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RESEARCH ARTICLE

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LINC02535 promotes cell growth in poorly differentiated gastric cancer

Jianzhong Wu[1,2] | **Ling Gao**[1] | **Hong Chen**[1] | **Xiaojun Zhou**[1] | **Xialiang Lu**[3] | **Zhongqi Mao**[1]

[1]Department of General Surgery, The First Affiliated Hospital of Soochow University, Suzhou, China

[2]Department of Gastroenterology Surgery, Suzhou Ninth People's Hospital Affiliated to Soochow University, Suzhou, China

[3]Department of Pathology, Suzhou Ninth People's Hospital Affiliated to Soochow University, Suzhou, China

Correspondence
Zhongqi Mao, Department of General Surgery, The First Affiliated Hospital of Soochow University, 188 Shizi Street, Suzhou 215006, Jiangsu, China.
Email: Maozq31@126.com

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Abstract

Background: Abnormal long non-coding RNA (lncRNA) expression plays important roles in gastric cancer. However, the functions of many lncRNAs in poorly differentiated gastric cancer (PDGC) remain unknown.

Methods: Three sets of paired tissues from patients with PDGC were used, and transcriptome sequencing was performed, followed by the construction and sequencing of a library and mapping of the reads. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and protein-protein interaction (PPI) networks were analysed, and canonical pathway significance was calculated among the differentially expressed genes (DEGs; $p < 0.05$). Gene expression in 30 paired PDGC specimens and four cell lines was validated through quantitative PCR. Cell proliferation, migration, invasion, apoptosis, and wound healing were analysed.

Results: A total of 499 upregulated DEGs and 627 downregulated DEGs were identified between peritumoral and gastric cancer tissues. The proportions of positive and negative correlations between LINC02535 and the DEGs were 98.40% and 92.66%, respectively, while the Spearman's correlation coefficient was greater than 0.5. The PPI network showed that approximately 73.15% of the top five genes were directly correlated with LINC02535 according to the STRING database. Based on KEGG analysis, the functions of LINC02535 target genes were enriched in signalling pathways related to cancer cell growth. Furthermore, cell function studies showed that LINC02535 upregulation contributed to cell proliferation, migration, invasion, and wound healing and that its inhibition facilitated cell apoptosis.

Conclusion: LINC02535 expression was upregulated in PDGC and contributed to cell proliferation, migration, invasion and wound healing, whereas its inhibition in PDGC facilitated cell apoptosis.

KEYWORDS
apoptosis, invasion, LINC02535, migration, PDGC, proliferation

1 | INTRODUCTION

Gastric cancer is one of the most prevalent malignant tumours in China and around the world.[1,2] The prognosis of PDGC is worse than that of moderately and highly differentiated gastric adenocarcinoma.[3] PDGC is associated with high mortality and morbidity. The main reason for this high mortality is that the early stages of gastric cancer are difficult to detect in a timely manner and drug treatment is not satisfactory.[4,5] Therefore, further exploration of the aetiology and mechanism underlying PDGC is important for clinical practice and for identifying new diagnostic and therapeutic targets.

In recent years, a large number of studies on long non-coding RNAs (lncRNAs) have found that lncRNAs have very important regulatory functions in cells and are involved in various biological regulatory processes and pathways.[6–8] lncRNAs participate in tumour signalling pathways, such as the Notch, mTOR, NF-κb, and Wnt pathways,[7,8] which control cell proliferation, migration, apoptosis, invasion, tumorigenicity, cell cycle progression, and metastasis. lncRNAs modulate the expression of protein-coding genes through cis-regulation of neighbouring genes and trans-regulation of distal genes.[9] Thus, the expression level of lncRNAs is correlated with that of potential target genes, and the function of lncRNAs can be predicted based on the associated mRNAs in lncRNA–mRNA coexpression networks.[10] Abnormal lncRNA expression has important clinical significance in the diagnosis of gastric cancer and is associated with the metastasis, invasion, prognosis, and other clinicopathological factors of this type of cancer.[11–13] Nonetheless, many lncRNAs remain unknown, and their functions in gastric cancer, especially in PDGC, require further studies.

We aimed to understand the clinical significance of LINC02535 in PDGC using high-throughput sequencing and to evaluate its functions in vitro.

2 | MATERIALS AND METHODS

2.1 | Patients

This study included 30 patients with PDGC who were enrolled at the First Affiliated Hospital of Soochow University between July 1, 2019, and March 30, 2020. Cancer tissues and paracancerous tissues from three patients were used for transcriptome sequencing, and tissues from 30 patients were used for gene expression verification. None of these patients had received treatments before surgery. All the patients provided written, informed consent to participate in the study, which was approved by the Ethics Committee at The First Affiliated Hospital of Soochow University (Ethics No. 2019-026). The study was conducted in strict accordance with the Declaration of Helsinki (2013).

2.2 | RNA extraction, library construction and sequencing

RNA for quantitative PCR (qPCR) was extracted from cancer tissues using the FastPure® Cell/Tissue Total RNA Isolation Kit V2 (Vazuume, Nanjing, China) according to the manufacturer's instructions. The library was constructed using the Total RNA-seq (H/M/R) Library Prep Kit for Illumina® (Vazyme) and subsequently sequenced with PE150 using the NovaSeq6000 platform (Illumina, San Diego, CA, USA). The data from this study were deposited in the NCBI Sequence Read Archive (SRA Accession No: PRJNA663723).

