Long non-coding RNA MALAT1 activates autophagy and promotes cell proliferation by downregulating microRNA-204 expression in gastric cancer

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Abstract. Gastric cancer (GC) is one of the major diseases that threaten human health. Although the development of novel drugs has significantly improved the efficacy of GC chemotherapy, the 5-year survival rate of patients with GC remains unsatisfactory. In the present study, the role and mechanism of the long non-coding RNA (lncRNA) metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) in GC proliferation was investigated. Clinical specimens and cancer cells were analyzed by western blotting or immunofluorescence. Reverse transcription-quantitative polymerase chain reaction analysis of 57 paired GC and non-tumorous tissues revealed elevated expression of *MALAT1* in GC tissues compared with controls. In addition, increased *MALAT1* was associated with elevated levels of microtubule-associated protein 1 light chain 3β (LC3B) and antigen Ki67, which are autophagy and proliferation markers, respectively. MTT and colony formation assay results demonstrated that *MALAT1* promoted GC cell proliferation. To the best of our knowledge, the present study was the first to demonstrate that upregulated *MALAT1* was associated with increased autophagy activation in GC tissues. Furthermore, this study reported that *MALAT1* increased cell proliferation and enhanced autophagy activation in GC cells. In addition, the results revealed that *MALAT1* inhibited microRNA (miR)-204 expression in GC cells. The present study also demonstrated that miR-204 repressed autophagy through the downregulation of *LC3B* and transient receptor potential melastatin 3 expression in GC cells. These results indicated that *MALAT1* activated autophagy and promoted cell proliferation by downregulating miR-204 expression in GC.

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Introduction

Gastric cancer (GC) is the fifth most frequent cancer (1,033,701 new cases in 2018) and the third leading cause of cancer-associated mortality 782,685 deaths in 2018) worldwide (1). Incidence rates are the highest in East Asia, particularly in Japan and China (1). Despite comprehensive use of surgical treatment, chemotherapy, radiotherapy and molecular-targeted treatment, the 5-year survival rate of patients with GC remains <20% in China (2-4). Further investigation is therefore required to fully elucidate the molecular mechanisms underlying the progression of GC and to identify novel therapeutic targets.

Autophagy is a highly conserved catabolic process in which cytoplasmic material and organelles are delivered to lysosomes for degradation. Autophagy is a multi-step and complex process that is regulated by ~30 autophagy-associated proteins and multiple signaling pathways, including AMPK, PERK (5,6). Dysregulation of autophagy has been detected in numerous human diseases, including cancer (7). In addition, increasing evidence indicates that autophagy serves a crucial role in tumor cell proliferation and differentiation, and that it affects the efficacy of anti-tumor drugs (8,9). Although a number of studies have investigated autophagy in GC (10-15), the precise role of autophagy in the development of GC remains to be completely identified.

Long non-coding RNAs (lncRNAs) serve crucial roles in various biological processes, including immune responses, angiogenesis, cell proliferation, differentiation, apoptosis and autophagy (16-18). Previous studies have also reported the abnormal expression and roles of lncRNAs in cancer (19-21). Investigation of the tumor-associated molecular mechanisms of lncRNAs may therefore contribute to the prevention and treatment of cancer. Metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) is a lncRNA discovered in 2003 that is closely associated with the clinical outcome and progression of lung cancer (22). *MALAT1* expression has been reported to be significantly upregulated in lung cancer,

hepatocellular carcinoma, bladder cancer and other types of malignancy (23-25). A recent study reported that *MALAT1* may be used as a diagnostic marker of GC metastasis (26). However, the precise mechanism of *MALAT1* in the development of GC remains not fully understood.

MicroRNA (miR)-204 is a well-studied tumor suppressor, which is commonly downregulated in breast and prostate cancer, renal cell carcinoma and GC (27-32). Numerous studies have indicated that miR-204 can repress the development of GC (31,33,34). Furthermore, loss of miR-204 leads to upregulated expression of transient receptor potential melastatin 3 (TRPM3), which stimulates oncogenic autophagy by regulating microtubule-associated protein 1 light chain 3α (MAP1LC3A, also known as LC3A) and LC3B and promotes cancer growth (33,35). However, in cholangiocarcinoma and lung cancer, miR-204 is negatively regulated by *MALAT1* (36,37).

The present study aimed to clarify the association between *MALAT1*, miR-204, and GC progression, and to investigate the underlying molecular mechanism of GC.

Materials and methods

Clinical samples. A total of 57 human GC tissues and corresponding non-cancerous adjacent tissues (5 cm from the edge of tumor) were collected from 18 women and 39 men who received surgery at Jiangyin Hospital Affiliated to Nantong University (Jiangyin, China) between September 2017 and June 2018. The mean age of patients was 56.3 years (age range, 37-72 years). All patient materials were obtained with written informed consent in accordance with the requirements of the Clinical Research Ethics Committees of Jiangyin Hospital Affiliated to Nantong University; The experimental protocols were approved by the Research Ethics Committee in Jiangyin Hospital Affiliated to Nantong University (approval no. JY1608AC97). All methods used in this study were in accordance with the approved guidelines.

