Research Square

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Long Non-coding RNA LINC02679 Regulates TRIML2 Expression to Promote the Proliferation and Invasion of Gastric Cancer Cells by Targeting miR-5004-3p

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Abstract

Long non-coding RNAs (lncRNAs) play a key role in regulating the development, invasion and metastasis of gastric cancer. However, the specific clinical value and mechanism of LINC02679, a lncRNA family member, is not clear. This study explored the mechanism of action of LINC02679 by measuring LINC02679 expression in gastric tissues and cells and explored the role it played in gastric cancer in a series of experiments, including bioinformatics, quantitative reverse transcription PCR (qRT-PCR), cell activity, invasion and migration, protein expression and dual-luciferase reporter gene analyses. The bioinformatics and verification test results indicated that LINC02679 expression in gastric cancer tissue and cells was higher than that in normal tissue and cells and that LINC02679 overexpression was a marker of a poor prognosis for patients with gastric cancer. By regulating the expression of LINC02679 in cells, cell proliferation, invasion, and migration and related gene expression changed. The results of the in vivo experiments were consistent with those of the cell experiments. Further exploration revealed that LINC02679 carried out its functional role by regulating the miR-5004-3p/tripartite motif family-like 2 (TRIML2) pathway via sponge.This study demonstrated that LINC02679 targeted TRIML2 to promote the proliferation, invasion and migration of gastric cancer cells and that LINC02679 might serve as a target gene in the treatment of gastric cancer.

Full Text

Gastric cancer is one of the most common gastrointestinal malignancies worldwide. The latest data from GLOBOCAN 2020 indicated that the number of newly diagnosed patients with gastric cancer exceeded 1 million worldwide. Its global incidence ranks fifth among tumors and first among upper gastrointestinal tumors. East Asia has the highest incidence of gastric cancer. In China, there are numerous patients with gastric cancer, and most are in the progressive stage [1-3]. Currently, comprehensive treatment based on surgery is the main therapeutic approach for gastric cancer (including chemotherapy, radiotherapy, targeted therapy, immunotherapy, and traditional Chinese medicine). Although there have been continuous improvements in surgical techniques and instruments (such as staplers), new chemotherapy drugs, and rapid developments in immunotherapy that have brought a variety of treatment options to patients in recent years, the overall 5-year survival rate for patients with gastric cancer remains less than 40%, mainly due to pre-operative micrometastasis, post-operative recurrence/metastasis and drug resistance [4-8]. Therefore, there is an urgent need to find a highly sensitive and specific molecular target that aids in the diagnosis, treatment and prognostic evaluation of gastric cancer.

With the recent rapid development of genomics and molecular biology, non-coding RNA (ncRNA) has attracted increasing attention. Long non-coding RNA (lncRNA) and microRNA (miRNA) are two important members of the ncRNA family [9-11]. lncRNA is a non-coding transcript greater than 200 nt in length that regulates gene expression at various levels (epigenetic, transcription, and post-transcription) [12-15]. miRNA is a non-coding single-stranded RNA molecule 18-22 nt in length that is encoded by endogenous genes; it binds to and breaks down target mRNAs, thereby regulating the post-transcription expression of target genes [16-19]. In many previous studies, lncRNA and miRNA have been shown to play roles in the

occurrence, development, metastasis, drug resistance and prognosis of gastric cancer; thus, they have potential to become novel targets for the diagnosis, therapy and prognosis of gastric cancer [20-23]. LINC02679 is a member of the lncRNA family whose specific clinical value and mechanism of action in gastric cancer are still unclear. Using bioinformatics and survival analyses, we found that LINC02679 was highly expressed in gastric cancer and was a marker of poor prognosis. We then measured LINC02679 expression in gastric cancer tissues and cells and performed a series of experiments to investigate the mechanism of action of LINC02679.

1. Materials And Methods

1.1 Clinical tissue specimens

A total of 48 patients with gastric cancer who underwent radical surgery at the Third Department of Surgery, Fourth Hospital of Hebei Medical University, China, from January 2019 to June 2021 were included in this study. Diagnosis of gastric adenocarcinoma was confirmed by post-operative pathology; none of the patients received pre-operative chemotherapy, radiotherapy, immunotherapy or traditional Chinese medicine therapy or had a second tumour. After a specimen was taken from the body and necrotic tissue and mucus on the surface were cleared, tumour tissue and paracancerous tissue were collected and placed in cryogenic vials, flash frozen in liquid nitrogen and stored in the -80℃ freezer. This study was approved by the Medical Ethics Committee of the Fourth Hospital of Hebei Medical University, and all patients signed an informed consent form.

1.2 Bioinformatics analysis

1.2.1 Acquisition of LINC02679 expression data and differential expression analysis: The Stomach Adenocarcinoma (STAD) dataset in The Cancer Genome Atlas (TCGA) database (http://cancergenome.nih.gov/) was used for this study. T tests and the fold-change method were used to assess differential expression, and BH correction was used to calculate the significance of the differential expression of each gene and lncRNA. |LogFoldChange|>1 and false discovery rate (FDR)<0.05 were used as thresholds to screen genes and lncRNAs with significant differential expression.

1.2.2 Clinical data and survival analysis: The STAD clinical dataset was downloaded from the TCGA database. The R packages "survival" and "survminer" were used to conduct survival analysis. Multivariate Cox regression was performed to evaluate the association between the survival rate for patients and the expression level of each lncRNA-gene pair. Then, a mathematical equation was established to predict survival rates, and the influence of each lncRNA-gene pair was considered. The risk score was calculated as follows: , where α and β are the Cox regression coefficients of lncRNAs and genes, respectively, in the dataset. Patients in the training dataset were assigned to high-risk or low-risk groups based on the median risk score, and the difference in survival between the 2 subsets was compared using Kaplan-Meier survival curves; the log-rank test was performed to assess statistical significance. In addition, a

nomogram, drawn using the R package "Rms," was used to predict the survival rate at different time nodes.

1.3 Cell lines and main reagents

Human gastric cancer cell lines (MKN28, HGC27, AGS, and MKN45) and a gastric epithelial cell line (GES-1) were purchased from the China Center for Type Culture Collection (CCTCC). RPMI-1640 culture medium and trypsin (Gibco, USA); foetal bovine serum and phosphate buffered saline (PBS) (BI company, Israel); double antibiotic (penicillin-streptomycin) (Beyotime, Shanghai); methylthiazolyldiphenyl-tetrazolium bromide (MTT) (Sigma, USA); and Lipofectamine^{TM}2000 and Trizol reagent (Invitrogen, USA) were used in this study. The PCR primers were synthesized by Sangon Biotech Co., Ltd., Shanghai.

1.4 Cell culture

All cells were cultured in RPMI-1640 medium containing 10% foetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in an incubator (37°C and 5% CO_2). Cell growth and bacterial contamination were monitored daily. The medium was changed every 1 or 2 days. For passage or subsequent experiments, cells were digested with 0.25% trypsin. Cells in the logarithmic growth phase were used for subsequent experiments such as PCR and MTT.

1.5 Real-time fluorescence quantitative PCR

Total RNA was extracted from tissues or cells using Trizol, and the purity and concentration of the extracted RNA were measured by spectrophotometry. Total RNA (2 μg) was reverse transcribed to synthesize template cDNA (reverse transcription reaction conditions: 42℃ for 60 min, 72℃ for 15 min). Primers (see Table 1 for sequences) were added to single-stranded cDNA for qPCR amplification (95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s). The mRNA expression level of each target gene was calculated using $2^{-\triangle\triangle Ct}$. U6 was uesd as the reference gene for micro RNA, β-actin was used as an internal reference gene for others.