2.3 | Bioinformatics analysis

The reads were initially mapped to the latest UCSC transcript set using Bowtie2 version 2.1.0. The lncRNAs were identified by using Lncipedia (http://www.lncipedia.org). The expression values of these lncRNAs were estimated by using RSEM v1.3.3[14] and normalized based on the trimmed means of M-values (TMM). Differentially expressed genes (DEGs) were identified using the edgeR software package.[15] Genes with altered expression with cut-off values of $p < 0.05$ and >1.5-fold changes were considered significant. Pathways and networks were analysed using the Ingenuity Pathway Analysis (IPA) tool (Qiagen, Germantown, MD, USA). Fischer's exact test with the FDR option was used to calculate the significance of the canonical pathway. The correlation was analysed by calculating the Spearman's correlation coefficient between LINC02535 and the DEGs annotated by ENCODE, and the absolute value of the correlation coefficient (|correlation|, |cor|) ≥0.5 and $p < 0.05$ were selected for the LINC02535-DEG pair.

2.4 | Verification of LINC02535 expression using quantitative PCR

We verified the LINC02535 expression levels in 30 pairs of tissues using qPCR. The conditions for qPCR were as follows: 1 cycle of 95°C for 5 min and 40 cycles of 95°C for 10 sec and 60°C for 30 s. The primers for LINC02535 were forward primer 5′- AAGGAGCTCTGTTCTCCAGG-3′ and reverse primer 5′-GCCTCTATGTAGGGCGCTTT-3′. The primers for the housekeeping gene β-actin were forward primer 5′-AGCGAGCATC CCCCAAAGTT-3′ and reverse primer 5′-GGGCACGAAGGCTCATCATT- 3′.

2.5 | siRNAs for LINC02535

Two pairs of siRNAs, si-LINC02535-1 and si-LINC02535-2, specific for LINC02535 were designed. The siRNA sequences used for the negative control and LINC02535 are listed in Table S1.

2.6 | Cell proliferation assays

The gastric cancer cell lines MGC803 and SGC7901 (purchased from the American Type Culture Collection, ATCC) were selected to assess the effects of LINC02535 in vitro. Cells in the logarithmic phase of growth were cultured to a density of 5×10^5 cells/ml.

Cell suspensions (100 μl/well) and blanks were incubated in 96-well plates overnight at 37°C. The medium was then replaced with serum-free medium. Two hours later, 1 μl of serum-free medium was diluted with 10 μl opti-MEM and mixed with 10 μl of Lipofectamine™ 2000, incubated at 25°C for 5 min, and mixed again. Thereafter, 20 μl of this mixture was added to the wells, and the plates were gently shaken. The cells were transfected with the negative control, si-LINC02535-1 and si-LINC02535-2 for 0, 24, 48, and 72 h, and cell viability was determined using Cell Counting Kit-8 (CCK-8) (Beyotime Biotechnology, Shanghai, China).

2.7 | Migration and invasion assays

Gastric cancer cells were treated with small interfering (si) RNA and digested with 0.25% trypsin, centrifuged at 600 g, and washed with PBS. The cells were resuspended in serum-free RPMI-1640 medium and counted. The cells were diluted to 2×10^4/ml, and Matrigel was diluted to 1 mg/ml in serum-free RPMI-1640 medium. Chilled MEM containing 10% foetal bovine serum (FBS, 800 μl) and penicillin-streptomycin was added to 24-well plates, and Transwell chambers were then added to the plates. Matrigel (100 μl) at a final concentration of 1 mg/ml was added to the bottom centre of the upper Transwell chamber and incubated for 24 h at 37°C in a 5% CO_2 atmosphere. The cells were stained with 0.5% crystal violet, washed with phosphate-buffered saline (PBS) and examined using microscopy.

2.8 | Wound healing assays

Cells were transfected for 24 h, digested with 0.25% trypsin, resuspended in complete RPMI-1640 medium, seeded in 6-well plates and cultured overnight at 37°C in a 5% CO_2 atmosphere. When the cell density reached ~90% and the wells were covered with cells, a horizontal line was scratched in the cell surface. The cells were washed with PBS, and serum-free RPMI-1640 medium was added. The cells were photographed at 0 h and after 24 h of incubation at 37°C in a 5% CO_2 atmosphere.

2.9 | Cell apoptosis assays

SGC-7901 and MGC-803 cells were suspended in complete RPMI 1640 medium containing 10% FBS and 1% penicillin-streptomycin, seeded in Petri dishes, and incubated at 37°C in a 5% CO_2 atmosphere. When the confluence reached 80%, cells in the logarithmic phase of growth were adjusted to a concentration of 1×10^5 cells/ml. Cell suspensions (2 ml/well) were cultured in 6-well plates for 48 h at 37°C in a 5% CO_2 atmosphere along with a blank control. Cell apoptosis was determined by flow cytometry after being processed with Annexin V-APC/7-AAD apoptosis detection kits (Yeasen Biotech Co., Shanghai, China).

