CircTMC5 promotes gastric cancer progression and metastasis by targeting miR-361-3p/RABL6

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Abstract
Background Gastric cancer (GC) is common in East Asia, yet its molecular and pathogenic mechanisms remain unclear. Circular RNAs (circRNAs) are differentially expressed in GC and may be promising biomarkers. Here, we investigated the role and regulatory mechanism of circTMC5 in GC.

Methods CircTMC5 expression was detected in human GC and adjacent tissues using microarray assays and qRT-PCR, while the clinicopathological characteristics of patients with GC were used to assess its diagnostic and prognostic value. The circTMC5/miR-361-3p/RABL6 axis was examined in vitro and vivo, and the immune roles of RABL6 were evaluated using bioinformatics analyses and immunohistochemistry (IHC).

Results CircTMC5 was highly expressed in GC tissues, plasma, and cell lines, and was closely related to histological grade, pathological stage, and T classification in patients with GC. CircTMC5 expression was also an independent prognostic factor for GC and its combined detection with carcinoembryonic antigen may improve GC diagnosis. Low circTMC5 expression correlated with good prognosis, inhibited GC cell proliferation, and promoted apoptosis. Mechanistically, circTMC5 over-expression promoted GC cell proliferation, invasion, and metastasis but inhibited apoptosis by sponging miR-361-3p and up-regulating RABL6 in vitro and vivo, whereas miR-361-3p up-regulation had the opposite effects. RABL6 was highly expressed in GC and was involved in immune regulation and infiltration in GC.

Conclusions CircTMC5 promotes GC and sponges miR-361-3p to up-regulate RABL6 expression, thus may have diagnostic and prognostic value in GC. RABL6 also displays therapeutic promise due to its role in the immune regulation of GC.

Keywords circTMC5 · miR-361-3p · RABL6 · Gastric cancer

Introduction
Despite new technologies and targeted therapies that have improved the early diagnosis and treatment of gastric cancer (GC), the 5-year overall survival rate of patients remains low [1–3]. Due to the poor prognosis of
patients with advanced GC, it is important to explore the mechanism underlying the progress of GC to improve its treatment [4].

Circular RNAs (circRNAs) are non-coding endogenous RNAs that are common in eukaryotic cells and are characterized by a covalent closed-loop structure, with neither 5′–3′ polarities nor a polyadenylic acid tail [5–7]. CircRNAs have multiple functions, including regulating alternative splicing or transcription, sponging miRNA, interacting with RNA-binding proteins, translating and transcribing pseudogenes, and transporting substances and information [8–10]. Studies have also shown that some circRNAs are differentially expressed in human GC tissues and cell lines, as well as being related to clinicopathological features [11–13]. Moreover, it has been shown that differentially expressed circRNAs in GC are related to diagnosis, prognosis, malignancy, and 5-year survival rate [14]; however, the mechanism and role of circRNAs in GC remain unclear.

In this study, we aimed to investigate the role and regulatory mechanism of circTMC5 in GC. We found that circTMC5 was significantly overexpressed in GC and was associated with a shorter patient survival time. In addition, we showed that circTMC5 promoted GC progression and metastasis by targeting miR-361-3p/RABL6. Thus, our findings suggest that the circTMC5/miR-361-3p/RABL6 axis mediates the malignancy of GC, potentially by influencing tumor immune regulation.

Materials and methods

Patients and specimens

Plasma and tissue samples were taken from patients pathologically diagnosed with gastric adenocarcinoma who underwent radical resection at the First Affiliated Hospital of Anhui Medical University between January 2016 and December 2018. No patients had undergone chemotherapy or radiotherapy before surgery. Blood samples were also collected from healthy volunteers at the same hospital over the same period. Written consent was obtained from all participants. This study was conducted according to the revised Helsinki Declaration and was approved by Ethics Committee of First Affiliated Hospital of Anhui Medical University. Plasma was extracted from the peripheral blood samples and stored at −80°C. Tissue specimens and adjacent non-tumor tissues (5 cm from the tumor boundary) were preserved using RNA fixer reagent (Biotek, Beijing, China).

Cell culture

GC cell lines (MGC803, HGC27, AGS, MKN45) and normal gastric epithelial cells (GES-1) were purchased from the Cell Bank of the Chinese Academy of Sciences. All GC cells were grown in RPMI-1640 medium (Gibco, Paisley, Scotland, UK) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 μg/mL streptomycin, and 100 U/mL penicillin at 37°C and 5% CO2.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from GC tissues, paracancerous tissues, all cell lines, and plasma specimens using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, USA). Total RNA was extracted from plasma samples using a mirVana Paris kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. RNA concentration was determined at 260 and 280 nm using a spectrophotometer. Total RNA was reverse transcribed into complementary DNA (cDNA) using a PrimeScript RT reagent kit with gDNA Eraser (TaKaRa Bio, Shiga, Japan). All data were quantified using the 2−ΔΔCt method and normalized using GAPDH and U6 as endogenous controls. The primer sequences for qRT-PCR are shown in Supplementary Table 1.

Cell transfection

CircTMC5 was silenced using three different small interfering RNAs (circTMC5-si #1, circTMC5-si #2, and circTMC5-si #3) that were designed, verified, and inserted into GV298 plasmids by Gima (Shanghai, China). Lentiviral vectors were co-transfected with psPAX2 and pMD2G packaging plasmids (Gima) [15] and then the siRNA lentivirus was added to cultured GC cells to establish a stable cell line. CircTMC5-si #1 and circTMC5-si #2 with the best interference efficiency were selected for experiment, as evaluated by qRT-PCR analysis of circTMC5 expression. Control cells were transfected with lentivirus scrambled control siRNA (NC-si).