1.6 Detection of cell viability using the MTT assay

Single-cell suspensions of each group of transfected cells (5×10^4 cells/ml) were added to each well of a 96-well plate. The cells were incubated at 37°C and 5% CO_2, and 20 μl of MTT (5 mg/ml) was added 4 hours before the end of the incubation. After the incubation period, the medium was removed, 150 μl of dimethyl sulfoxide (DMSO) was added to each well, and the plate was shaken for 15 min. The optical density (OD) at 570 nm was measured using a microplate reader. A cell growth curve was drawn, with time as the horizontal axis and OD value as the vertical axis.

1.7 Cell scratch assay

Straight lines were drawn every 0.5 cm on the back of a 24-well plate using a marker. Single-cell suspensions (5×10^4 cells/ml) were added to each well of the 24-well plate, and the cells were incubated

at 37°C and 5% CO_2. When reaching a confluence of 70%-80%, the cells were transfected. After transfection, the cells were incubated until forming a monolayer. Scratches were made by using a 200 μl pipette tip following the pre-drawn lines as a guide (to ensure that the width of each scratch was the same). The cells were gently washed 3 times with PBS to remove unadhered cells, after which serum-free culture medium was added to each well, and the cells were incubated. The scratches were observed under a microscope at 0, 12, 24, and 48 hours; the width of scratches was measured, and the healing rate was calculated. The assay was performed in triplicate.

1.8 Transwell chamber invasion assay

Single-cell suspensions (1×10^6 cells/ml) were prepared. Matrigel was thawed at 4°C overnight and diluted to 1 mg/ml with 4°C serum-free RPMI 1640 medium in a low temperature environment. The transwell chamber was placed in a sterile 24-well plate. Matrigel (100 μL) was added to the upper chamber and solidified at 37°C in an incubator. Then, 100 μl of cell suspension was added to the upper chamber, and 600 μl of RPMI 1640 medium containing 10% foetal bovine serum was added to the lower chamber. After the cells incubated for 48 hours, the chamber was removed, and the cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature, air-dried and stained with crystal violet for 20 minutes. The cells in the chamber were observed under an inverted phase contrast microscope coupled with a camera (×200). Five fields of view were randomly selected to count the number of penetrating cells.

1.9 Western blot detection of target protein expression

Cells of each group were collected to extract total protein; the cells were lysed with RIPA and PMSF at a ratio of 100:1. The total protein concentration was measured using the bicinchoninic acid (BCA) method. After boiling the samples for 5 minutes to denature the protein, 60 μg of protein was separated by 10-12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was then blocked with 5% skimmed milk powder in TBS for 1 hour at room temperature. Primary antibodies were added, and the membranes were incubated overnight at 4°C. The membranes were then washed 3 times with TBST, after which the corresponding secondary antibodies were added. The membrane was incubated for 1 hour at room temperature and then washed 3 times with TBST. The Odyssey dual-colour infrared fluorescence scanning system was used for imaging and analysis. The ratio of grey scale values for the target protein band and the internal reference protein band was used as the relative expression level of the target protein. This experiment was performed in triplicate.

1.10 Cell transfection

Single-cell suspensions (1×10^6 cells/mL) were prepared. The cells were added to each well of a 6-well plate and incubated overnight until completely adhered. Diluted siRNA and constructed plasmids (3-5 μL) was mixed with in 100 μL of RPMI 1640 medium. Lipofectamine^{TM} 2000 (5 μL) was mixed with 100 μL of RPMI1640 medium. The two solutions were mixed well and allowed to rest at room temperature for 20

min, after which 800 μL of antibiotic-free medium was added. The solutions were mixed well and then added to each well of the 6-well plate. After the plate had incubated for 6 hours, the medium was replaced with RPMI1640 containing 10% foetal bovine serum. The cells were collected after 48 hours.

1.11 Establishment of a tumour-bearing mouse model of gastric cancer

Single-cell suspensions (5×10^6/ml) of stably transfected cancer cells were prepared and injected subcutaneously (200 μL) into the bilateral armpits of 4-to-5-week-old BALB/c nude mice. The longest diameter of the subcutaneous tumour (a) and the distance (b) perpendicular to the longest diameter were measured and recorded every 3 days, and the tumour volume was calculated (volume (mm^3)=$0.5\times a\times b^2$). At the end of the experiment, the mice were sacrificed, and the subcutaneous transplanted tumour was harvested for subsequent experiments.

1.12 Dual-luciferase reporter assay

Potential binding sites of LINC02679 and miR-5004-3p were identified using bioinformatics software (Starbase v2.0 miRcode and RNAhybrid). Primers were designed based on the predicted sites. Amplified fragments were inserted into the luciferase reporter gene plasmid to establish the reporter vector LINC02679-wt. The predicted fragment of miR-5004-3p was altered to construct the mutant reporter vector LINC02679-mt. The wild-type and mutant reporter vectors, miR-5004-3p-5p-mimic and NC-mimic were co-transfected into gastric cancer cells using Lipofectamine^{TM}2000. The cells were collected 48 hours after transfection to detect the luciferase activity, followed by statistical analysis. The same method was used to analyse and verify the regulatory relationship with tripartite motif family-like 2 (TRIML2).

1.14 Statistical analysis

The data are expressed as`x±s. Analysis of variance (ANOVA) and Dunnett’s t test were carried out using SPSS 22.0 software. The effects of LINC02679 on MKN28 and HGC27 cells and the underlying molecular mechanism were comprehensively analysed. $P<0.05$ was considered statistically significant.

2. Results

2.1 LINC02679 and miR-5004-3p expression in gastric tissue and bioinformatic analysis

(1)The qRT-PCR results indicated that LINC02679 expression in human gastric cancer tissue was significantly higher than that in paracancerous tissue ($P<0.01$) . (Fig. 1 A.B).

(2)Bioinformatics results indicated that LINC02679 expression in human gastric cancer tissue was significantly higher than that in paracancerous tissue. Prognostic analysis suggested that high LINC02679 expression indicated a poor prognosis for patients with gastric cancer .(Fig. 1 C.D).

(3) The qRT-PCR results indicated that LINC02679 expression in GES-1 cells was lower than that in gastric cancer cells; HGC27 cells had the highest LINC02679 expression, and MKN28 cells had the lowest LINC02679 expression (P<0.01). (Fig. 1 E).

2.2 Effects of LINC02679 inhibition on the activity, invasion and migration of HGC27 cells

(1) The qRT-PCR results indicated that after knocking down LINC02679 in HGC27 cells, LINC02679 expression was significantly reduced. siRNA3, which had the most inhibitory effect, was selected for subsequent experiments (P<0.01)(Fig. 2 A).

(2) The MTT results indicated that after LINC02679 was inhibited in HGC27 cells, cell activity significantly decreased (P<0.01)(Fig. 2 B).

(3)After LINC02679 was inhibited in HGC27 cells, genes and proteins involved in cell cycle significantly changed.(Fig. 2 C).

(4) After LINC02679 was inhibited in HGC27 cells, cell invasion and migration significantly decreased (P<0.01).(Fig. 2 D).

(5)After LINC02679 was inhibited in HGC27 cells, genes and proteins involved in cell invasion and migration significantly changed (P<0.01) (Fig. 2 E).

2.3 Effects of LINC02679 overexpression on the activity, invasion, and migration of MKN28 cells

(1) The qRT-PCR results indicated that after LINC02679 overexpressed in MKN28 cells, LINC02679 significantly increased. The optimal concentration was selected for subsequent experiments (P<0.01) (Fig. 3 A).