2.10 | Statistics

All the statistical analyses were conducted with SPSS 22.0 software (IBM Corporation, Armonk, NY, USA) and GraphPad Prism 5.0 (GraphPad Inc., La Jolla, CA, USA). Differences between groups were analysed utilizing Student's *t*-test or one-way analysis of variance (ANOVA). *p*-values <0.05 were considered to be statistically significant. The *t*-test was used to analyse the qPCR data.

3 | RESULTS

3.1 | DEG selection and correlation with LINC02535

After data preprocessing, the expression matrix of 16,375 genes in three paired samples was determined. With the threshold of |log2FC| ≥ 0.5, a total of 1126 DEGs were selected for subsequent analysis, including 499 upregulated genes and 627 downregulated genes. Moreover, LINC02535, a previously unknown functional lncRNA in PDGC, was selected from the top 10 upregulated genes for further analysis (see Table S2). The proportions of positive and negative correlations between LINC02535 and the DEGs were 98.40% and 92.66%, respectively, while the Spearman's correlation coefficient was greater than 0.5 (Figure 1, Table S3). These results demonstrated that the DEGs mentioned above, such as LINC02535 target genes, were coexpressed with LINC02535.

3.2 | Functional enrichment of the DEGs correlated with LINC02535

Kyoto Encyclopedia of Genes and Genomes pathway analyses were performed to determine the roles and corresponding molecular functions of the DEGs that were correlated with LINC02535 in the pathways. The cell cycle, cAMP signalling pathway and homologous recombination were enriched in the KEGG pathway analysis (Figure 2A, Table S4). These findings suggest that LINC02535 target genes are closely related to cell growth–related pathways in PDGC progression, which is consistent with previous reports on the important role of LINC02535 in cervical cancer.[16] A heat map showing gene expression in paracancerous tissues, as well as in gastric cancer tissues, indicated that transcripts of ten genes (TM4SF20, CHRNA1, CEACAM18, RHCG, IL17C, SLC30A10, SAA1, SAA2, FEZF1, ALDH3B2 and LINC02535) that participate in cell growth were significantly increased in gastric cancer tissues compared with paracancerous tissues (Figure 2B). Furthermore, ten genes, PTGER3, HIF3A, UBE2QL1, RPRM, TMEM130, ARL4D, PPP1R1A, SLC8A2, CNTN2 and EPHA6, were significantly decreased in gastric cancer tissues (Figure 2B).

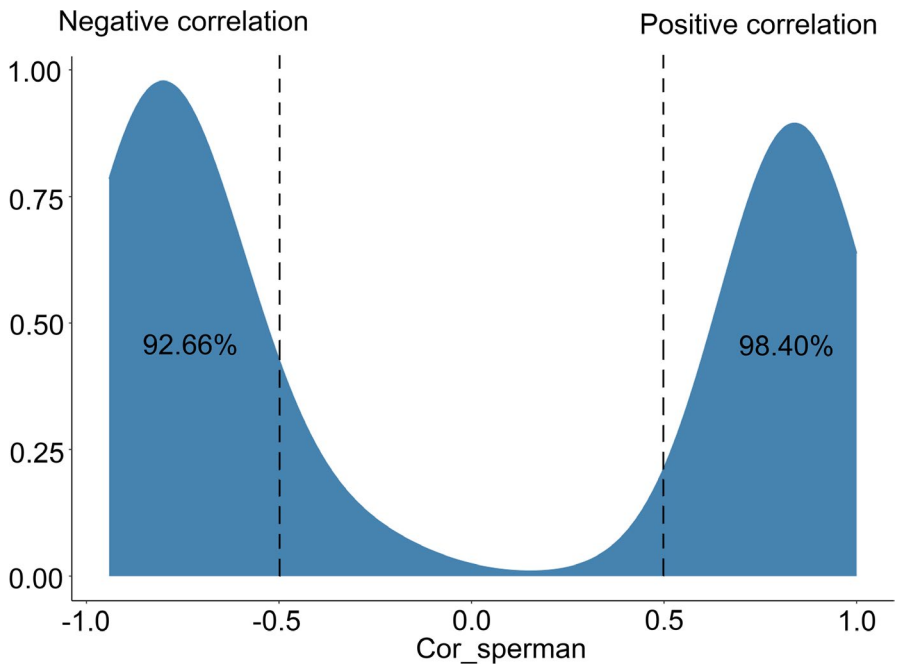


FIGURE 1 Correlation analysis between LINC02535 and DEGs was performed by calculating Spearman's correlation coefficient