CircTMC5 overexpression sequences and RABL6 sequences (NM_001173988.2) were verified by Sangon (Shanghai, China). A cDNA3.1 vector expressing circTMC5 or RABL6 was constructed by Sangon. MiR-361-3p mimic and NC were purchased from Sigma–Aldrich (Shanghai, China). GC cell lines were cultured at 37°C in a 5% CO2 incubator until they
reached 70–80% confluence before transfection using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Cells were transfected with empty vectors as a negative control. The Sequences of gene-specific siRNAs and overexpression sequences are shown in Supplementary Table 2.

**Cell cycle assays**

Apoptotic cells were detected using flow cytometry with an Annexin V-FITC/Phosphatidylinositol Apoptosis Detection Kit (BD Biosciences, Bedford, MA, USA) according to the manufacturer's instructions.

**Cell proliferation assays**

Cell proliferation was detected using cell counting kit-8 (CCK-8; Beijing, China). Briefly, $1 \times 10^3$ GC cells were inoculated into each well of a 96-well plate and, 48 h after transfection, 10 μL of CCK-8 reagent was added to each well and incubated 37 °C for 2 h. Relative proliferation was calculated from the absorbance of each well at 450 nm using a spectrophotometer.

Cell proliferation was also evaluated using a 5-Ethynyl-2'-deoxyuridine (EdU) Cell Proliferation Kit (Sangon, Shanghai, China) according to the manufacturer's instructions. Briefly, $1 \times 10^5$ cells were inoculated into each well of a 6-well plate and cultured for 48 h. EdU positive cells were then observed under a fluorescence microscope.

**Fluorescence in situ hybridization (FISH)**

Cy3- and Cy5-labeled circTMC5 probes were designed and synthesized by GenePharma (Shanghai, China) to hybridize to the post-splice junction. GC cells were cultured to 50% confluence in a confocal petri dish, washed with PBS, and fixed with 4% paraformaldehyde for 15 min. After the probe mixture had been added, the petri dish was incubated in the dark at 73 °C for 5 min and then at 37 °C for 15 h. The nucleus was stained with 4', 6-dimidyl-2-phenylindole (DAPI) and probe signals were processed using a FISH kit (GenePharma) according to the manufacturer's instructions. Images were obtained using a Zeiss LSM 800 confocal microscope (Carl Zeiss, Jena, Germany).

**Dual-luciferase reporter assay**

To detect circTMC5 and miR-361-3p, we inserted a circTMC5 fragment containing miR-361-3p complementary binding sites into a luciferase reporter vector. Wild-type pMIR-REPORT-circTMC5 was synthesized by Obio Technology (Shanghai, China). GC cells were spread on a 96-well plate, cultured to 60–70% confluence, and transfected with 200 ng circTMC5 luciferase reporter vector, 50 mM miR-361-3p, and NC using Lipofectamine 3000 (Invitrogen).

To detect RABL6 and miR-361-3p, the 3' UTR of human RABL6 containing a putative miR-361-3p binding site was cloned into a vector. Wild-type pMIR-REPORT-RABL6-3' UTR was synthesized by Obio Technology. Cells were transfected with 200 ng RABL6 3' UTR, 50 mM miR-361-3p, and an NC vector. Luciferase activity was measured after 48 h using a Dual Luciferase Reporter Assay kit (Promega, Beijing, China) according to the manufacturer's instructions. Luminescence was calibrated using the firefly luciferase sequence and standardized against Renilla luciferase.

**Transwell migration/invasion assay**

Transwell plates coated with matrix gel (Corning, Kennebunk, USA) were used to detect invasion, while those without matrix gel were used to detect migration. Briefly, $1 \times 10^5$ transfected cells were inoculated into Transwell plates with 1640 serum-free medium in the upper chamber and 1640 medium containing 10% FBS in the lower chamber. After incubation for 24 h at 37 °C with 5% CO$_2$, migrated cells were imaged and counted using an inverted microscope (Olympus, Tokyo, Japan).

**Immunohistochemistry (IHC)**

According to standard IHC procedures and evaluation methods [16], tissues fixed in formalin and embedded in paraffin were incubated with polyclonal antibodies against human RABL6 (Abcam, USA, 1:100 dilution), anti-IL-6 (Abcam, USA, 1:200 dilution), anti-IL-8 (Thermofisher, USA, 1:200 dilution), anti-CCL14 (Thermofisher, USA, 1:100 dilution), anti-CCL22 (Thermofisher, USA, 1:100 dilution), anti-CXCL3 (Thermofisher, USA, 1:100 dilution) at 4 °C overnight,
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Identification of circTMC5 as a clinical biomarker and its biological function in GC. a Heat map showing the clustering results of circRNA expression in GC tissues ($n = 3$) and adjacent normal tissues ($n = 3$) using circRNA microarrays. Color indicates circRNA expression as log10 (circRNA + 0.000001). b Volcano plot showing differences in circRNA expression in GC tissues and adjacent normal tissues screened using fold change ≥ 1.5 and FDR < 0.05. c Heat map showing the clustering results of the top ten highly expressed circRNAs in GC tissues screened using fold change ≥ 1.5 and FDR < 0.05. d–e Specific circTMC5 primers (ID: chr16:19,483,409–19,483,456) were verified by Sanger sequencing (d) and agarose gel electrophoresis (e), the red box indicates the special reverse splicing of circTMC5 cDNA verified using Sanger sequencing. f CircTMC5 expression in 96 GC tissues compared to normal adjacent tissues as well as plasma samples from 139 patients with gastric GC and 116 healthy volunteers measured using qRT-PCR. g CircTMC5 expression in GC and normal gastric epithelial (GES-1) cell lines detected by qRT-PCR. h Receiver operating characteristic curves for circTMC5, CEA, or their combination. i Kaplan–Meier analysis revealing that patients with low circTMC5 expression display a better OS than those with high circTMC5 expression. j CircTMC5 expression was significantly lower in MGC803 and HGC27 cell lines transfected with circTMC5-si #1 or circTMC5-si #2 than NC-si, as measured using qRT-PCR. k CCK-8 analysis showing that circTMC5 silencing can inhibit MGC803 and HGC27 cell proliferation. l EdU analysis of the percentage of positively stained MGC803 and HGC27 cells showing that circTMC5 knockout inhibited MGC803 and HGC27 cell proliferation. m Flow cytometry analysis showing that silencing circTMC5 in GC cells promotes apoptosis. n Bax, Bcl-2, caspase-3, and caspase-9 protein expression detected in NC-si and circTMC5-si #1 or circTMC5-si #2 transfected GC cell lines measured using Western blotting followed by secondary antibodies at 37 °C, the intensity of immunostaining was evaluated according to the previously reported method [16].