Cell lines and transfection. The human normal gastric epithelial cell line GES-1 and the GC cell line MKN45 were purchased from the American Type Culture Collection. The gastric adenocarcinoma-derived circulating tumor cell lines CTC141 and CTC105 (38) were kindly donated by the Laboratory of Stem Cell Biology of Sichuan University (Chengdu, China). All cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Sigma-Aldrich; Merck KGaA) and placed at 37˚C in a humidified incubator containing 5% CO_2. Cells were passaged every 2-3 days.

For the transfection, the small interfering (si)RNA were obtained from GenSript. Sequences for the siRNAs were designed as follows: Negative control siRNA (si-NC), 5'-CUUGCCUGGACCAGCUUAAdTdT-3'; si-*MALAT1*-1, 5'-CAGCCCGAGACTTCTGTAAdTdT-3'; and si-*MALAT2*, 5'-AGCCCGAGACTTCTGTAAAdT dT-3'. The pcDNA vector and *MALAT1* overexpression plasmid (pcDNA-*MALAT1*) were obtained from Nanjing KGI Biological Technology Development Co., Ltd. miR-204 mimic (cat. no. miR10022693-1-5) and mimic control were purchased from Guangzhou RiboBio Co., Ltd. Transcription efficiency was evaluated by quantitative PCR.

CTC 141 and CTC105 cells were transiently transfected with siRNA (100 nM/1x10^5 cells), plasmid (1 μg/1x10^5 cells) or mimic (100 nM/1x10^5 cells) using Lipofectamine® 3000 (12 μl/1x10^5 cells; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Following 48 h transfection, cells were subjected to experiments. The transcription efficiency was evaluated by quantitative real-time PCR.

MTT assay. CTC 141 and CTC105 cell proliferation was measured with MTT assay (Sigma-Aldrich). Cells were seeded into 96-well plates (3x10^3 cells/well) for 24, 48 or 72 h and were treated for 3 h with 2 mM 3-methyladenine (3-MA; Selleck, cat. no. S2767), used as a cell autophagy inhibitor, before assessing cell proliferation. MTT (20 μl) was then added to each well for 4 h at 37˚C. Cell viability was assessed by detecting absorbance at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.). All tests were performed in quadruplicate.

Colony formation assay. Transfected CTC105 cells were plated in a 6-well plate (200 cells/well) and cultured for 14 days under standard culture conditions. Cell culture medium was replaced every 2-3 days. Cells were fixed with methanol for 20 min, and stained with 0.5% crystal violet for 5 min at room temperature. Stained colonies (cells number >30) were imaged using a Nikon camera (Nikon, Tokyo, Japan). Each experiment was performed at least three times.

Immunofluorescence staining. Paraffin-embedded GC tissues were collected and fixed with 10% formalin overnight at room temperature. Section (0.5 μm thick) were dehydrated by increasing ethanol gradient (50, 70, 85, 95 and 100%) at room temperature for 2 h, deparaffinized with dimethylbenzene at room temperature for 15 min and rehydrated by decreasing gradient of ethanol (100, 95, 85 and 75%) for 5 min at room temperature. Tissue sections were incubated in 0.5 M sodium citrate buffer (JRDUN Biotechnology, Co., Ltd.) at 37˚C for 15 min and heated in a microwave at 92-98˚C for 20 min for antigen retrieval. Tissue sections were permeabilized with 0.1% Triton X-100 (Sangon Biotech Co. Ltd.) in PBS for 25 min at room temperature, and blocked with 2% bovine serum albumin (Sigma-Aldrich) in PBS for 1 h at room temperature. Tissue sections were then incubated with anti-LC3B (Cell Signaling Technology, Inc.; cat. no. 83506; 1:200) and anti-Ki67 antibodies (Cell Signaling Technology, Inc.; cat. no. 9449; 1:300) at 4˚C overnight. Tissue sections were then incubated with fluorescein isothiocyanate-labeled secondary anti-mouse antibody (Cell Signaling Technology Europe, B.V.; cat. no. 4410; 1:500) for 2 h at room temperature. Cell nuclei were stained with DAPI (1 μg/ml; Sigma-Aldrich; Merck KGaA) for 5 min at room temperature. Stained sections were observed under a CX41RF fluorescence microscope (Olympus Corporation) with x200 magnification.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). TRIzol® reagent (Takara Biotechnology Co., Ltd.) was used to extract total RNA from gastric tissues and cells. cDNAs were generated using the PrimeScript™ RT reagent kit (Takara Biotechnology Co., Ltd.) according to the