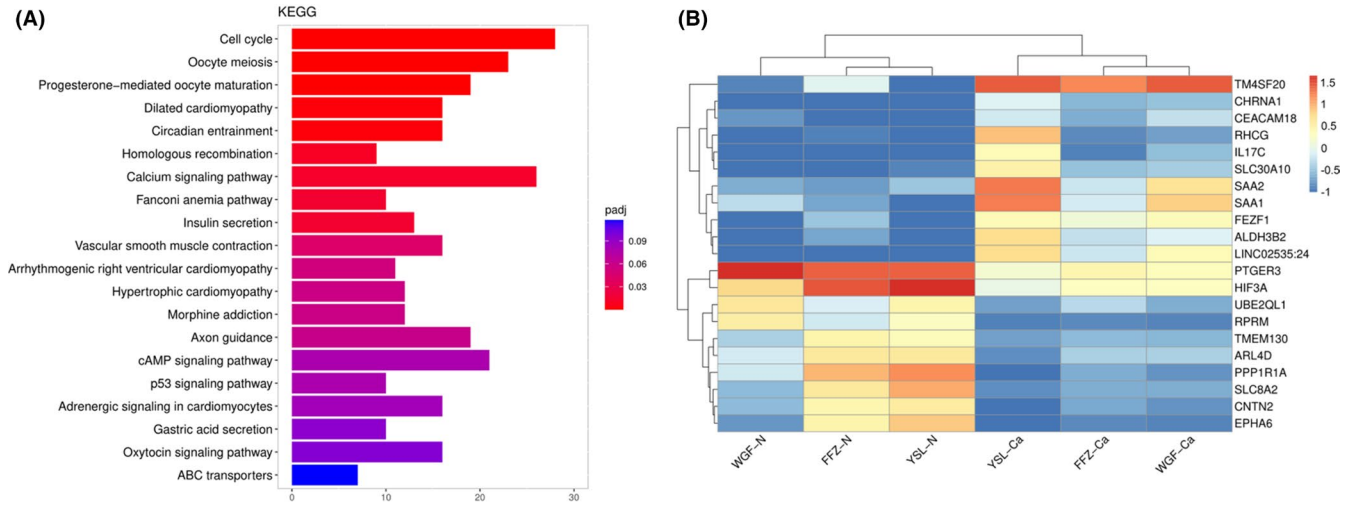


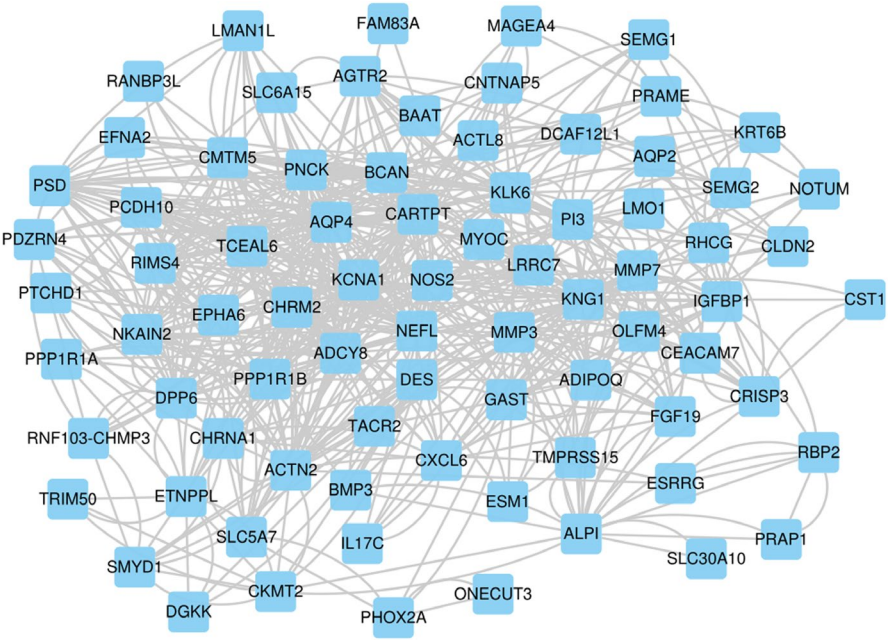
FIGURE 2 Gene expression profile of LINC02535 target genes. (A) Top 20 canonical pathways related to cancer cell proliferation enriched in the upregulated and downregulated genes, as determined by KEGG pathway analysis. (B) Heat map depicting the gene expression profiles of the cell cycle and homologous recombination in paired tissues resected from patients with PDGC. Red, high expression; yellow, intermediate expression; blue, low expression

3.3 | PPI analysis of the DEGs correlated with LINC02535

To further prove that LINC02535 plays a greater role in the process of PDGC development, a PPI network involving 108 genes, selected through Spearman's correlation coefficient positive and negative top 5 LINC02535 target genes calculating with the STRING database (see Table S5). Interestingly, in the network, approximately 73.15% (79/108 genes) of the target genes existed in the direct interaction relationship, indicating that LINC02535 was a key factor that affected the occurrence of PDGC (Figure 3, Table S6).