(2) The MTT results indicated that after LINC02679 overexpressed in MKN28 cells, cell activity significantly increased (P<0.01) (Fig. 3 B).

(3) After LINC02679 overexpressed in MKN28 cells genes and proteins involved in cell cycle significantly changed.(P<0.01). (Fig. 3 C).

(4) After LINC02679 overexpressed in MKN28 cells, cell invasion and migration significantly increased (P<0.01) (Fig. 3 D).

(5) After LINC02679 overexpressed in MKN28 cells, genes and proteins involved in cell invasion and migration changed significantly (P<0.01) (Fig. 3 E).

2.4 Effects of LINC02679 inhibition on subcutaneous transplanted tumours in nude mice

(1) The average weight and volume of transplanted tumours in the empty vector group were higher than those in the LINC02679-shRNA transfected group (P<0.01). (Fig 4 A.B).

(2) The Western blot results indicated that in the subcutaneous transplanted tumours, the protein expression levels of PCNA, MMP-2, and MMP-9 were significantly lower and those of p16 and p21 were significantly higher in the LINC02679-shRNA transfected group than in the empty vector group .(Fig 4 C.D).

2.5 miR-5004-3p expression in gastric cancer tissue and gastric cancer cells and the regulatory relationship between LINC0267 and miR-5004-3p in HGC27 and MKN28 cells

(1) The functional analysis results for LINC02679 indicated that LINC02679 may directly regulate the expression of miR-5004-3p. (Fig. 5 A).

(2) The miR-5004-3p expression in human gastric cancer tissue was significantly lower than that in paracancerous tissue ($P<0.01$) (Fig. 5 B).

(3) The qRT-PCR results indicated that miR-5004-3p expression in GES-1 cells was higher than that in gastric cancer cells; miR-5004-3p expression was lowest in HGC27 cells and highest in MKN28 cells ($P<0.01$) (Fig. 5 C).

(4)The qRT-PCR results indicated that after LINC02679 overexpressed, miR-5004-3p significantly decreased in MKN28 cells ($P<0.01$) (Fig. 5 D).

(5) The qRT-PCR results indicated that after knocking down LINC02679, miR-5004-3p significantly increased in HGC27 cells ($P<0.01$) (Fig. 5 E).

(6) Dual-luciferase reporter genes analysis revealed that LINC02679 can directly regulate miR-5004-3p expression, a finding that was consistent with the bioinformatics results (Fig. 5 F).

2.6 TRIML2 expression in gastric cancer tissue and effects of overexpression of miR-5004-3p in HGC27 cells and inhibition of miR-5004-3p in MKN28 cells on Triml2 expression

(1) The predictive analysis of target gene binding site sequences revealed that a miR-5004-3p binding site exists in the 3'UTR region of TRIML2 mRNA. (Fig. 6 A).

(2) The qRT-PCR and Western blot results indicated that TRIML2 expression in human gastric cancer tissues was significantly higher than that in paracancerous tissue ($P<0.01$) (Fig. 6 B).

(3) The qRT-PCR and Western blot results indicated that TRIML2 expression in GES-1 cells was lower than that in gastric cancer cells; HGC27 cells had the highest TRIML2 expression, and MKN28 cells had the lowest TRIML2 expression ($P<0.01$) (Fig. 6 C).

(4) miR-5004-3p expression significantly increased after a miR-5004-3p mimic was transfected into HGC27 cells ($P<0.01$), and TRIML2 mRNA and protein significantly decreased after miR-5004-3p mimic transfection ($P<0.01$) (Fig. 6 D).

(5) The qRT-PCR results indicated that the transfection of a miR-5004-3p inhibitor into MKN28 cells led to a reduction in miR-5004-3p expression (P<0.01) and an increase in TRIML2 mRNA and protein expression (P<0.01) (Fig. 6 E).

(6)Dual-luciferase reporter gene analysis revealed that miR-5004-3p has a direct regulatory effect on TRIML2 mRNA (Fig. 6 F).

2.7 Effects of LINC02679 and miR-5004-3p inhibition on TRIML2 expression and cell activity, invasion and migration of HGC27 cells

(1) The qRT-PCR and Western blot results indicated that after knocking down LINC02679 in HGC27 cells, TRIML2 expression significantly decreased (P<0.01); in contrast, knocking down miR-5004-3p increased TRIML2 expression (P<0.01) (Fig. 7 A).

(2) The qRT-PCR and Western blot results indicated that after overexpressing LINC02679 in MKN28 cells, TRIML2 expression significantly increased (P<0.01); in contrast, overexpressing miR-5004-3p significantly reduced TRIML2 expression (P<0.01) (Fig. 7 B)

(3) The MTT and cell invasion and migration assay results indicated that after knocking down LINC02679 in HGC27 cells, cell activity, invasion and migration significantly decreased (P<0.01); in contrast, knocking down miR-5004-3p increased cell activity, invasion and migration (P<0.01) (Fig. 7 C).

(4) The MTT and invasion and migration assay results indicated that after overexpressing LINC02679 in MKN28 cells, cell activity, invasion and migration significantly increased (P<0.01); in contrast, overexpressing miR-5004-3p significantly reduced cell activity, invasion and migration (P<0.01) (Fig. 7 D).

3. Discussion

Cancer has become the main cause of death in various countries and is a serious threat to human health. The latest data from GLOBOCAN shows that the global death rate of gastric cancer is the fourth highest among common cancers. In 2020, there were 768,793 deaths from gastric cancer; i.e., 1 in 13 deaths from all causes was attributed to gastric cancer [1]. Therefore, research into the pathogenesis of gastric cancer and the identification of highly sensitive and specific targets that can simultaneously guide the diagnosis, treatment and prognostic analysis of gastric cancer have become a hot topic as well as a challenge.

The occurrence, development and metastasis of gastric cancer is a multi-factor, multi-step, multi-stage process involving various aspects of genetics and epigenetics. Previous studies of gastric cancer have focused on protein-coding genes; however, the Human Genome Project has revealed that protein-coding genes account for less than 3% of cases. Therefore, the transcription products of a large number of non-coding sequences are non-coding RNAs [24–26]. lncRNA and miRNA are important members of the ncRNA family. Increasingly more studies have shown that lncRNA and miRNA play key roles in the occurrence, development, metastasis, drug resistance and prognostic outcomes of gastric cancer [20–23,

27–30]. lncRNA HOTAIR is highly expressed in gastric cancer tissue and cells, participating in the proliferation, migration, and invasion of gastric cancer cells. Patients with high lncRNA HOTAIR levels have a poorer prognosis [31]. miR-145-5p is lowly expressed in gastric cancer cells. miR-145-5p overexpression inhibits the proliferation, migration and invasion of gastric cancer cells by suppressing ANGPT2 [32]. In this study, we found that LINC02679 upregulation is present in both human gastric cancer tissue and gastric cancer cell lines and is an indicator of a poor prognosis of patients with gastric cancer. Further cell experiments showed that knocking down LINC02679 reduced cell proliferation, migration and invasion. In animal experiments, the weight and volume of tumours also significantly decreased after downregulating LINC02679. Overall, our study demonstrated that LINC02679 plays an important role in the formation, development and metastasis of gastric cancer.