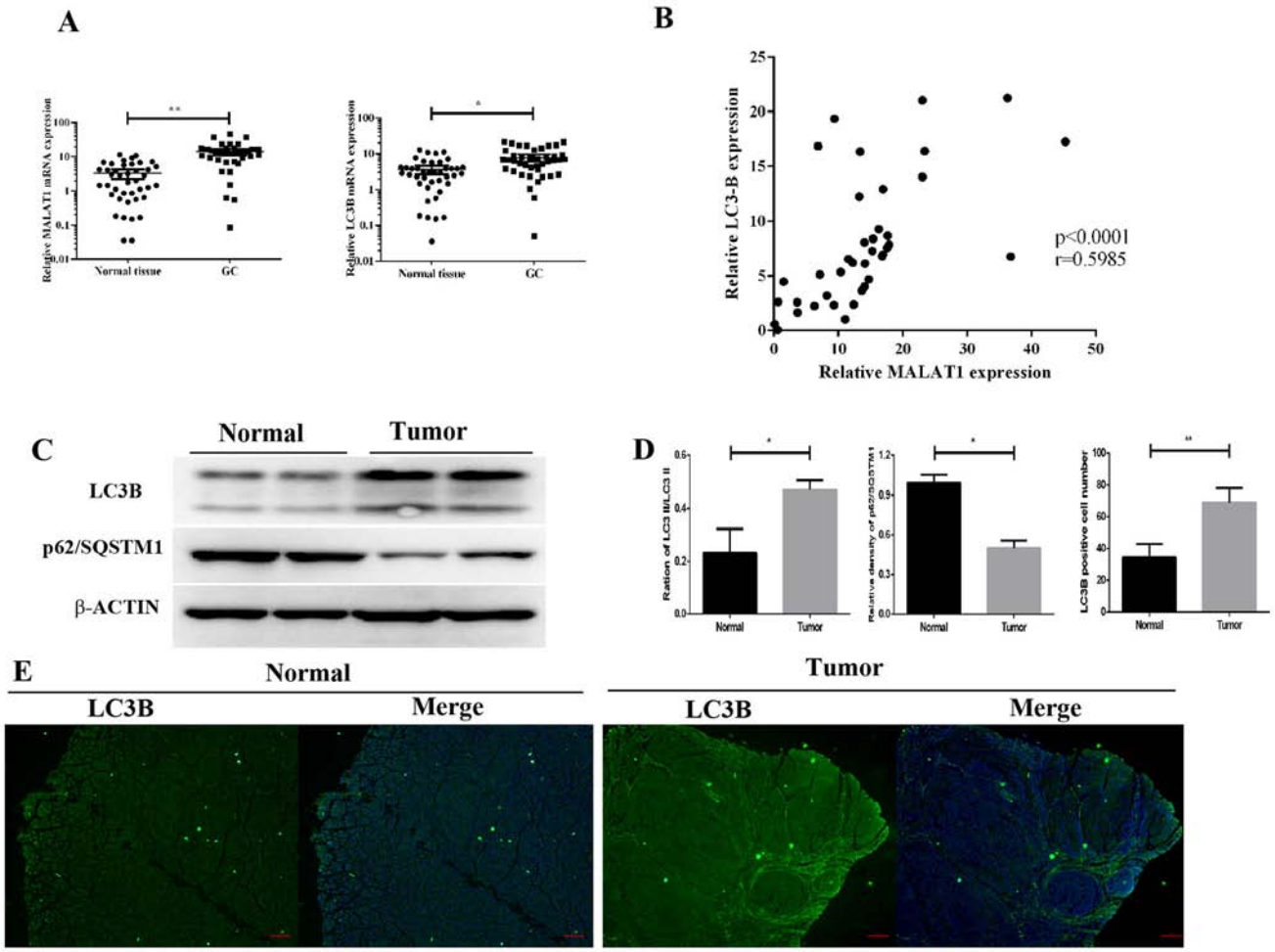

Figure 1. Upregulated *MALAT1* is associated with increased autophagy activation in GC tissues. (A) *MALAT1* expression levels in 57 paired GC tissues measured by reverse transcription-quantitative polymerase chain reaction. (B) Correlation analysis between *MALAT1* and *LC3B* mRNA expression in GC tissues compared with paired non-tumorous tissues. (C) Western blotting of LC3B and p62 protein levels in GC and paired non-tumorous tissues. (D) LC3B, p62 protein level and LC3B immunofluorescence quantification in clinic tissues. (E) Immunofluorescence detection of endogenous LC3B in GC and paired non-tumorous tissues. *P<0.05 and **P<0.01. GC, gastric cancer; *MALAT1*, metastasis-associated lung adenocarcinoma transcript 1.

manufacturer's instructions. SYBR® Premix Ex Taq (Takara Biotechnology Co., Ltd.) was used for qPCR according to the manufacturer's instructions. The thermocycling conditions were as follows: 95˚C for 10 sec, followed by 40 cycles of 95˚C for 5 sec, 60˚C for 15 sec, and 72˚C for 30 sec, the final extension was 72˚C for 5 min. Primer sequences used for qPCR were as follows: *MALAT1* forward, 5'-AGCGGAAGAACGAATGTAAC-3' and reverse, 5'-GAACAGAAGGAAGAGCCAAG-3'; *LC3B* forward, 5'-GATGTCCGACTTATTCGAGAGC-3' and reverse, 5'-TTGAGCTGTAAGCGCCTTCTA-3'; *TRPM3* forward, 5'-ATACCCAGCACCAAAGACC-3' and reverse 5'-TCTGAAGCACGGAGATACTG-3'; and *GAPDH* forward, 5'-TGAACGGGAAGCTCACTGG-3' and reverse, 5'-TCCACCACCCTGTTGCTGTA-3'. The relative expressions levels were normalized to the endogenous control *GAPDH* and calculated using the $2^{-\Delta\Delta Cq}$ method (39).