Tissues fixed were incubated with polyclonal antibodies against human CD68 (Thermofisher, USA, 1:200 dilution), anti-CD20 (Thermofisher, USA, 1:200 dilution), anti-CD8 (Thermofisher, USA, 1:200 dilution), anti-CD177 (Thermofisher, USA, 1:100 dilution), anti-S100 (Thermofisher, USA, 1:100 dilution), the density of infiltrating lymphocytes in gastric cancer tissues and adjacent tissues was evaluated, the tumor nest and corresponding regions around the tumor were measured at 400 × magnification, and then the number of nucleated lymphocytes in each region was counted manually and the average value was calculated [17].

Bioinformatics analyses

CircRNA–miRNA interactions were predicted using RNAhybrid [19], miRNA-gene interactions were predicted using DIANA-TarBase v8 [20]. GC gene expression data were downloaded from Oncomine (https://www.oncomine.org/) and UALCAN [21]. Linked Omics [22] were used to analyze RAB6 gene expression and functions in GC. The relationships between RAB6 expression, immune system interaction, and tumor-infiltrating immune cells in human GC were analyzed using TISIDB [23] and TIMER [24], respectively.

In vivo assays

Female nude mice (4 weeks old) were purchased from the animal center of Anhui Medical University and randomly divided into three groups: NC-si, circTMC5-si #1, and circTMC5-si #2 (MGC803 and HGC27; $n = 5$ per group). The mice were subcutaneously injected with 100 μL of cell suspension ($2 \times 10^6$ cells) containing either NC-si, circTMC5-si #1, and circTMC5-si #2. Tumor volume and body weight were measured from the 7th day after injection, tumor volume was then checked every three days, while body weight was checked once a week. All mice were sacrificed after 31 days. All animal procedures were approved by the ethics committee of the First Affiliated Hospital of Anhui Medical University.
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Statistical analysis

Statistical analyses were performed using SPSS 23.0 software (SPSS, Chicago, IL, USA), GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA), and R software (v3.6.1). Linear correlation was evaluated using Pearson correlation analysis. Nonlinear correlation was evaluated using Spearman’s rank correlation analysis. Data expressed as the mean ± standard deviation were compared using Student’s t tests or one-way analysis of variance (ANOVA). Categorical variables were analyzed using chi-squared test. Survival was analyzed using the Kaplan–Meier method with Log rank and univariate or multivariate Cox regression tests. Bar graph represents the mean ± SD of three independent experiments. P values < 0.05 were considered statistically significant: *p < 0.05, **p < 0.01, and ***p < 0.001.

Results

CircTMC5 is up-regulated in GC tissues and cell lines

First, we identified circRNAs participating in GC using a circRNA microarray (Biomarker Technologies, Beijing, China). A total of 2,490 circRNAs were identified in GC tissues (n = 3) compared to matched adjacent non-cancerous tissues (n = 3; Fig. 1a,b) and the top 10 up-regulated circRNAs in GC were examined further (Fig. 1c). The expression level of circTMC5 (ID: chr16:19,483,409–19,483,565, hsa_circ_0038320 from Circular RNA Interactome [25]) on circRNA microarray was significantly different (p = 0.0029), and its annotated gene was TMC5. In particular, we confirmed the end-to-end splicing of the circTMC5 cDNA qRT-PCR product using Sanger sequencing and agarose gel electrophoresis (Fig. 1d,e).

Although, circTMC5 has not been reported in GC, we found that circTMC5 was significantly up-regulated in 96 cancer tissues compared to paracancerous tissues (Fig. 1f; p < 0.001) and was highly expressed in the plasma of 139 patients with GC and compared to 116 healthy volunteers using a Roche E601 instrument (Basel, Switzerland) with a cut-off value of 5 ng/mL. ROC curve analysis revealed that the Area Under Curve (AUC) for CEA as a diagnostic marker was 0.910 (sensitivity, 0.863; specificity, 0.793; p < 0.001), whereas circTMC5 had an AUC of 0.821 (sensitivity, 0.619; specificity, 0.922; p < 0.001). However, when CEA and circTMC5 were combined, the AUC was 0.941 (sensitivity, 0.791; specificity, 0.948; p < 0.001; Fig. 1h), suggesting that CEA and circTMC5 could be a combined diagnostic marker for GC.

CircTMC5 displays prognostic value in GC

To analyze the relationship between circTMC5 and the clinicopathological features of GC, we divided patients into two groups based on median circTMC5 expression. Interestingly circTMC5 expression was closely related to histological grade, pathological stage, and T classification (p < 0.001), but not age, gender, alcohol, smoking, CEA levels, lymph node metastasis, Helicobacter pylori (HP), or tumor size (Table 1, p > 0.05). Subsequent Kaplan–Meier survival analysis revealed that higher circTMC5 expression predicted a lower survival rate (Fig. 1i, p = 0.002), while uni- or multivariate Cox regression analyses confirmed that circTMC5 expression, histological grade, and pathological stage were independent prognostic factors in patients with GC (Table 2, p < 0.05), indicating the potential of circTMC5 as a prognostic biomarker in GC.