The lncRNA located in the nucleus can directly bind to DNA sequences to inhibit the transcription process; or it can bind transcription factors, methyltransferases, RNA polymerase complexes, and affect the transcription process through histone modifications[33–36]. lncRNA has the characteristics of free shuttle inside and outside the nucleus, it can also participate in the regulation process in the cytoplasm, for example: ceRNA mechanism [37–39]. lncRNA can also affect the exercise of intracellular physiological functions through the variable shearing, positioning and stability of mRNA. In addition, lncRNA can also promote the cleavage of pri-miRNA, or become a precursor of miRNA. After being cut into miRNA, lncRNA can inhibit the expression level of target gene mRNA. lncRNA can also participate in the process of protein translation, combining with mRNA 5'UTR to promote translation; binding to specific proteins, targeting mRNA and inhibiting translation[40–41]. In this study, the bioinformatics analysis of the function of LINC02679 revealed that LINC02679 may directly regulate the expression of miR-5004-3p, LINC02679 is highly expressed in human gastric cancer tissue and cell lines and miR-5004-3p is lowly expressed; and LINC02679 and miR-5004-3p expression in tissues are negatively correlated (LINC02679 knockdown significantly increased miR-5004-3p in HGC27 cells, and LINC02679 overexpression significantly decreased miR-5004-3p in MNN28 cells). Furthermore, the dual-luciferase reporter gene analysis revealed that LINC02679 directly regulates miR-5004-3p expression, a finding that is consistent with the bioinformatics results. These results indicate that LINC02679 is involved in the occurrence and development of gastric cancer by regulating the expression of miR-5004-3p.

TRIML2 is a member of the tripartite motif (TRIM) protein family and involved in the regulation of p53-mediated apoptosis [42]. Previous studies have shown that 2 C alleles encoding single nucleotide polymorphisms (SNPs) (rs79698746 and rs2279551) in TRIML2 may increase susceptibility to Alzheimer's disease in Koreans [43]. Recent studies have shown that TRIML2 plays an important role in tumour cell growth. TRIML2 is highly expressed in oral squamous cell carcinoma tissue. The inhibition of TRIML2 leads to the downregulation of CDK2, CDK4 and cyclin D1 and upregulation of p21 and p27, which arrests the cell cycle in the G0/G1 phase, thereby inhibiting cell proliferation [44]. The role of TRIML2 in gastric cancer has not been reported before. This study found that TRIML2 is highly expressed in gastric cancer tissues and cells and that miR-5004-3p and TRIML2 mRNA are negatively correlated in gastric cancer tissues: miR-5004-3p overexpression reduces TRIML2 mRNA and protein expression and miR-5004-3p inhibition increases the TRIML2 mRNA and protein expression. Furthermore, dual-luciferase

reporter gene analysis revealed that miR-5004-3p can directly regulate the expression of TRIML2, indicating that TRIML2 is the target gene of miR-5004-3p. In addition, we also found that in tissues, LINC02679 and TRIML2 mRNA were positively correlated and miR-5004-3p and TRIML2 mRNA were negatively correlated; miR-5004-3p inhibition can attenuate the influences of LINC02679 inhibition on the downregulation of TRIML2 mRNA and protein and cell activity, migration and invasion; miR-5004-3p overexpression can attenuate the effects of LINC02679 overexpression on the upregulation of reduced TRIML2 mRNA expression and cell activity, migration and invasion. It is proposed that the lncRNA LINC02679 regulates TRIML2 expression by targeting miR-5004-3p to influence the proliferation and invasion of gastric cancer cells. Bioinformatics predicts that that LINC02679 involved in the proliferation and invasion of gastric cancer cells by regulating the miR-5004-3p/TRIML2, results that were confirmed by the luciferase reporter gene analysis.

In conclusion, this study found that LINC02679 expression in gastric cancer tissues and cells is increased and that LINC02679 overexpression is an indicator of a poor prognosis for patients with gastric cancer. Regulating LINC02679 expression in cells changed the proliferation, invasion and migration behaviours of cells and the expression of related genes, findings that were consistent with the results of in vivo experiments. Further experiments found that LINC02679 regulates the miR-5004-3p/TRIML2 pathway. This study demonstrated that LINC02679 targets TRIML2 to promote the proliferation, invasion and migration of gastric cancer cells and that LINC02679 could serve as a target gene for gastric cancer treatment.

4. Declarations

4.1 Ethics approval and consent to participate

The institutional review board of the Fourth Hospital of Hebei Medical University provided approval to conduct this study and signed informed consent.

4.2 Consent for publication

Applicable.

4.3 Availability of data and materials

The data that support the findings of this study are available from database of the Fourth Hospital of Hebei Medical University, but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of the the Fourth Hospital of Hebei Medical University.

4.4 Competing interests

The authors declare that they have no competing interests.

4.5 Funding

Not applicable.

4.6 Authors' contributions

Yong Li, Liqiao Fan, Qun Zhao in the design of the study, carried out the acquisition of data, participated in the manuscript drafting. Bibo Tan, Zihao Chen and Yijie Zhao participated in the design of the study and the acquisition of data. Bibo Tan, Zihao Chen, Yijie Zhao, Ming Tan, Yuxiang Xia, Wenbo Liu conceived of the study, participated in the design and coordination, drafted the manuscript. All authors read and approved the final manuscript.

4.7 Acknowledgments

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Tables

Table I. Sequences of each primer.

Gene	Forward primer (5-3')	Reverse primer (5'-3')
PCNA	AAGCCGAAACCAGCTAGACTTTC	TGGCGGAGTGGCAACAA
MMP2	AGAAGGCTGTGTTCTTCGCA	AAAGGCAGCGTCTACTTGCT
MMP9	GGCACATAGTAGGCCCTTTAA	TCACTCCTTTCTTCCTAGCCA
Cyclin A	GCCTCTAAGATGAAGGAGACCAT	ATTTTGGAGAGGAAGTGTTCAAT
Cyclin D1	ACCTGAGGAGCCCCAACAAC	GCTTCGATCTGCTCCTGGC
E-Cadherin	GCTGCTCTTGCTGTTTCTTCG	CCGCCTCCTTCTTCATCATAG
Vimentin	GGACCAGCTAACCAACGACA	AATCCCATCACCATCTTCCAG
Cyclin E	GAACTGATGATGATGAAG	CCACTGATAACCTGAG
Zeb1	TAGTTGCTCCCTGTGCAGTTAC	GCATTCATATGGCTTCTCTCCACTG
β-actin	CATCCTCACCCTGAAGTACCCC	AGCCTGGATGCAACGTACATG
P21	ACTCAACCGTAATATCCCGACT	GCAGCAGATCACCAGATTAACCC
P16	ACCAGAGGCAGTAACCATGC	CCTGTAGGACCTTCGGTGAC
ICAM1	MMGNGMQMNNGQGQMQNNNG	QGMMNMMNNQNQGGGNNGGM

Table 2. Antibodies' Information

Gene	Product number
PCNA	SAB5701035
MMP2	SAB5701187
MMP9	SAB1402274
Cyclin A	SAB4503499
Cyclin D1	SAB5701174
E-Cadherin	SAB5700789
Vimentin	SAB5701293
Cyclin E	07-687
Zeb1	SAB5701068
β-actin	A1978
P21	SAB4500065
P16	SAB4500072
ICAM1	SAB4501486