To detect miRNA-204, reverse transcription and qPCR were performed using a Bulge-Loop™ miRNA qPCR Primer Set for hsa-miR-204 (Guangzhou RiboBio Co., Ltd.) and U6 snRNA (Guangzhou RiboBio Co., Ltd.) according to the manufacturer's instructions and as previously described (40). U6 served as an internal control.

Western blotting. Total cellular proteins from CTC105 and CTC141 cells were extracted using radioimmunoprecipitation assay buffer (Auragene). Protein concentration was determined with bicinchoninic acid assay (Thermo Fisher Scientific, Inc.). Proteins (20 μg) were separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% skimmed milk at room temperature for 1 h and incubated with primary antibodies against p62 (cat. no. 39749; 1:2,000; Cell Signaling Technology, Inc.), LC3B (cat. no. 3868; 1:1,000; Cell Signaling Technology, Inc.), Ki67 (cat. no. 13110; 1:5,000; Cell Signaling Technology, Inc.), β-actin (cat. no. 4970; 1:1,000; Cell Signaling Technology, Inc.) and TRPM3 (cat. no. ab56171; 1:1,000; Abcam) at room temperature for 2 h. Bands were detected using enhanced chemiluminescence substrate (Applygen Technologies, Inc.) according the manufacturer's protocol. Protein quantification was performed by ImageJ software (National Institutes of Health).

Statistical analysis. Data are presented as the mean ± standard deviation. SPSS 17.0 statistical software (SPSS, Inc.) was used for statistical analyses. Comparison between groups was