3.4 | LINC02535 expression was increased in PDGC tissues and cell lines

To investigate the expression level of LINC02535 in PDGC, LINC02535 expression was investigated in thirty pairs of gastric cancer tissues and paracancerous tissues using qRT-PCR. As depicted in Figure 4A, the $2^{-\Delta\Delta Ct}$ value of LINC02535 was significantly increased in gastric cancer tissues compared with paracancerous tissues ($p < 0.05$). This finding was consistent with an in vitro expression analysis comparing LINC02535 expression in MGC803, BGC823, HGC27, and SGC7901 cells to that in GES1 cells, an immortalized mammary epithelial cell line. LINC02535 expression



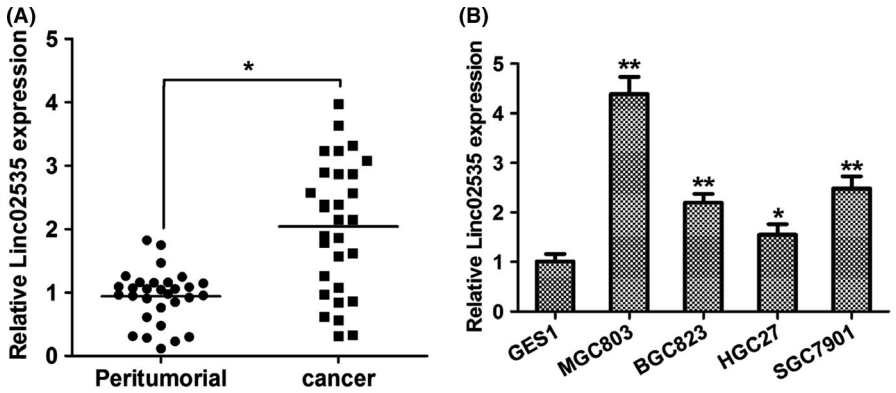


FIGURE 3 PPI network of the top five genes analysed by calculating Spearman's correlation coefficient in the STRING database. LINC02535 was selected as input

FIGURE 4 Expression of LINC02535 is upregulated in gastric cancer tissues and cell lines. (A) LINC02535 expression analysed in gastric cancer tissues and adjacent normal gastric tissues via qRT-PCR. (B) LINC02535 expression analysed in nonmalignant epithelial GES1 cells and four gastric cancer cell lines, HGC27, MGC803, SGC7901 and BGC823, by qRT-PCR. The average LINC02535 expression was normalized using GAPDH expression. $^{*}p < 0.05$, $^{**}p < 0.01$

was highest in SGC7901 and MGC803 cells and higher than that in BGC823 and HGC27 cells (Figure 4B). Therefore, SGC7901 and MGC803 gastric cancer cells were selected as the focus of this study.

3.5 | LINC02535 promotes gastric cancer cell proliferation, invasion and migration

To explore the possibility that LINC02535 affected gastric cancer cell tumorigenesis, the si-LINC02535-1 and si-LINC02535-2 were utilized to inhibit the expression of LINC02535 and the result showed that the expression of LINC02535 were remarkably decreased in SGC7901 cells under treated with si-LINC02535-1 and si-LINC02535-2 (Figure S1). Then, cell proliferation (Cell Counting Kit 8, CCK8), invasion and migration (Transwell) assays were performed on MGC803 and SGC7901 cells. The CCK-8 assays revealed that MGC803 (Figure 5A) and SGC7901 cells (Figure 5B) proliferation abilities were significantly inhibited following transfection with si-LINC02535-1 and si-LINC02535-2, indicating that LINC02535 promoted cell proliferation.

A Transwell assay revealed that the invasion of MGC803 and SGC7901 cells was greatly decreased when they were transfected with both si-LINC02535-1 and si-LINC02535-2 (Figure 6A). Furthermore, the migratory capabilities of MGC803 and SGC7901 cells were also decreased when LINC02535 was knocked down (Figure 6B). Furthermore, the effects of LINC02535 inhibition on the healing ability of SGC7901 and MGC803 cells were evaluated using wound healing assays. Both si-LINC02535-1 and si-LINC02535-2 inhibited SGC7901 and MGC803 cell healing compared with the negative control. The relative migration rate was significantly decreased (Figure 7A,B). Overall, these results suggest that LINC02535 exerts proliferative, invasive and migratory effects on gastric cancer cells and therefore may act as a tumour promoter in gastric cancer.