CircTMC5 affects GC cell proliferation and apoptosis

To examine the function of circTMC5 in GC cells, we detected circTMC5 expression in GC cell lines (Fig. 1g). Since circTMC5 expression was higher in GC cell lines than in normal gastric epithelial cells (p < 0.05), we selected the cell lines with the highest circTMC5 expression (MGC803 and HGC27) for further experiments. Transfecting GC cell lines with circTMC5-si #1 or circTMC5-si #2 significantly reduced circTMC5 expression compared to NC-si (Fig. 1j); therefore, we assessed cell proliferation, finding that silencing circTMC5 significantly inhibited cell proliferation (Fig. 1k,l; p < 0.01).
Moreover, knocking down circTMC5 promoted GC cell apoptosis (Fig. 1m; \( p < 0.01 \)). Furthermore, the expression of apoptosis-related proteins (Bax, caspase-3, and caspase-9) was increased in circTMC5-si-transfected cells compared to the NC-si group (\( p < 0.01 \)), while Bcl-2 expression was down-regulated (Fig. 1n; \( p < 0.01 \)). Interestingly, the protein expression of E-cadherin and vimentin (related to epithelial-mesenchymal transition (EMT)) increased and decreased in the circTMC5-si group, respectively (\( p < 0.05 \)), whereas the expression of the metastasis-related proteins MMP-2 and MMP-9 decreased (Fig. 2a, \( p < 0.01 \)). These findings indicated that TMC5 played a vital role in the progress of GC.

**CircTMC5 sponges miR-361-3p**

Previous RNAhybrid analyses showed that miR-361-3p may interact with circTMC5 in GC [19]; therefore, we detected miR-361-3p in GC and normal cell lines. We found that miR-361-3p expression was lower in GC cell lines than in GES-1 cells (Fig. 2b, \( p < 0.05 \)) and that circTMC5 and miR-361-3p expression were inversely

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**Table 1** Association of circTMC5 expression with clinicopathological features in gastric cancer patients’ tissue samples \([n, n (\%)]\)

| Characteristics                  | \( n = 96 \) | CircTMC5 Higher (\( n = 48 \)) | CircTMC5 Lower (\( n = 48 \)) | \( P \) value |
|----------------------------------|-------------|--------------------------------|-------------------------------|--------------|
| Age (years)                      |             |                                |                               |              |
| \( \geq 60 \)                    | 62          | 33 (34.37%)                    | 29 (30.21%)                   | 0.393        |
| \(< 60 \)                        | 34          | 15 (15.63%)                    | 19 (19.79%)                   |              |
| Gender                           |             |                                |                               |              |
| Male                             | 46          | 22 (22.92%)                    | 24 (25.00%)                   | 0.683        |
| Female                           | 50          | 26 (27.08%)                    | 24 (25.00%)                   |              |
| Alcohol                          |             |                                |                               |              |
| Yes                              | 38          | 18 (18.75%)                    | 20 (20.83%)                   | 0.676        |
| No                               | 58          | 30 (31.25%)                    | 28 (29.17%)                   |              |
| Smoking                          |             |                                |                               |              |
| Yes                              | 40          | 17 (17.71%)                    | 23 (23.96%)                   | 0.214        |
| No                               | 56          | 31 (32.29%)                    | 25 (26.04%)                   |              |
| CEA level                        |             |                                |                               |              |
| 0–5 \( \mu \)g/mL               | 32          | 15 (15.63%)                    | 17 (17.71%)                   | 0.665        |
| > 5 \( \mu \)g/mL               | 64          | 33 (34.37%)                    | 31 (32.29%)                   |              |
| Histological grade               |             |                                |                               |              |
| Well-moderately                  | 58          | 17 (17.71%)                    | 41 (42.71%)                   | <0.001       |
| Poorly -signet                   | 38          | 31 (32.29%)                    | 7 (7.29%)                     |              |
| Pathological stage               |             |                                |                               |              |
| I + II                           | 43          | 11 (11.46%)                    | 32 (33.33%)                   | <0.001       |
| III                              | 53          | 37 (38.54%)                    | 16 (16.67%)                   |              |
| Lymphnode metastasis             |             |                                |                               | 0.378        |
| N0                               | 30          | 13 (13.54%)                    | 17 (17.71%)                   |              |
| N1–N3                            | 66          | 35 (36.46%)                    | 31 (32.29%)                   |              |
| Helicobacter pylori (HP)         |             |                                |                               |              |
| Positive                         | 56          | 25 (26.04%)                    | 31 (32.29%)                   | 0.214        |
| Negative                         | 40          | 23 (23.96%)                    | 17 (17.71%)                   |              |
| T classification                 |             |                                |                               |              |
| T1–T2                            | 47          | 11 (11.46%)                    | 36 (37.50%)                   | <0.001       |
| T3–T4                            | 49          | 37 (38.54%)                    | 12 (12.50%)                   |              |
| Tumor size (cm)                  |             |                                |                               |              |
| \(< 3.5 \)                       | 53          | 30 (31.25%)                    | 23 (23.96%)                   | 0.151        |
| \( \geq 3.5 \)                   | 43          | 18 (18.75%)                    | 25 (26.04%)                   |              |
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Correlated in GC tumor tissue \((n = 96; r = -0.4158, p < 0.0001; \text{Fig. 2c})\). Since miR-361-3p mimic significantly inhibited circTMC5 WT luciferase reporter activity, we mutated the miR-361-3p target site of the circTMC5 sequence (Fig. 2d), which reversed reporter activity inhibition in MGC803 and HGC27 cells (Fig. 2e, \(p < 0.001\)). In addition, two-probe FISH assays revealed that circTMC5 and miR-361-3p co-localized in the cytoplasm of GC cells and that miR-361-3p expression was increased in the circTMC5-si #1 or circTMC5-si #2 group (Fig. 2f, g; \(p < 0.05\)). Together, these findings suggest that circTMC5 acts as a sponge for miR-361-3p.