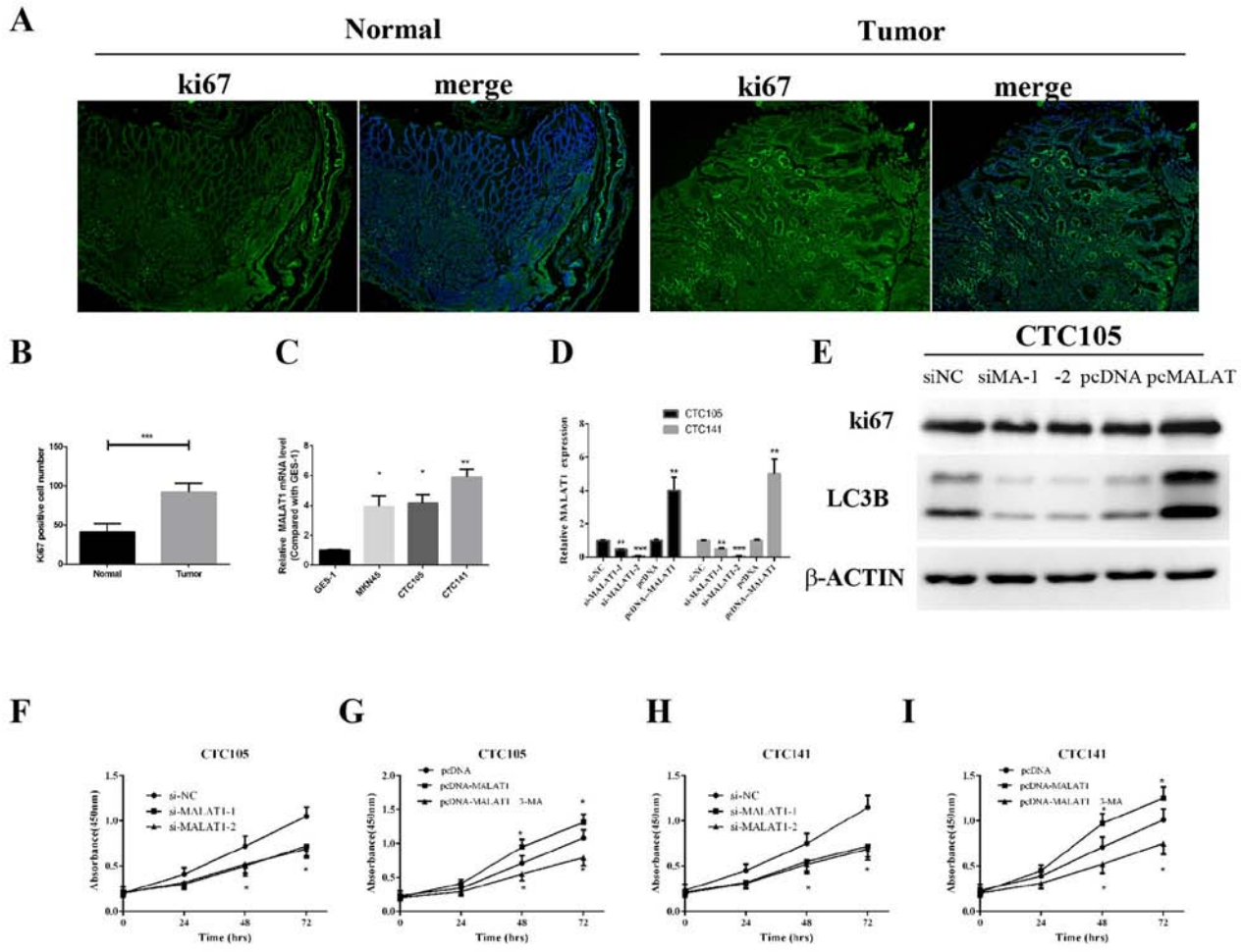

Figure 2. *MALAT1* promotes GC cell proliferation. (A and B) Immunofluorescence detection of Ki-67 in GC and non-tumorous tissues. (C) *MALAT1* expression levels in the normal human gastric epithelial cell line GES-1 and human GC cell lines MKN45, CTC105 and CTC141 were measured by RT-qPCR. (D and E) CTC105 and CTC141 cell lines were transfected with the *MALAT1* overexpression vector, *MALAT1* siRNAs or the indicated controls and examined by (D) RT-qPCR and (E) western blotting. (F-I) CTC141 and CTC105 cells were transfected with (F and H) *MALAT1* siRNAs, (H and I) the *MALAT1* overexpression vector or the indicated controls, and MTT assays were performed at 0, 24, 48 and 72 h, with or without 3-MA (2 mM, 3 h). *P<0.05, **P<0.01 and ***P<0.001 vs. the corresponding control. MA, *MALAT1*; *MALAT1*, metastasis-associated lung adenocarcinoma transcript 1; NC, negative control; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; si, small interfering.

performed with paired Student's t-test or analysis of variance followed by Holm-Sidak's or Dunnett's multiple comparisons test (GraphPad Software, Inc.). The expression correlation was analyzed using Pearson's correlation test. P<0.05 was considered to indicate a statistically significant difference.

Results

MALAT1 is associated with autophagy activation in GC tissues. To examine the role of *MALAT1* in GC and to identify potential molecular events, RT-qPCR was used to detect *MALAT1* expression levels in 57 GC and paired non-tumorous tissues. The results demonstrated that *MALAT1* expression was significantly increased in GC tissues compared with paired non-tumorous tissues (P<0.01; Fig. 1A). As a structural protein of autophagosome membranes (41), *LC3B* mRNA level was upregulated in GC tumors (P<0.05; Fig. 1A). Furthermore, the mRNA level of *LC3B* was positively correlated with *MALAT1*. As a marker of autophagy, the ratio of LC3-II/LC3-I was also increased in GC tissues compared with the control group (Fig. 1C and D). The results of the western blotting analysis demonstrated that p62 expression was lower in GC tissues compared with the control group (Fig. 1C and D). In addition, fluorescence microscopy revealed that the number of FITC-LC3 puncta was higher in GC tissues compared with controls (Fig. 1D and E). These results demonstrated that upregulated *MALAT1* levels were associated with increased expression of autophagy markers in GC tissues.

MALAT1 enhances GC cell proliferation. To examine whether *MALAT1* may be associated with GC cell proliferation, Ki67 expression levels were assessed in clinical specimens by immunofluorescence analysis. Ki67 expression levels in GC tissues were demonstrated to be higher compared with the control group (Fig. 2A and B), which indicated increased proliferation in GC samples.

MALAT1 expression levels in GC cells lines were evaluated, and RT-qPCR results demonstrated that *MALAT1* expression was significantly upregulated in CTC105 and CTC141 cells compared with normal gastric epithelial GES-1 cells (Fig. 2C). To evaluate the function of *MALAT1* in GC-derived circulating cells, *MALAT1* overexpression plasmid and siRNAs targeting *MALAT1* were used. *MALAT1* mRNA expression increase in