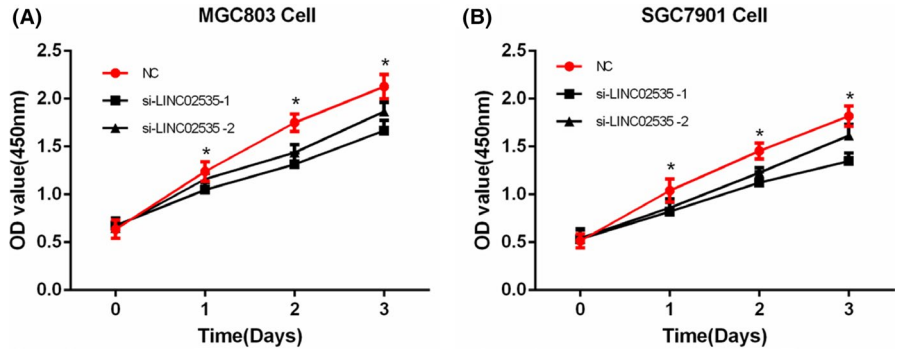
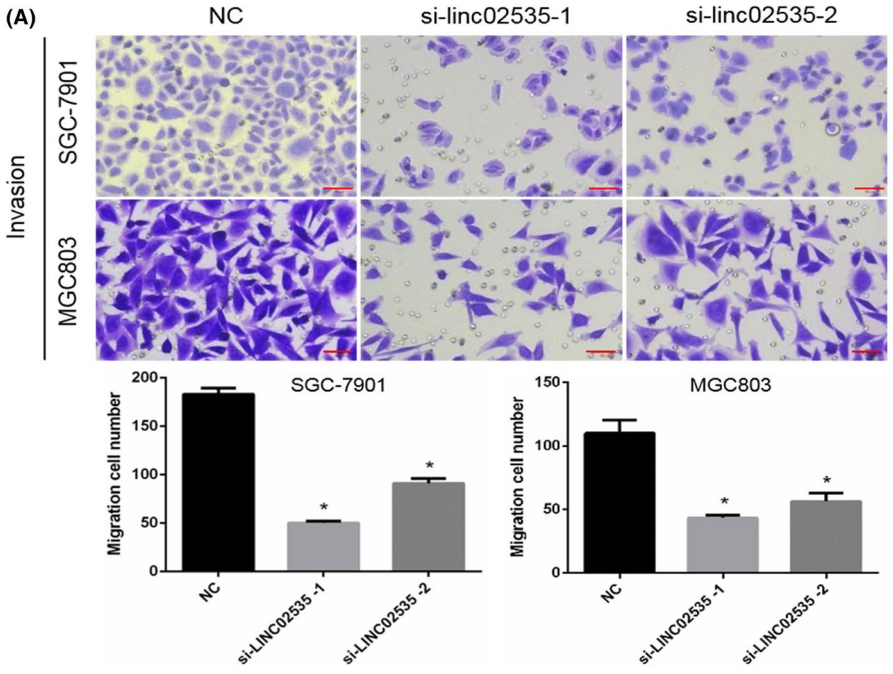


FIGURE 5 The CCK-8 assay was performed to monitor the proliferation level of (A) SGC7901 cells and (B) MGC803 cells after treatment with si-LINC02535-1 and si-LINC02535-2 for 0, 24, 48, and 72 h. *$p < 0.05$



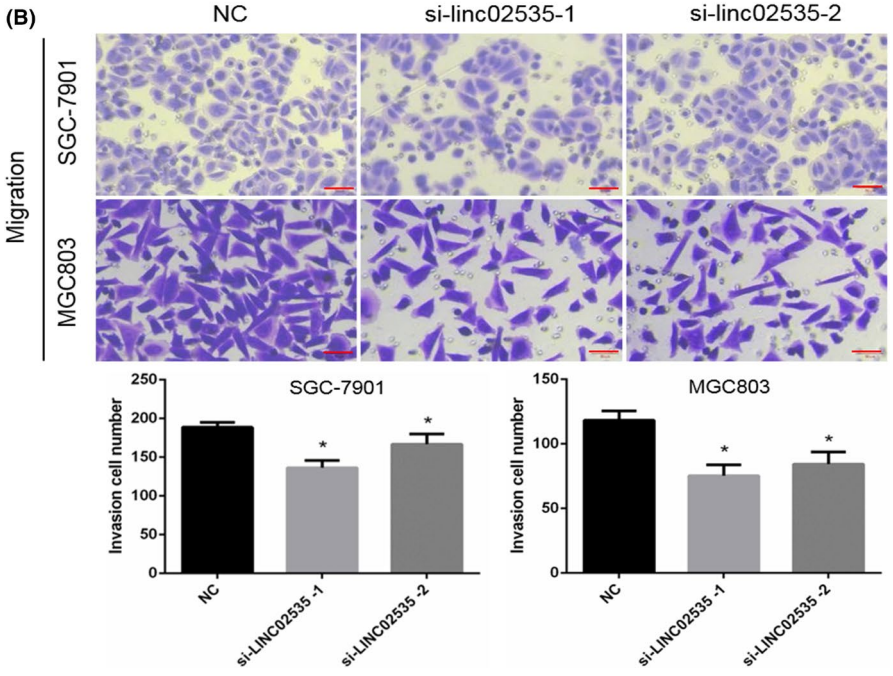


FIGURE 6 Transwell analyses of the migrated (A) SGC7901 cells and (B) MGC803 cells treated with i-LINC02535-1 and si-LINC02535-2. The cells were allowed to migrate for 12 h. *$p < 0.05$

