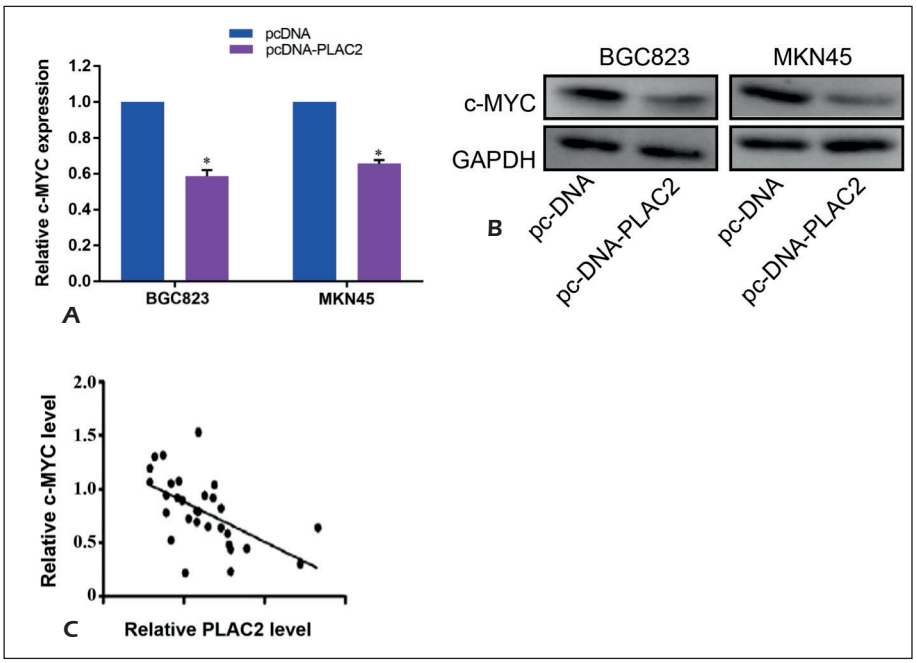

Figure 4. PLAC2 regulates c-Myc expression. **A**, The c-Myc expression is detected by qRT-PCR after overexpression of PLAC2. **B**, The c-Myc expression is detected by Western blotting after overexpression of PLAC2. **C**, The c-Myc expression in GC tissues is determined using qRT-PCR, and the association between PLAC2 and c-Myc expressions is explored using Pearson correlation analysis.

cinoma cells[19]. LncRNAs can be involved in regulating the c-Myc expression as an important regulator[20]. The *in vitro* assays in this study revealed that lncRNA PLAC2 suppressed the expression of c-Myc, but the specific molecular mechanism of PLAC2 in regulating c-Myc expression was not further explored. It is reported in the literature that lncRNAs can regulate the expressions of downstream target genes by carrying apparent complexes, such as PRC2, LSD1, and DNMT1[21], regulate the gene expression at the post-transcriptional level as ceRNAs[22], and also bind to protein to prevent mRNA degradation, thereby regulating the gene expression[23]. How PLAC2 regulates the c-Myc expression will be further clarified in subsequent investigations.

Conclusions

This research revealed that the expression of PLAC2 is downregulated in GC, and it promotes apoptosis and inhibits migration and invasion of GC by regulating the c-Myc expression. This study provides a theoretical basis for the clinical development of targeted drugs for GC.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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