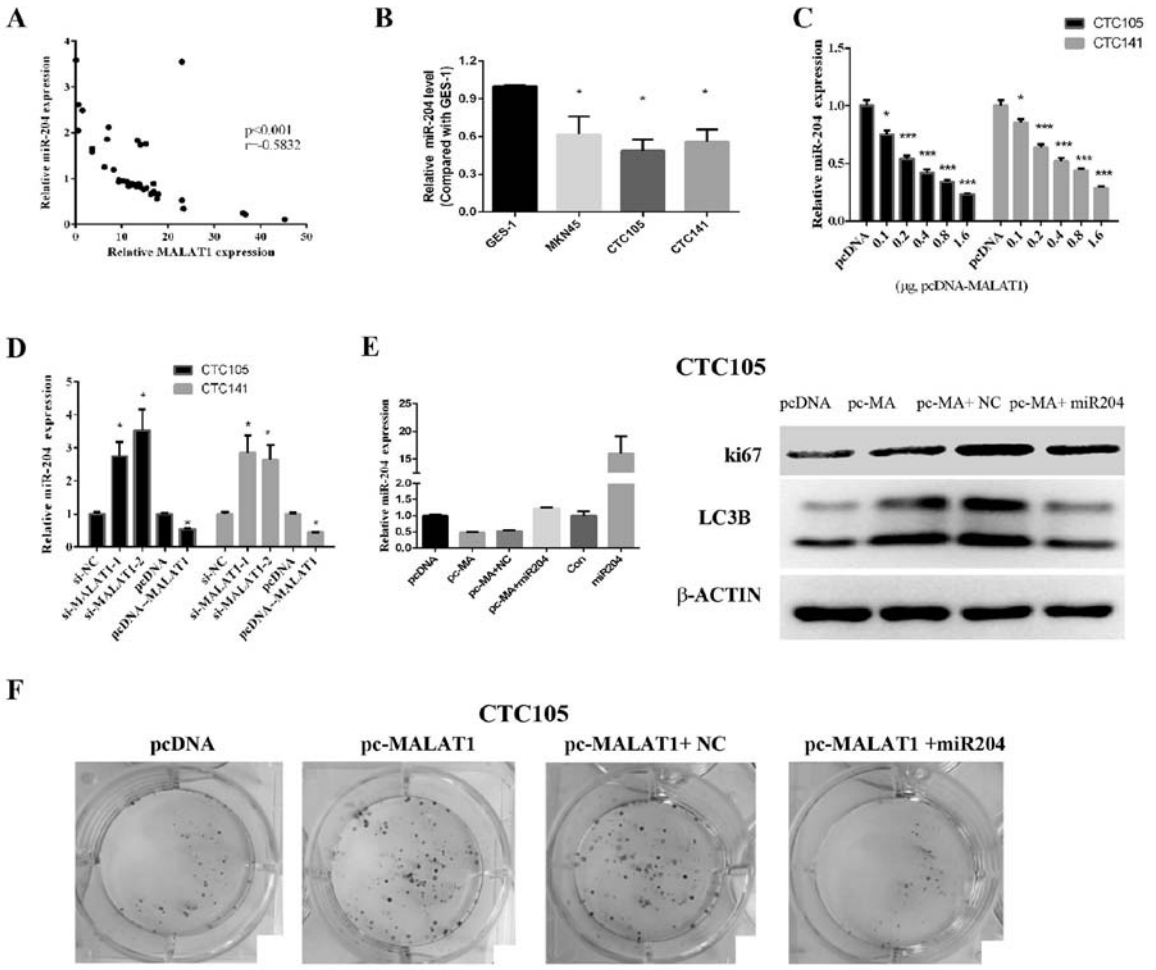

Figure 3. *MALAT1* regulates miR-204 levels in GC cells. (A) *MALAT1* expression was negatively correlated with miR-204 expression in GC tissues. (B) RT-qPCR analysis of miR-204 expression in GC cells compared with GES-1 cells. (C and D) miR-204 levels were detected in GC cell lines transfected with (C) *MALAT1* overexpression vector, (D) siRNAs, or the appropriate controls as indicated. (E) After the miR-204 expression was detected by RT-qPCR, western blot analysis of LC3B and Ki67 was performed in CTC105 cells transfected with the MALAT1 overexpression vector or miR-204 mimic as indicated. (F) Colony formation assay of CTC105 cells transfected with the *MALAT1* overexpression vector or miR-204 mimic as indicated. *P<0.05 and ***P<0.001 vs. the corresponding control. MA, *MALAT1*; *MALAT1*, metastasis-associated lung adenocarcinoma transcript 1; miR-204, microRNA-204; NC, negative control; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

cells transfected with the *MALAT1* overexpression plasmid and decrease in si-*MALAT1*- and si-*MALAT2*-transfected cells were confirmed (Fig. 2D). Similar results were obtained by western blotting in CTC105 cells (Fig. 2E).

MALAT1 downregulation significantly decreased CTC105 and CTC141 cell proliferation compared with controls (Fig. 2F and H), whereas upregulated *MALAT1* enhanced CTC105 and CTC141 cell proliferation, and the increased proliferation was inhibited by the autophagy inhibitor, 3-methyladenine (3-MA) (Fig. 2G and I). These results demonstrated that *MALAT1* may increase GC cell proliferation through autophagy.