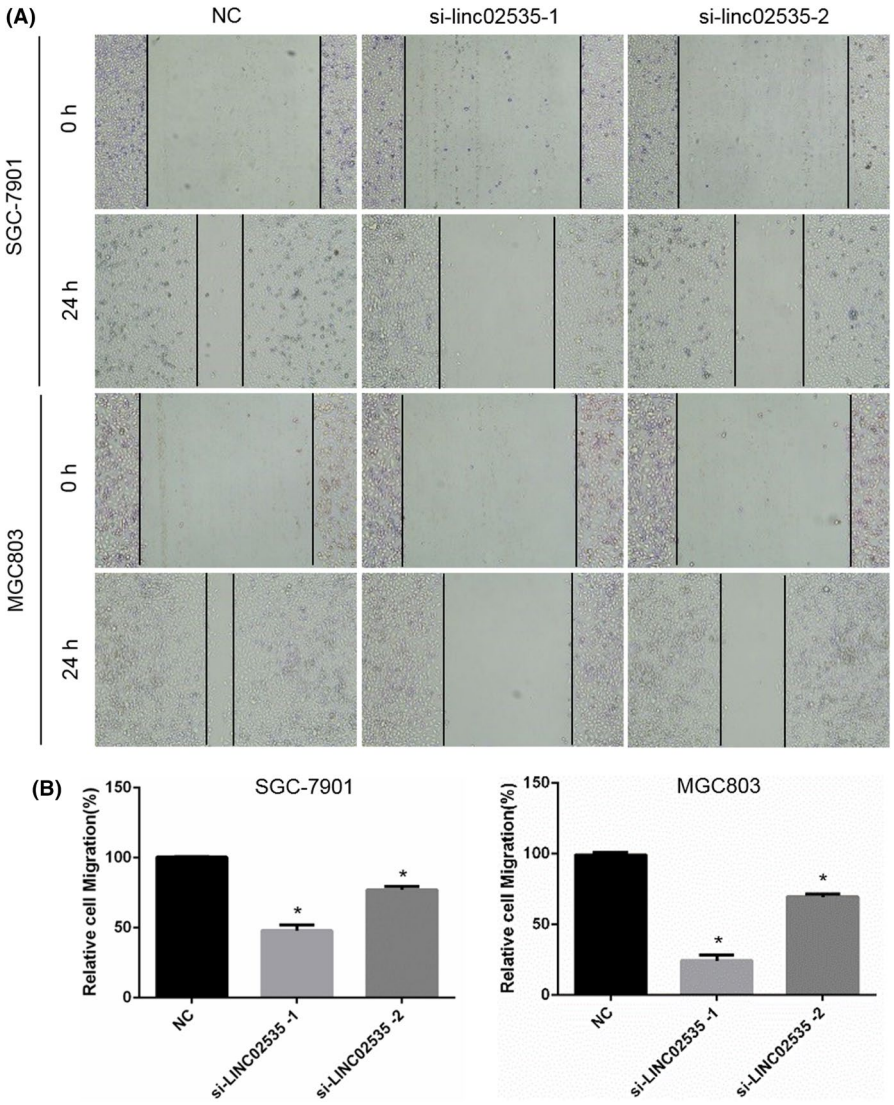


FIGURE 7 Flow cytometry was used to analyse (A) SGC7901 cell and (B) MGC803 cell apoptosis after treatment with i-LINC02535-1 and si-LINC02535-2. $^{*}p < 0.05$

3.6 | LINC02535 inhibits apoptosis in gastric cancer cells

A flow cytometry assay was performed to further detect the effect of LINC02535 on gastric cancer cell apoptosis. The results demonstrated that the group transfected with si-LINC02535-1 and si-LINC02535-2 had a significantly increased proportion of cells undergoing early and late apoptosis than the group transfected with the NC, and similar results were obtained with both MGC803 and SGC7901 cells (Figure 8A,B). Taken together, these data indicate that LINC02535 could inhibit gastric cancer cell apoptosis.

4 | DISCUSSION

Investigations of gastric cancer–related long lncRNAs have significantly progressed in the last decade. The abnormal expression of lncRNAs is associated with the occurrence and development of gastric cancer by interacting with target genes or other non-coding (nc) RNAs and by regulating gene expression transcriptionally or post-transcriptionally.[17–19] A recent study showed that LINC00319 was highly expressed in gastric cancer and sponged miR-335-5p, which targets ADCY3 to regulate the expression of ADCY3, thus participating in the growth and metastasis of gastric cancer.[20] Similar research found that the differential expression and survival analysis of LINC02407 in gastric cancer were significantly different, and high expression of LINC02407 had a negative effect on patient prognosis.[6]

In this study, a total of 499 upregulated and 627 downregulated genes were identified in PDGC through high-throughput sequencing. The proportions of positive and negative correlations between LINC02535 and the DEGs were 98.40% and 92.66%, respectively, while the Spearman's correlation coefficient was greater than 0.5. The PPI network showed that approximately 73.15% of the top five genes analysed by calculating Spearman's correlation coefficient were directly correlated with LINC02535 in the STRING database. In addition, through KEGG analysis, the functions of LINC02535 target genes were enriched in signalling pathways related to cancer cell growth, including cell cycle progression, homologous recombination, and calcium and cAMP signalling pathways.