### MiR-361-3p targets RABL6 in GC

Previous DIANA-TarBase v8 analyses have indicated that miR-361-3p targets RABL6 in GC [20]. Here, we found that RABL6 mRNA expression was higher in GC cell lines than in GES-1 cells (Fig. 2h, \(p < 0.05\)) and that miR-361-3p correlated inversely with RABL6 mRNA expression in GC tumor tissues (Fig. 2i, \(r = -0.3356\)). Therefore, we mutated the RABL6 mRNA target site of the miR-361-3p sequence (Fig. 2j), finding that the mutation abolished miR-361-3p wild-type luciferase reporter plasmid activity inhibition by miR-361-3p mimic (Fig. 2k). When GC cell lines were transfected with miRNA NC and miR-361-3p mimic (Fig. 2l, \(p < 0.05\)), RABL6 mRNA and protein expression were reduced in the miR-361-3p mimic group compared to the miRNA NC group (Fig. 2m,n; \(p < 0.05\)), indicating that miR-361-3p may target RABL6 in GC.

### RABL6 is involved in the immune regulation of GC

After analyzing RABL6 expression in GC and non-tumor tissues using network tools, Oncomine (https://www.oncomine.org/) and UALCAN [21] revealed that RABL6 expression was significantly up-regulated in GC (Fig. 3a,b; \(p < 0.05\)). IHC verification showed that RABL6 was highly expressed in GC tissues (Fig. 3c, \(n = 96; p < 0.001\)).

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### Table 2: Univariate and multivariate COX regression proportional hazards models to predict the overall survival of patients with GC

| Characteristics          | Univariate analysis | Multivariate analysis |
|--------------------------|---------------------|-----------------------|
|                          | HR value (95% CI)   | P value               | HR value (95% CI)   | P value               |
| Gender                   |                     |                       |                     |                       |
| (Male/Female)            | 1.245 (0.698–2.221) | 0.458                 | 0.815 (0.383–1.731) | 0.594                 |
| Age                      |                     |                       |                     |                       |
| (≥ 60/< 60 years)        | 1.131 (0.613–2.088) | 0.694                 | 1.048 (0.528–2.079) | 0.894                 |
| Smoking                  |                     |                       |                     |                       |
| (Yes/No)                 | 1.017 (0.561–1.844) | 0.955                 | 0.834 (0.423–1.647) | 0.602                 |
| Alcohol                  |                     |                       |                     |                       |
| (Yes/No)                 | 1.415 (0.793–2.525) | 0.240                 | 1.617 (0.753–3.471) | 0.218                 |
| CEA level                |                     |                       |                     |                       |
| (> 5 µg/mL /<5 µg/mL)    | 0.710 (0.385–1.307) | 0.271                 | 0.683 (0.353–1.320) | 0.256                 |
| Histological grade       |                     |                       |                     |                       |
| (Poorly -signet/Well-moderately) | 3.327 (1.837–6.025) | <0.001               | 2.569 (1.187–6.094) | 0.015                 |
| Pathological stage       |                     |                       |                     |                       |
| (III/I+II)               | 3.780 (1.990–7.181) | <0.001               | 2.598 (1.250–5.4)   | 0.011                 |
| Helicobacter pylori      |                     |                       |                     |                       |
| (Positive/Negative)      | 0.980 (0.549–1.750) | 0.946                 | 0.889 (0.446–1.772) | 0.737                 |
| Tumor size (cm)          |                     |                       |                     |                       |
| (≥3.5/<3.5)              | 1.254 (0.697–2.2567) | 0.449               | 1.517 (0.795–2.896) | 0.206                 |
| CircTMC5                 |                     |                       |                     |                       |
| (Higher/Lower)           | 5.051 (2.657–9.603) | <0.001               | 2.690 (1.187–6.094) | 0.018                 |
| T classification         |                     |                       |                     |                       |
| (T3–T4/T1–T2)            | 2.007 (1.111–3.628) | 0.021                | –                  | –                     |
| Lymphnode metastasis     |                     |                       |                     |                       |
| (Yes/No)                 | 1.079 (0.580–2.006) | 0.810                | –                  | –                     |

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Linked Omics was also used to analyze the expression of RABL6-related genes in TCGA_STAD (n = 415; Fig. 3d) [22]. Gene Ontology (GO: biological process, cellular component, and molecular function) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were carried out on these genes (FDR < 0.05 and 1000 simulations) using Gene Set Enrichment Analysis (GSEA; Fig. 3e–i). The immunological effect of RABL6 in GC involved IL-6 production (GO:0,032,635, Normalized Enrichment Score (NES) = –1.7521, p < 0.0001), leukocyte activation involved in inflammation (GO:0,002,269, NES = –1.9271, p < 0.0001), leukocyte migration (GO:0,050,900, NES = –1.7145, p < 0.0001), neuroinflammatory response (GO:0,150,076, NES = –1.9301, p < 0.0001), macrophage activation (GO:0,042,116, NES = –1.7378, p < 0.0001), and IL-8 production (GO:0,032,615, NES = –1.7365, p < 0.0001; Fig. 3j).