Figures

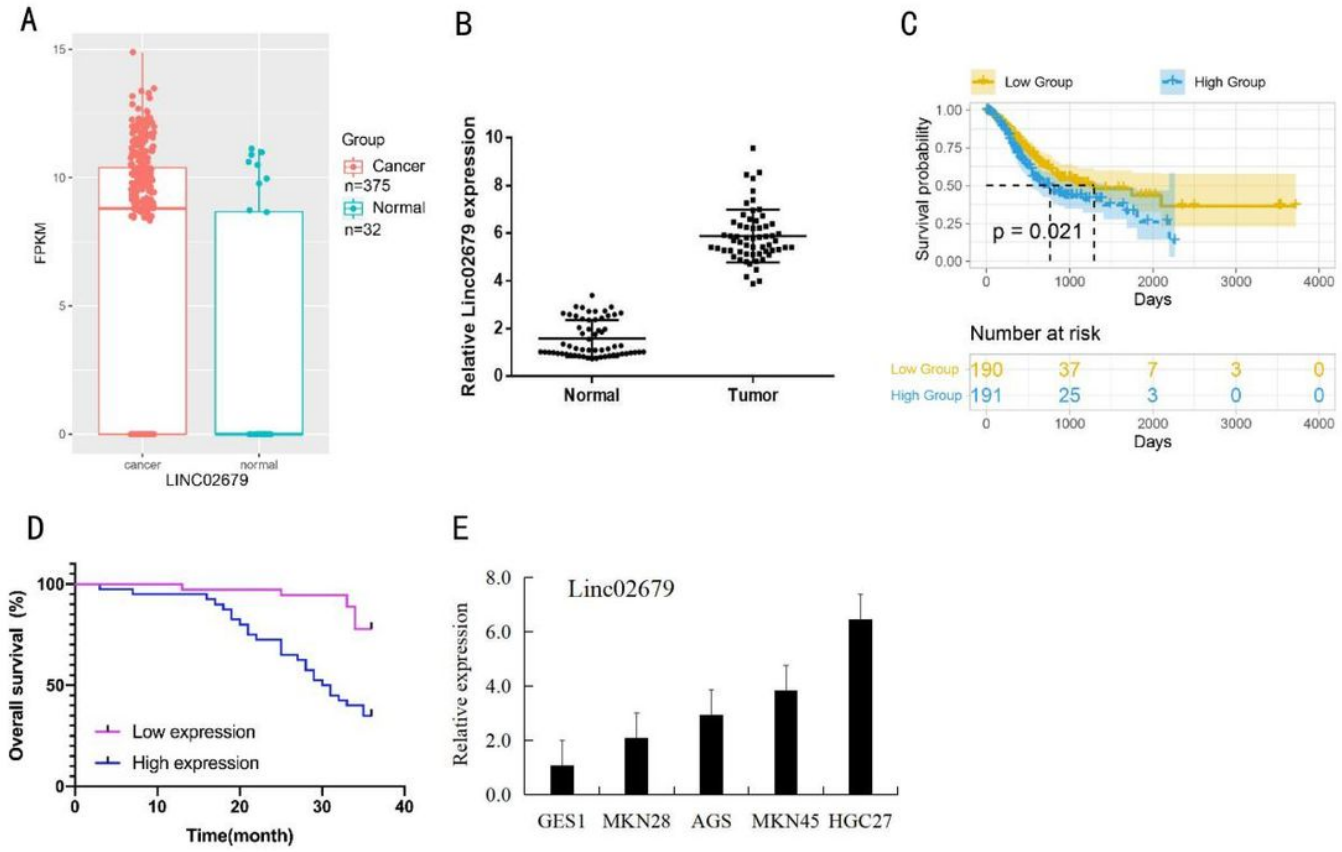

Figure 1

LINC02679 and miR-5004-3p expression in gastric tissue and bioinformatic analysis. A.B The qRT-PCR results indicated that LINC02679 expression in human gastric cancer tissue was significantly higher than that in paracancerous tissue (P<0.01) . C.D Bioinformatics results indicated that LINC02679 expression in human gastric cancer tissue was significantly higher than that in paracancerous tissue. Prognostic analysis suggested that high LINC02679 expression indicated a poor prognosis for patients with gastric cancer . E The qRT-PCR results indicated that LINC02679 expression in GES-1 cells was lower than that in gastric cancer cells; HGC27 cells had the highest LINC02679 expression, and MKN28 cells had the lowest LINC02679 expression (P<0.01).

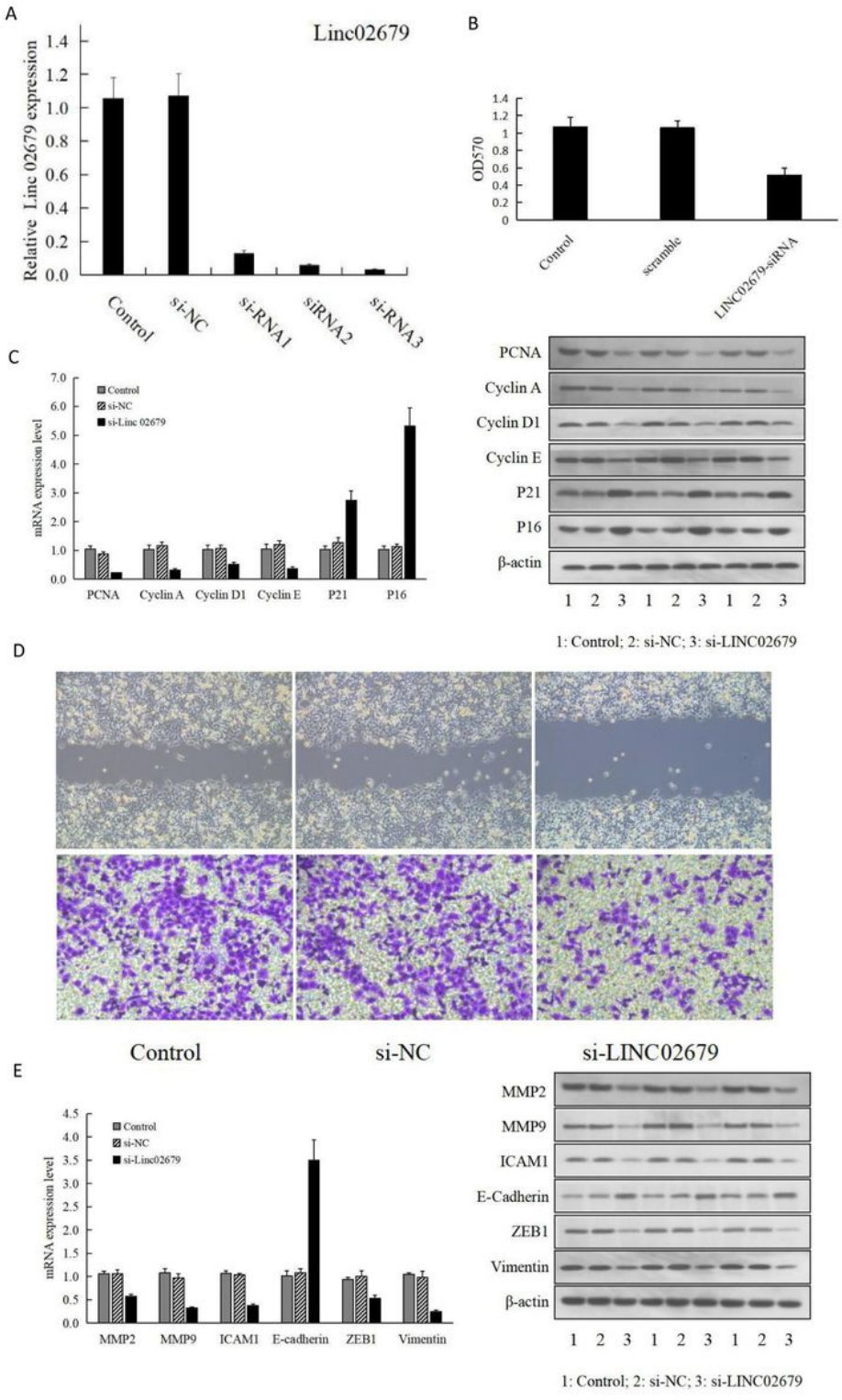

Figure 2

Effects of LINC02679 inhibition on the activity, invasion and migration of HGC27 cells . A The qRT-PCR results indicated that after knocking down LINC02679 in HGC27 cells, LINC02679 expression was significantly reduced. siRNA3, which had the most inhibitory effect, was selected for subsequent experiments (P<0.01) . B The MTT results indicated that after LINC02679 was inhibited in HGC27 cells, cell activity significantly decreased (P<0.01) . C After LINC02679 was inhibited in HGC27 cells, genes and

proteins involved in cell cycle significantly changed. D After LINC02679 was inhibited in HGC27 cells, cell invasion and migration significantly decreased (P<0.01). E After LINC02679 was inhibited in HGC27 cells, genes and proteins involved in cell invasion and migration significantly changed (P<0.01).