MALAT1 regulates miR-204 in GC. A previous study reported that *MALAT1* exerted its effects by binding and inhibiting miR-204 (36). The results from the present study demonstrated that miR-204 and *MALAT1* expression levels were negatively correlated in GC tissues (Fig. 3A), which suggested that *MALAT1* may inhibit miR-204 expression in GC. In addition, miR-204 expression levels in CTC105 and CTC141 cells were significantly lower compared with those in GES-1 cells (Fig. 3B), and the levels or miR-204 decreased following *MALAT1* overexpression (Fig. 3C). Furthermore, following *MALAT1* downregulation in CTC105 and CTC141 cells, miR-204 expression level was increased, whereas miR-204 expression level was decreased in CTC105 and CTC141 cells overexpressing *MALAT1* (Fig. 3D).

Furthermore, *MALAT1* overexpression increased LC3B and Ki67 expression in CTC105 cells, which was abolished following miR-204 mimic transfection (Fig. 3E). In addition, colony formation assays indicated that *MALAT1* promoted GC cell proliferation and that miR-204 rescued the effect of *MALAT1* on cancer cells (Fig. 3F). These results suggested that *MALAT1* may induce miR-204 downregulation and that *MALAT1*-induced cell proliferation and autophagy marker upregulation was blocked by miR-204. These results suggested that *MALAT1* may work by negatively regulating miR-204.

miR-204 inhibits MALAT1-induced regulation of LC3B and TRPM3 in GC. The regulatory effects of *MALAT1* and miR-204 on *TRPM3*, which is an autophagy activator, were evaluated. The results demonstrated that *MALAT1* increased *LC3B* and *TRPM3* mRNA expression levels, whereas miR-204