Further TISIDB analysis [23] showed that RABL6 expression correlated positively with the levels of chemokine (C–C motif) ligand 20, chemokine (C–X–C motif) ligand 1, chemokine (C–X–C motif) ligand 2, chemokine (C–C motif) ligand 3, and chemokine (C–X–C motif) ligand 5, but negatively with chemokine (C–C motif) ligand 2, chemokine (C–C motif) ligand 4, chemokine (C–C motif) ligand 5, chemokine (C–C motif) ligand 11, chemokine (C–C motif) ligand 13, chemokine (C–C motif) ligand 14, chemokine (C–C motif) ligand 17, chemokine (C–C motif) ligand 19, chemokine (C–C motif) ligand 21, chemokine (C–C motif) ligand 22, chemokine (C–C motif) ligand 23, chemokine (C–C motif) ligand 10, chemokine (C–X–C motif) ligand 12, chemokine (C–X–C motif) ligand 13, chemokine (C–X–C motif) ligand 14, chemokine (C–X–C motif) ligand 17, and chemokine (C–Motif) Ligand 2. We found that RABL6 expression correlated positively with chemokine (C–C motif) ligand 14 (CCL14; r = –0.33, p < 0.0001), chemokine (C–C motif) ligand 22 (CCL22; r = –0.2, p < 0.0001) and chemokine (C–X–C motif) ligand 3 (CXCL3; r = 0.166, p = 0.000688). (Fig. 3k, p < 0.05).

IL-6 and IL-8 were involved in leukocyte activation in inflammatory response [26–29], chemokine (C–C motif) ligand 14 (CCL14) was a chemokine involved in leukocyte migration [30], chemokine (C–C motif) ligand 22 (CCL22) was an important chemokine involved in macrophage activation [31, 32], and chemokine (C–X–C motif) ligand 3 (CXCL3) was considered to be a chemokine involved in neuroinflammatory response [33, 34].

In 96 cases of gastric cancer, we found that the expression of circTMC5 was positively correlated with the expression of RABL6 mRNA by qRT-PCR (Fig. 4a, r = 0.247, p = 0.015). Furthermore, we detected the expression of inflammatory cytokines and chemokines (IL-6, IL-8, CCL14, CCL22, CXCL3) in 96 cases of gastric cancer tissues and paracancerous tissues by immunohistochemistry, showing that IL-6, IL-8, CCL22 and CXCL3 were highly expressed in GC tissues, and CCL14 was low expressed in GC tissues (Fig. 4b and Supplementary Fig. 1a; p < 0.05).

In gastric cancer tissues, the relationship between the expression of RABL6 and expression of inflammatory cytokines and chemokines (IL-6, IL-8, CCL14, CCL22, CXCL3) were analyzed by IHC. The results showed that the expression of RABL6 was positively correlated with the expression of inflammatory cytokines and chemokines (IL-6, IL-8, CXCL3) in GC tissues, and negatively correlated with CCL14 and CCL22 expression in GC tissues (Fig. 4c, p < 0.05). These findings verified that RABL6 may be involved in IL-6 production, leukocyte activation involved in inflammation, leukocyte migration, neuroinflammatory response, macrophage activation, and IL-8 production by inflammatory cytokines and chemokines.

We used TIMER [24] to study the association between RABL6 expression and immune infiltrates, finding that RABL6 expression correlated inversely with macrophages, B cells, CD8 + T cells, neutrophils, and dendritic cells infiltration (Fig. 4d, p < 0.05). In 96 cases of gastric cancer, we found that the expression of circTMC5 was positively correlated with the expression of RABL6 mRNA by qRT-PCR (Fig. 4a, n = 96; r = 0.245, p = 0.015). Furthermore, we detected the expression of immune cell markers (CD68, CD20, CD8, CD177, S100) in 96 cases of gastric cancer tissues and paracancerous tissues by immunohistochemistry (Fig. 4e and Supplementary Fig. 1b, n = 96). CD68 was an immune marker for macrophages [35], CD20 was an immune marker for B cells [36], CD8 was an immune marker for CD8 + T cells [37], CD177 was an immune marker for neutrophils [17], and S100 was an immune marker for dendritic cells [38]. The results showed that the expression of RABL6 was negatively correlated with the expression of CD68 + macrophages, CD20 + B cells, CD8 + T cells, CD177 + neutrophils, and S100 + dendritic cells (Fig. 4f, p < 0.05). In addition, the TISIDB database [23] also found a negative correlation between RABL6 and the vast majority of lymphocytes in human GC samples by

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**a**

|       | MGC603 | HGC27 |
|-------|--------|-------|
| MMP-2 (63KD) | ![Image](image1) | ![Image](image2) |
| MMP-9 (73KD) | ![Image](image3) | ![Image](image4) |
| E-cadherin (120KD) | ![Image](image5) | ![Image](image6) |
| Vimentin (54KD) | ![Image](image7) | ![Image](image8) |
| GAPDH (37KD) | ![Image](image9) | ![Image](image10) |

**b**

- Relative expression of miR-361-3p in MGC603 and HGC27.

**c**

- Relative level of miR-361-3p in GC tissues.

**d**

|        | MGC603-NC | MGC603-miR-361-3p | HGC27-NC | HGC27-miR-361-3p |
|--------|-----------|------------------|-----------|------------------|
| circTMC5-NC | 0.1         | 0.2              | 0.1        | 0.2              |
| circTMC5-NC | 0.3         | 0.4              | 0.3        | 0.4              |
| miR-361-3p | 0.5         | 0.6              | 0.5        | 0.6              |
| miR-361-3p | 0.7         | 0.8              | 0.7        | 0.8              |
| RABL6-NC  | 0.9         | 1.0              | 0.9        | 1.0              |
| RABL6-NC  | 0.1         | 0.2              | 0.1        | 0.2              |

**e**

- Luciferase activity of circTMC5-NC and miR-361-3p.

**f**

- DAPI, circTMC5, miR-361-3p, Merge images for MGC603 and HGC27.

**g**

- Relative level of miR-361-3p in MGC603 and HGC27.

**h**

- Relative expression of RABL6 in MGC603 and HGC27.