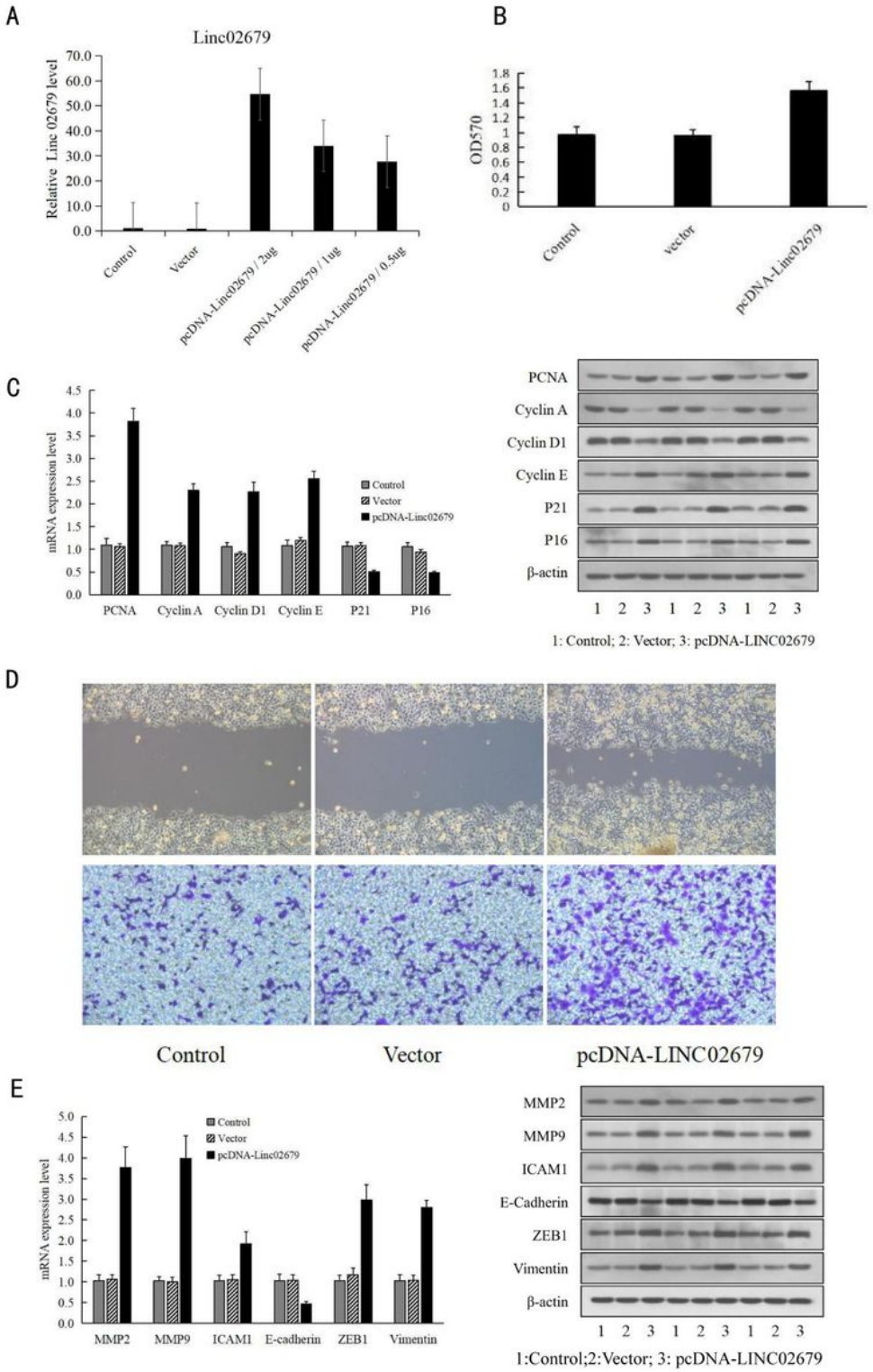

Figure 3

Effects of LINC02679 overexpression on the activity, invasion, and migration of MKN28 cells. A The qRT-PCR results indicated that after LINC02679 overexpressed in MKN28 cells, LINC02679 significantly increased. The optimal concentration was selected for subsequent experiments (P<0.01). B The MTT results indicated that after LINC02679 overexpressed in MKN28 cells, cell activity significantly increased (P<0.01) . C After LINC02679 overexpressed in MKN28 cells genes and proteins involved in cell cycle significantly changed.(P<0.01). D After LINC02679 overexpressed in MKN28 cells, cell invasion and migration significantly increased (P<0.01) . E After LINC02679 overexpressed in MKN28 cells, genes and proteins involved in cell invasion and migration changed significantly (P<0.01).

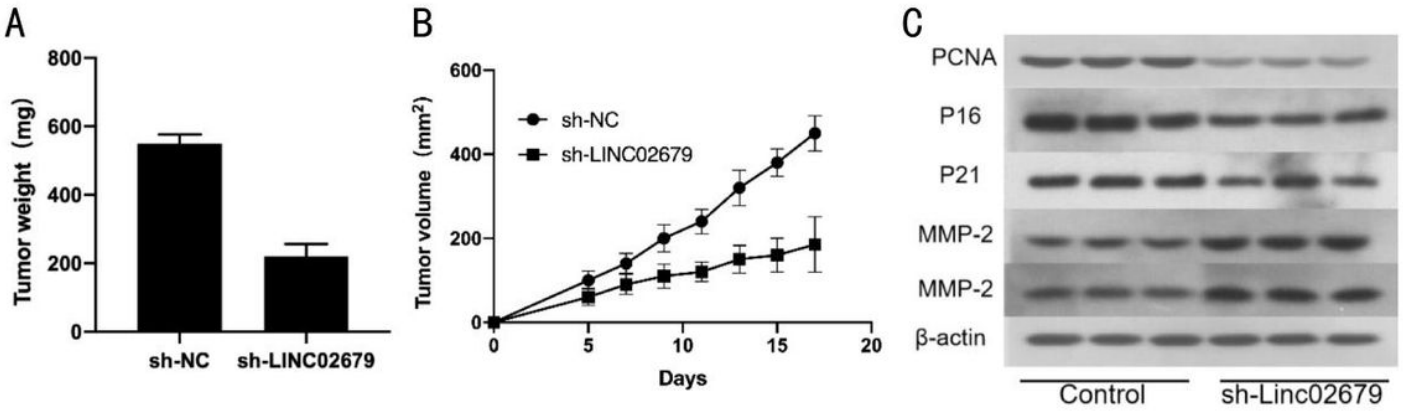

Figure 4

Effects of LINC02679 inhibition on subcutaneous transplanted tumours in nude mice. A.B The average weight and volume of transplanted tumours in the empty vector group were higher than those in the LINC02679-shRNA transfected group (P<0.01). C The Western blot results indicated that in the subcutaneous transplanted tumours, the protein expression levels of PCNA, MMP-2, and MMP-9 were significantly lower and those of p16 and p21 were significantly higher in the LINC02679-shRNA transfected group than in the empty vector group.