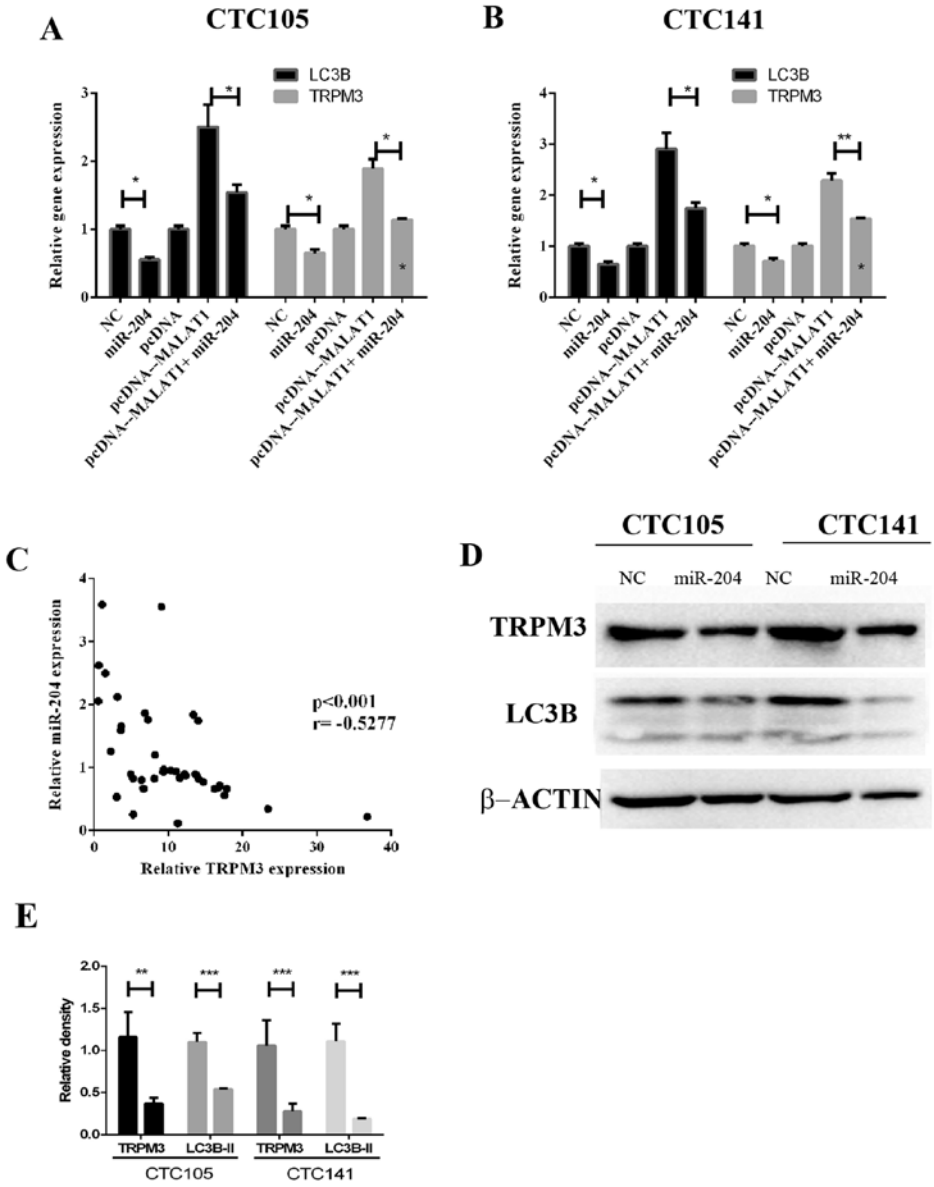

Figure 4. miR-204 reverses *MALAT1*-induced regulation of *LC3B* and *TRPM3* in GC. (A and B) Reverse transcription-quantitative polymerase chain reaction analysis of *LC3B* and *TRPM3* mRNAs in (A) CTC105 and (B) CTC141 cells transfected as indicated. (C) Correlation analysis of miR-204 and TRPM3 expression in GC tissues. (D) LC3B and TRPM3 western blotting in GC cells transfected with miR-204. (E) LC3B and TRMP3 level quantification. *P<0.05, **P<0.01 and ***P<0.001. *MALAT1*, metastasis-associated lung adenocarcinoma transcript 1; MA, *MALAT1*; *miR-204*, microRNA-204; NC, negative control; TRPM3, transient receptor potential melastatin 3.

co-expression eliminated these effects in CTC105 (Fig. 4A) and CTC141 (Fig. 4B) cell lines. In addition, *TRPM3* and miR-204 expression levels were negatively correlated GC tissues (Fig. 4C). Western blotting demonstrated that LC3B and TRPM3 protein levels were significantly decreased in CTC105 and CTC141 cells transfected with the miR-204 mimic (Fig. 4D and E).

Discussion

Numerous studies have demonstrated that lncRNAs serve crucial roles in the development of various types of cancer (16-21). However, the function of lncRNAs in GC remains unclear. Identification of cancer-associated lncRNAs and their targets is therefore critical for understanding their roles in tumorigenesis and for the development of novel targets for GC therapy. The present study investigated the role of lncRNA *MALAT1* in GC and its potential underlying mechanisms.

MALAT1 has recently been proposed as a marker of GC (26). Previous studies have reported that *MALAT1* promotes GC cell proliferation and metastasis (42,43). In addition, *MALAT1* serves a crucial role in the regulation of autophagy, and its overexpression is associated with chemoresistance (44). Although these studies indicate that *MALAT1* is involved in GC progression and autophagy-associated chemoresistance, the correlations between *MALAT1*, autophagy and proliferation *in vivo* remain unclear.

In the present study, the correlation between *MALAT1* expression levels and autophagy activation in GC cell lines and tissues and the association between *MALAT1* expression and GC proliferation were examined. The results demonstrated that *MALAT1* upregulation was associated with

increased autophagy in GC tissues. *MALAT1* expression levels were significantly increased in GC tissues, and *LC3B* expression was proportional to the level of *MALAT1*. In addition, expression levels of LC3B were also significantly higher in GC tissues compared with non-tumor tissues. In the pcDNA negative control group, LC3B was downregulated compared with the siNC group, which may be due to endotoxin effect following plasmid extraction that may have affected cell autophagy (45). However, this did not affect our conclusion.

However, p62 expression was lower in GC tissues compared with the control group, p62, which is a scaffolding/adaptor protein, is involved in numerous physiological processes, including inflammation, mitosis and autophagy and is a crucial factor during tumorigenesis (46,47). These findings demonstrated that upregulated *MALAT1* was associated with increased autophagy activation in GC. The results observed in GC cell lines were consistent with these findings. In addition, the present study demonstrated that *MALAT1* overexpression increased GC cell proliferation.

As a target of *MALAT1*, miR-204 prevents tumor development (33,36,37,48,49) and regulates *TRPM3*-induced oncogenic autophagy (50,51). The results of the present study revealed that the miR-204 mimic reversed the effects of *MALAT1* overexpression on *LC3B* and *TRPM3* in GC cells, which indicated that *MALAT1* may exert its roles in GC by negatively regulating miR-204. Furthermore, the results suggested that miR-204 may regulate the expression of the autophagy markers LC3B and TRPM3 in GC cells. The results of the present study demonstrated that *MALAT1* overexpression resulted in decreased miR-204 levels, and that *MALAT1* and miR-204 expression levels were negatively correlated in GC tissues, which further indicated that *MALAT1* may negatively regulate miR-204 in GC cells. Although the *MALAT1*/miR-204/*LC3B* axis has been reported to regulate autophagy in myocardial ischemia-reperfusion injury (52), the effect of the microenvironment on cell autophagy through this axis remains unclear. Preliminary data from a mouse model of GC indicated that this axis was involved in *Helicobacter pylori*-induced GC and regulated the infection by causing autophagy (data not shown). Identifying how the *MALAT1*/miR-204/*LC3B* axis regulates the *H. pylori*-induced infection and tumorigenesis through autophagy will be the aim of our further study.

The present study explored the molecular mechanism of *MALAT1* in the occurrence and development of GC. The results may provide potential novel targets for the development of molecular targeted therapy of GC.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

GS, ZZ and WZ analyzed and interpreted the patient data. GS, ZZ and GH performed the cellular and molecular experiments, LZ, WL, CX and XZ analyzed the data. WZ and CX were major contributors in writing the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of Jiangyin Hospital Affiliated to Nantong University (approval no. JY1608AC97). All patients provided written informed consent for the present study.

Patients consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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