**i**

- Relative level of miR-361-3p in GC tissues.

**j**

- RABL6-NC | 0.1         | 0.2              | 0.1        | 0.2              |
| RABL6-NC | 0.3         | 0.4              | 0.3        | 0.4              |
| miR-361-3p | 0.5         | 0.6              | 0.5        | 0.6              |
| miR-361-3p | 0.7         | 0.8              | 0.7        | 0.8              |
| RABL6-MUT | 0.9         | 1.0              | 0.9        | 1.0              |
| RABL6-MUT | 0.1         | 0.2              | 0.1        | 0.2              |

**k**

- Luminescence activity of circTMC5-NC and miR-361-3p.

**l**

- Relative level of miR-361-3p in MGC603 and HGC27.

**m**

- Relative RABL6 mRNA level.

- Relative RABL6 protein level.
CircTMC5 regulates GC cell proliferation, migration, invasion, and apoptosis by targeting miR-361-3p/RABL6

To verify the relationship between TMC5 and miR-361-3p/RABL6 in GC, we found that circTMC5 is low expressed in AGS and MKN45 cell lines by qRT-PCR(Fig. 1g), so we selected AGS and MKN45 gastric cancer cell lines to evaluate the effect of circTMC5 overexpression. We detected circTMC5, miR-361-3p, and RABL6 mRNA and protein expression in GC cell lines from the following groups: oe-NC, oe-circTMC5, oe-circTMC5 + miR-361-3p mimic, oe-circTMC5 + miR-361-3p mimic + oe-RABL6 (Fig. 5a, b). Up-regulating circTMC5 expression decreased miR-361-3p expression and increased RABL6 expression, whereas up-regulating circTMC5 with miR-361-3p mimic exerted the opposite effect. Thus, up-regulating circTMC5 may significantly promote RABL6 expression and miR-361-3p mimic may weaken this effect. In addition, up-regulating circTMC5 promoted GC cell proliferation, migration, and invasion while inhibiting apoptosis (Fig. 5c–f), whereas miR-361-3p mimic attenuated these effects. Moreover, RABL6 overexpression partly rescued the inhibitory effect of miR-361-3p mimic on the biological function of circTMC5 in GC cells. In summary, these results indicate that circTMC5 overexpression promotes GC progression via the miR-361-3p/RABL6 pathway.

CircTMC5 affects tumor growth in vivo

Finally, we confirmed our in vitro findings using nude mice infected with NC-si, circTMC5-si #1 and circTMC5-si #2 in MGC803 and HGC27 GC cell lines. Tumor volume and weight were significantly lower in the circTMC5-si group (Fig. 5g–k and Supplementary Fig. 1d–h), indicating that circTMC5-si significantly inhibited GC cell proliferation in vivo. HE staining revealed tumor tissue morphology and TUNEL staining differed significantly between the circTMC5-si #1, circTMC5-si #2 and NC-si groups (Fig. 5l and Supplementary Fig. 1i, p < 0.01). Consistently, circTMC5 and RABL6 mRNA or protein expression were decreased in the circTMC5-si #1 or circTMC5-si #2 group, and miR-361-3p expression were increased (Fig. 5m–n and Supplementary Fig. 1j–k, p < 0.05). The results of the study had verified that down-regulating of circTMC5 expression inhibited tumor growth by regulating the expression of miR-361-3p/RABL6 in vivo.
CircTMCS promotes gastric cancer progression and metastasis by targeting miR-361-3p/RABL6.
circTMC5 and CEA may improve the diagnosis of GC.

Interestingly, we found that the combined detection of the plasma of patients with GC and normal volunteers required for GC, we analyzed circTMC5 expression in tissues. Since non-invasive biomarkers are urgently required for GC, we analyzed circTMC5 expression in the plasma of patients with GC and normal volunteers. Interestingly, we found that the combined detection of circTMC5 and CEA may improve the diagnosis of GC.

When we analyzed the clinicopathological characteristics and circTMC5 expression in patients with GC, we found that circTMC5 expression was related to histological grade, pathological stage, and T classification. Moreover, low circTMC5 expression was associated with a better prognosis in GC and circTMC5 was suggested as an independent prognostic risk factor for GC. Together, these findings indicate that circTMC5 may promote GC and be related to its degree of malignancy.

We also found that silencing circTMC5 inhibited GC cell proliferation and promoted apoptosis. To elucidate the regulatory mechanism of circTMC5 in GC, we performed bioinformatics analyses using RNAhybrid and DIANA-TarBase v8, finding that circTMC5 may interact with miR-361-3p in GC [19], which may also target RABL6 in GC [20]. We further demonstrated that circTMC5 acts as a sponge for miR-361-3p, which targets RABL6 in GC, and verified the relationship between TMC5 and miR-361-3p/RABL6 in GC, finding that circTMC5 regulates GC cell proliferation, migration, invasion, and apoptosis by targeting miR-361-3p/RABL6. The relationship between circTMC5, miR-361-3p and RABL6 were also verified in gastric cancer tissues by qRT-PCR.

RABL6, also known as RBEL1 or C9orf86, is a novel protein in the Ras superfamily [43–45], whose members play important roles in tumorigenesis by promoting tumor growth and invasion and regulating cell cycle progression, proliferation, apoptosis, migration, and survival [44–46]. Here, we found that RABL6 was highly expressed in GC tissues from Oncomine and UALCAn [21], consistent with previous studies that have reported RABL6 overexpression in various human cancers and its association with aggressive disease and poor prognosis [43–45]. GO analysis of the Linked Omics database suggested that RABL6 is involved in immune regulation in GC, as well as being associated with IL-6 production, leukocyte activation in inflammation, leukocyte migration, neuroinflammatory responses, macrophage activation, and IL-8 production [22]. TISIDB database also found correlation between RABL6 and chemokines in human GC samples by Spearman correlation, including chemokines CCL14, CCL22, and CXCL3 [23].