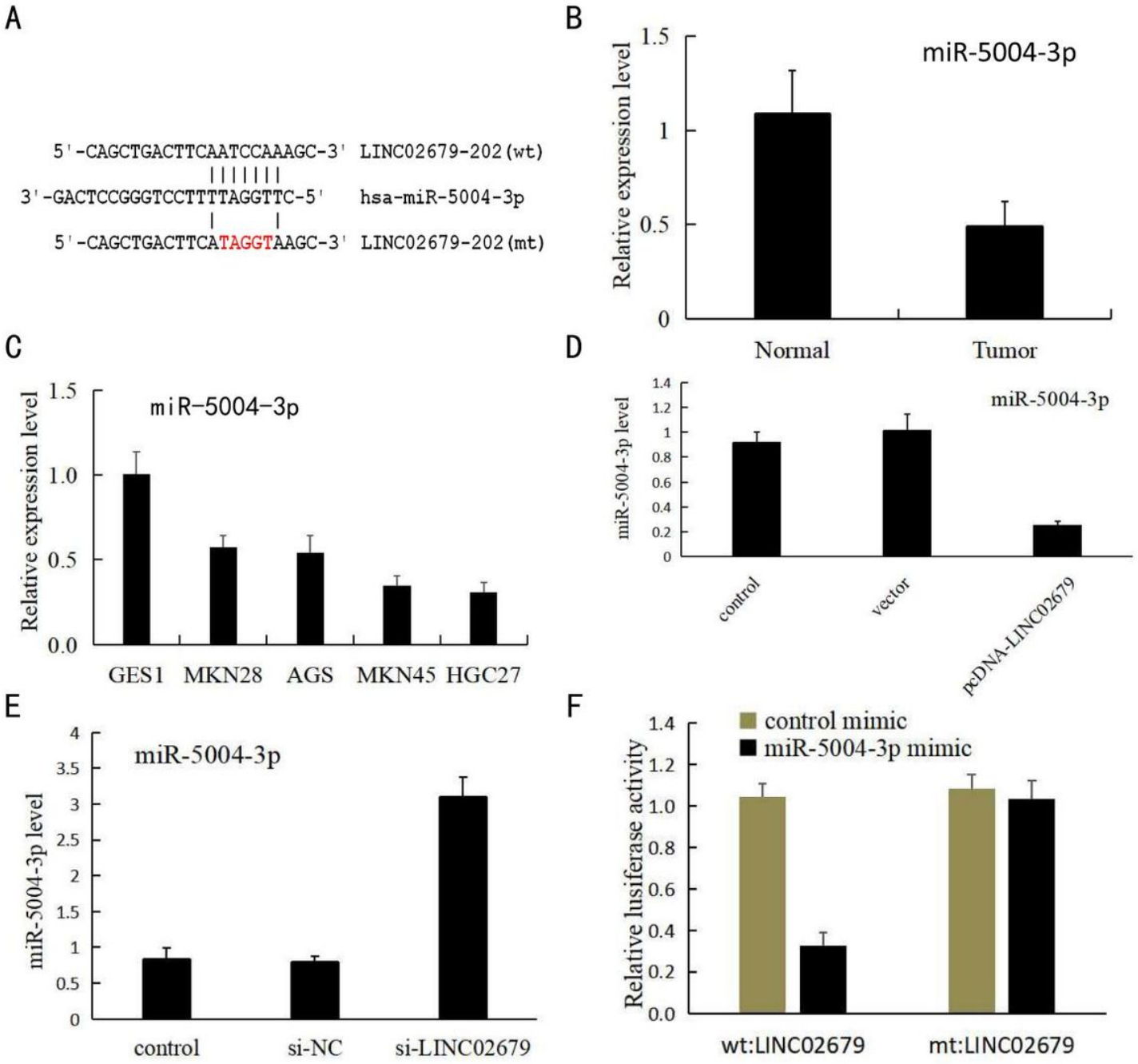

Figure 5

miR-5004-3p expression in gastric cancer tissue and gastric cancer cells and the regulatory relationship between LINC0267 and miR-5004-3p in HGC27 and MKN28 cells. A The functional analysis results for LINC02679 indicated that LINC02679 may directly regulate the expression of miR-5004-3p. B The miR-5004-3p expression in human gastric cancer tissue was significantly lower than that in paracancerous tissue ($P<0.01$) . C The qRT-PCR results indicated that miR-5004-3p expression in GES-1 cells was higher than that in gastric cancer cells; miR-5004-3p expression was lowest in HGC27 cells and highest in MKN28 cells ($P<0.01$) . D The qRT-PCR results indicated that after LINC02679 overexpressed, miR-5004-3p significantly decreased in MKN28 cells ($P<0.01$) . E The qRT-PCR results indicated that after knocking

down LINC02679, miR-5004-3p significantly increased in HGC27 cells (P<0.01) . F Dual-luciferase reporter genes analysis revealed that LINC02679 can directly regulate miR-5004-3p expression, a finding that was consistent with the bioinformatics results.