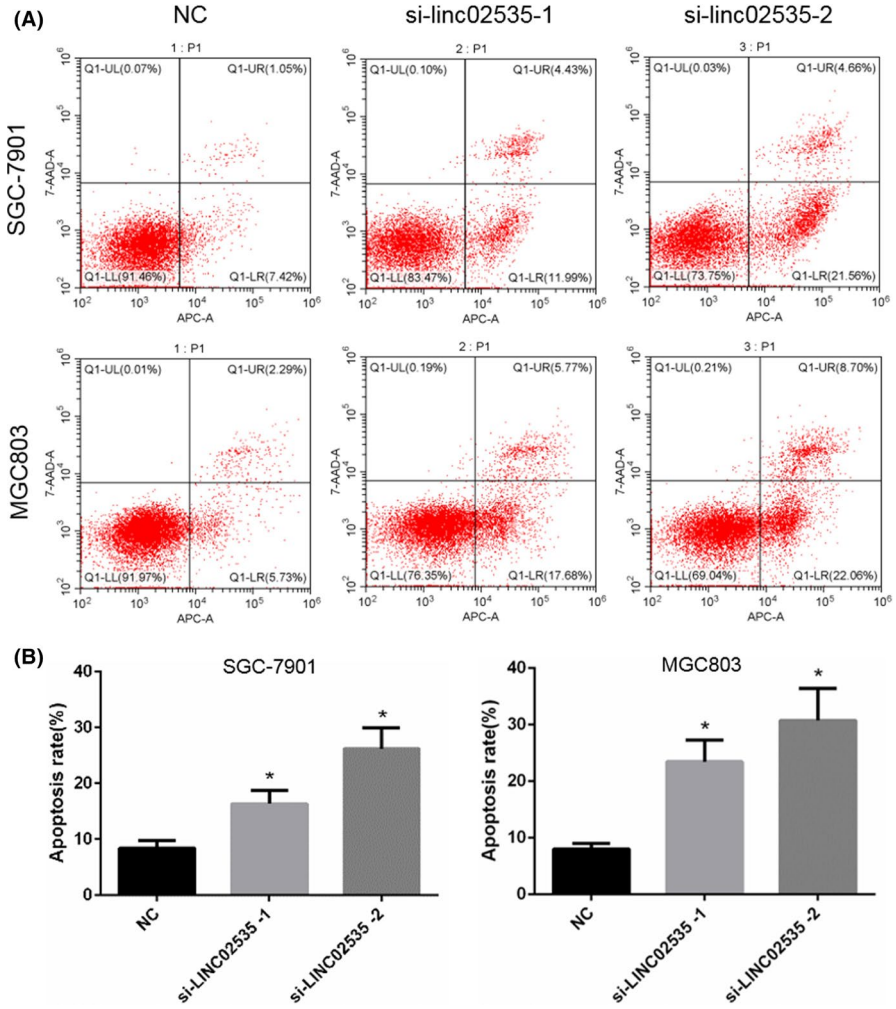


FIGURE 8 A wound healing assay was performed to monitor the migration of (A) SGC7901 cells and (B) MGC803 cells after treatment with si-LINC02535-1 and si-LINC02535-2 for 0 and 24 h. *$p < 0.05$

Cell division is a key biological process, and abnormalities in this process can lead to the development of cancer. Rapid cell growth and division are common features of malignant cells, and abnormal cell division cycles play an important role in cancer cell proliferation. The abnormal expression of genes associated with cell cycle regulation might cause cancer.[21] We found that the DEGs correlated with LINC02535 were mainly enriched in cell cycle–related pathways, indicating that this pathway plays a crucial role in PDGC. lncRNAs regulate cellular cAMP through their signalling pathways to trigger a downstream cascade of reactions, thus affecting metabolism, cell proliferation, differentiation, and apoptosis.[22–24] Therefore, the cAMP signalling pathway is closely associated with tumour occurrence and progression. We identified many genes associated with the cAMP signalling pathway that might be involved in the progression of gastric cancer.

Differences in the expression of lncRNAs, especially LINC02535, were observed. LINC02535 expression was obviously upregulated in PDGC. We also assessed the functions of the novel lncRNA LINC02535 and found that LINC02535 promoted gastric cancer cell progression in vitro. To the best of our knowledge, only one other similar study showed that LINC02535 was correlated with cervical cancer.[16] The study found significantly higher LINC02535 and poly(C)-binding protein 2 (PCBP2) expression in cervical cancer tissues and that LINC02535 and PCBP2 costabilized RRM1 mRNA to facilitate DNA repair damage and promote cell proliferation and epithelial-to-mesenchymal transition. Our findings of upregulated LINC02535 gene expression in PDGC tissues are consistent with these findings. The expression and functions of LINC02535 in PDGC have been revealed for the first time. Further investigation is needed to elucidate the role of LINC02535 in PDGC.

There is a limitation to our investigation. Although expression and functional studies on LINC02535 were performed, we did not investigate the mechanism by which LINC02535 functions in PDGC, and this mechanism awaits further studies.

In conclusion, we found that upregulated LINC02535 expression in PDGC contributed to cell proliferation, migration, invasion and wound healing and that its inhibition facilitated the apoptosis of gastric cancer cells. Our functional studies provide important clinical information and warrant further investigation in vivo.

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CONFLICTS OF INTEREST

The authors declare that there are no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

JZW and ZQM conceived and designed the study; JZW, LG, HC, and XLL conducted the experiments; JZW, LG, XJZ, and XLL collected, analysed and interpreted the data. All the authors discussed the results and contributed to the final manuscript. All the authors read and approved the manuscript and agreed to the order of authors and to submit the manuscript for publication.

DATA AVAILABILITY STATEMENT

All data are included in this article.

ORCID

Zhongqi Mao https://orcid.org/0000-0001-5767-264X

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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