IL-6 and IL-8 were involved in leukocyte activation in inflammatory response [26–29]. CCL14 was a chemokine involved in leukocyte migration [30], and CCL22 was an important chemokine involved in macrophage activation [31, 32], and CXCL3 was considered to be a chemokine involved in neuroinflammatory response [33, 34]. We also verified that the expression...
CircTMC5 promotes gastric cancer progression and metastasis by targeting miR-361-3p/RABL6.
of RABL6 was positively correlated with the expression of inflammatory cytokines and chemokines (IL-6, IL-8, CXCL3) in GC tissues, and negatively correlated with CCL14 and CCL22 expression in GC tissues by IHC. IL-6 and IL-8 have been found to promote tumorigenesis and development [47, 48]. CXCL3 has also been found to promote tumor metastasis and progression [34, 49]. In liver cancer, CCL14 was found to have a tumor suppressor effect by reducing the activation of the Wnt/catenin pathway [50]. CCL22 was found to have an anti-cancer suppressor effect by causing the infiltration of anti-cancer tumor-infiltrating lymphocytes (TIL) into the tumor [51–54]. The tumor microenvironment is the site where tumor cells interact with the host immune system, including the effects of inflammatory factors and chemokines, which can recruit different immune cell subgroups into the tumor microenvironment, which is unique to tumor progression and treatment results influences [55].

Using TIMER [24], we revealed that RABL6 expression correlated negatively with the infiltration of macrophages, B cells, CD8 + T cells, neutrophils, and dendritic cells. We also verified that the expression of RABL6 was negatively correlated with the expression of CD68 + macrophages, CD20 + B cells, CD8 + T cells, CD177 + neutrophils, and S100 + dendritic cells by IHC. Recently, increasing evidence has suggested that tumor immune infiltration is a promising target for tumor therapy [56, 57]. Studies have demonstrated that tumor hypertrophy is an anti-tumor mechanism promoted by T cells, while CD8 + T cells play important roles in tumor immunotherapy; therefore, combining checkpoint blockade with therapies targeting this pathway could represent a potential treatment strategy [58]. B cell, macrophage, CD4 + T cell, and dendritic cell infiltration exert anti-tumor effects and neutrophils play key roles in different aspects of cancer development and progression, emphasizing the relationship between tumor angiogenesis and metastasis [57, 59–62].

TISIDB analysis [23] also showed that RABL6 expression correlated negatively with most lymphocytes in human GC and is expressed differently in different molecular and immune subtypes of GC. Similarly, Kim et al. found that the molecular subtype of GC correlates with immune subtype [63], while previous studies have demonstrated that the molecular and immune subtypes have clinical significance for the prognosis and outcomes of immune targeted therapy in patients with GC [64, 65]. Therefore, we speculate that RABL6-mediated tumor promotion is partially achieved by immune-related regulation, which may be due to circTMC5/miR-361-3p axis regulation; however, the specific mechanism underlying RABL6 immune regulation in GC requires further study. In conclusion, we demonstrate that the circTMC5/miR-361-3p/ RABL6 axis plays a vital role in GC occurrence and development, and can be influenced by the tumor immune microenvironment (Supplementary Fig. 1 l). Future studies should explore the roles of the circTMC5/miR-361-3p/ RABL6 axis in tumor growth and immune infiltration to determine their therapeutic value. Furthermore, we found that RABL6 is involved in the immune regulation of GC, suggesting that novel RABL6-related therapies could be used to treat GC.
CircTMCS promotes gastric cancer progression and metastasis by targeting miR-361-3p/RABL6
Fig. 5 CircTMC5 regulates GC cell proliferation, migration, invasion, and apoptosis by targeting miR-361-3p/RABL6 in vitro and in vivo. a–b Relative circTMC5, miR-361-3p, and RABL6 mRNA or protein expression detected using qRT-PCR or western blotting in the following AGS and MKN45 GC cell line groups: oe-NC, oe-circTMC5, oe-circTMC5+miR-361-3p mimic, and oe-circTMC5+miR-361-3p mimic+oe-RABL6. c–f GC cell proliferation, apoptosis, migration, and invasion were detected in the oe-NC, oe-circTMC5, oe-circTMC5+miR-361-3p mimic, and oe-circTMC5+miR-361-3p mimic+oe-RABL6 groups using CCK-8, Edu, flow cytometry, and Transwell assays in AGS and MKN45 GC cell lines, respectively. g–k Tumor volume and tumor weight were significantly lower in the circTMC5-si #1 or circTMC5-si #2 group than in the NC-si group in vivo (MGC803), but body weight was not statistically significant. l HE staining showing tumor tissue morphology and TUNEL staining showing higher expression in the circTMC5-si #1 or circTMC5-si #2 group than in the NC-si group in vivo (MGC803). m–n circTMC5, miR361-3p, and RABL6 mRNA or protein expression in the circTMC5-si #1 or circTMC5-si #2 and NC-si groups detected using qRT-PCR or western blotting (MGC803).

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Author contributions Peng Xu, XiaoLan Xu, Jie Yao, and AMAn Xu carried out the main work, and contributed equally to the study design, as well as drafting and revision of the manuscript. Peng Xu, Xiao Wu, LiXiang Zhang, and Lei Meng were responsible for clinical specimen collection and experimental operation. XiaoLan Xu, ZhangMing Chen, and WenXiu Han performed the analysis. All authors read and approved of the final manuscript.

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Declarations

Conflict of interest The authors declare that they have no potential conflict of interest.

Ethics approval and consent to participate The study was approved by the ethics committee of the First Affiliated Hospital of Anhui Medical University. Each patient provided signed informed consent.

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