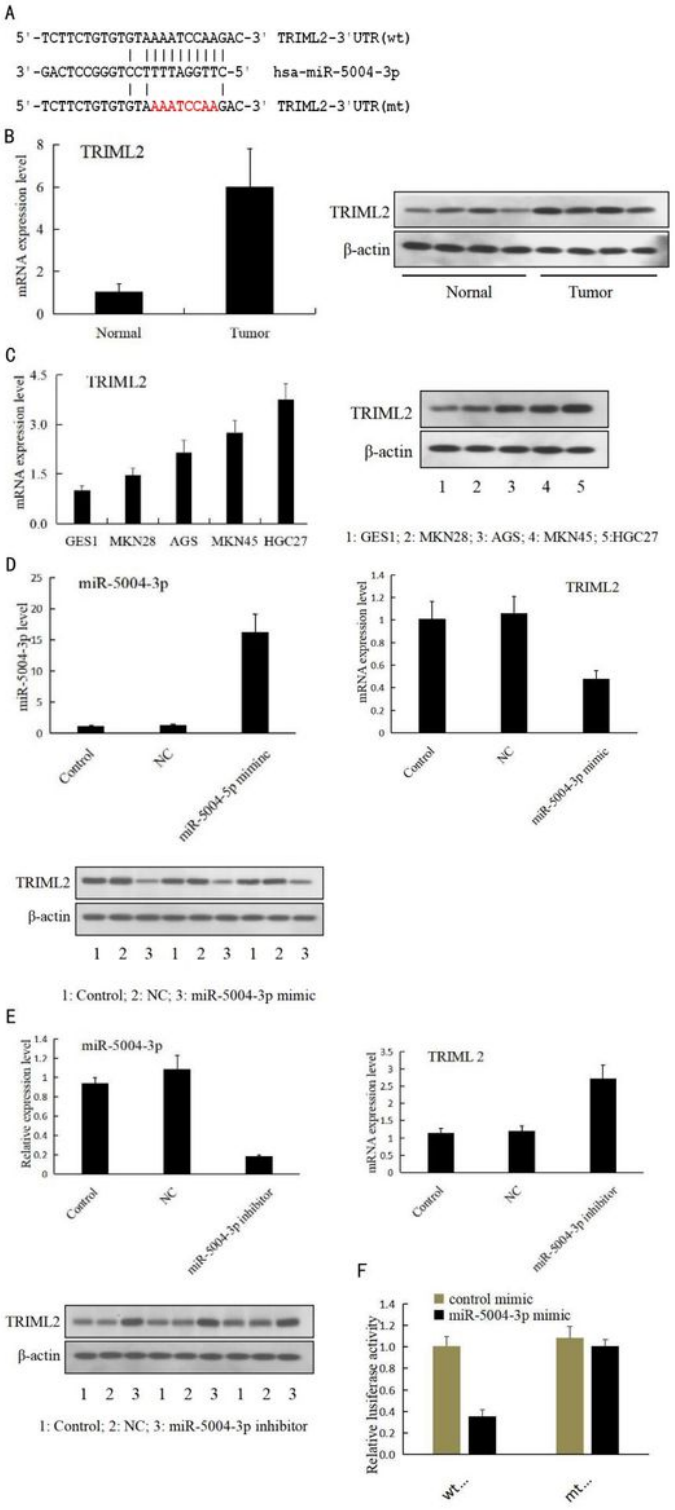

Figure 6

TRIML2 expression in gastric cancer tissue and effects of overexpression of miR-5004-3p in HGC27 cells and inhibition of miR-5004-3p in MKN28 cells on Triml2 expression. A The predictive analysis of target gene binding site sequences revealed that a miR-5004-3p binding site exists in the 3'UTR region of TRIML2 mRNA. B The qRT-PCR and Western blot results indicated that TRIML2 expression in human gastric cancer tissues was significantly higher than that in paracancerous tissue ($P<0.01$) . C The qRT-PCR and Western blot results indicated that TRIML2 expression in GES-1 cells was lower than that in gastric cancer cells; HGC27 cells had the highest TRIML2 expression, and MKN28 cells had the lowest TRIML2 expression ($P<0.01$) . D miR-5004-3p expression significantly increased after a miR-5004-3p mimic was transfected into HGC27 cells ($P<0.01$), and TRIML2 mRNA and protein significantly decreased after miR-5004-3p mimic transfection ($P<0.01$) . E The qRT-PCR results indicated that the transfection of a miR-5004-3p inhibitor into MKN28 cells led to a reduction in miR-5004-3p expression ($P<0.01$) and an increase in TRIML2 mRNA and protein expression ($P<0.01$) . F Dual-luciferase reporter gene analysis revealed that miR-5004-3p has a direct regulatory effect on TRIML2 mRNA.

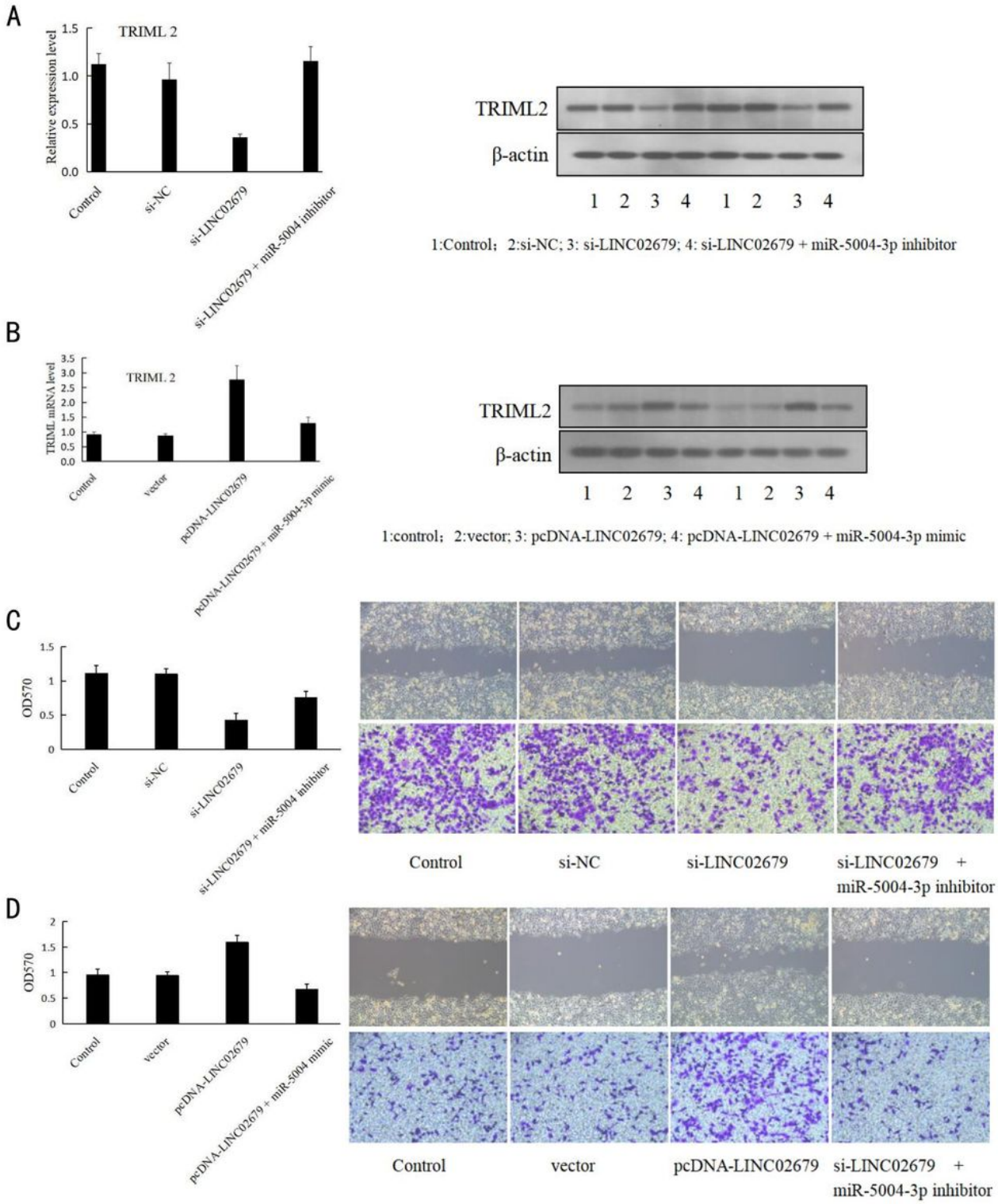

Figure 7

Effects of LINC02679 and miR-5004-3p inhibition on TRIML2 expression and cell activity, invasion and migration of HGC27 cells . A The qRT-PCR and Western blot results indicated that after knocking down LINC02679 in HGC27 cells, TRIML2 expression significantly decreased (P<0.01); in contrast, knocking down miR-5004-3p increased TRIML2 expression (P<0.01) . B The qRT-PCR and Western blot results indicated that after overexpressing LINC02679 in MKN28 cells, TRIML2 expression significantly increased

(P<0.01); in contrast, overexpressing miR-5004-3p significantly reduced TRIML2 expression (P<0.01) . C The MTT and cell invasion and migration assay results indicated that after knocking down LINC02679 in HGC27 cells, cell activity, invasion and migration significantly decreased (P<0.01); in contrast, knocking down miR-5004-3p increased cell activity, invasion and migration (P<0.01) . D The MTT and invasion and migration assay results indicated that after overexpressing LINC02679 in MKN28 cells, cell activity, invasion and migration significantly increased (P<0.01); in contrast, overexpressing miR-5004-3p significantly reduced cell activity, invasion and migration (P<0.01).