Silencing of circRACGAP1 sensitizes gastric cancer cells to apatinib via modulating autophagy by targeting miR-3657 and ATG7

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Abstract
The positive results of the apatinib phase III trial have cast new light on treatment for patients with advanced gastric cancer (GC). However, in terms of safety, apatinib toxicities may lead to a dose modification or treatment interruption. Therefore, proper intervention is urgently needed to help patients benefit from apatinib treatment. In this study, we found that apatinib promoted autophagy activation via upregulation of ATG7 expression and autophagy inhibition enhanced apatinib-induced apoptosis. With microRNA and circular RNA-sequencing analyses of GC xenograft models, we demonstrated that circRACGAP1 functioned as an endogenous sponge for miR-3657 to inhibit its activity and further upregulate ATG7 expression. Silencing of circRACGAP1 inhibited apatinib-induced autophagy, which was rescued by miR-3657. Moreover, knockdown of circRACGAP1 sensitized GC cells to apatinib via autophagy inhibition in vitro and in vivo. These findings provided the first evidence that the circRACGAP1-miR-3657-ATG7 axis mediates a novel regulatory pathway critical for the regulation of apatinib sensitivity in GC. Thus, specific blockade of circRACGAP1 may be a potential therapeutic strategy to reduce the toxicities of apatinib and enhance its therapeutic effect in human GC.

Introduction
Gastric cancer (GC) is the fifth most common cancer and the third leading cause of cancer-related deaths worldwide1. The morbidity of GC have already risen to the second place in China2. Despite certain advances in chemotherapy regimens and targeted therapies3, the 5-year survival of patients with advanced GC is disappointing4. Thus, further studies are needed to identify predictive biomarkers for selecting appropriate and effective treatments for patients with advanced GC.

Up to now, the results of most phase III studies of targeted therapies for advanced GC have been unsatisfactory, except for ToGA5, RAINBOW6, REGARD7, and the phase III trial of apatinib8. Apatinib is a novel and highly selectively inhibitor of the vascular endothelial growth factor receptor-2 (VEGFR-2) tyrosine kinase9 and has shown antitumor effects in various tumors10,11. Li et al.8 reported that apatinib treatment significantly improved overall survival and progression-free survival in patients with advanced GC refractory to two or more lines of previous systemic chemotherapies. However, a dose reduction is required to prevent apatinib toxicity such as grade 3–4 hand–foot skin reaction, grade 3–4 hypertension, and proteinuria (8.5%, 4.5%, and 2.3%, respectively)8,12. Therefore, certain measures, such as...
co-treatment, should be taken to reduce toxicity and help patients benefit from apatinib treatment.

The relationship between autophagy and apoptosis is complex. Autophagy can either promote or inhibit apoptosis under certain circumstances\(^1\), the mechanisms of which are related to the degradation of different pro-apoptotic or anti-apoptotic regulators by autophagy\(^2\). The role of autophagy in protecting cells from undergoing programmed cell death explains why the inhibition of autophagy has been shown to improve the response to other agents in some clinical trials\(^3\). In addition, concurrent autophagy inhibition has been reported to overcome the resistance of some tyrosine kinase inhibitors in human lung cancer, thyroid cancer, and bladder cancer\(^4\). Thus, our study was aimed at the potential role of autophagy inhibition in the co-treatment with apatinib.

Circular RNAs (circRNAs) were first discovered nearly 40 years ago\(^5\). Recently, with the development of deep RNA-sequencing technology, a growing number of studies have demonstrated that circRNAs are involved in biological processes and disease\(^6,7\). circRNAs are characterized by a covalently closed loop structure with the 3′-end of the RNA joined to the 5′-end\(^8,9\). circRNAs are more stable than their linear counterparts and the expression of circRNAs does not often correlate well with the expression of linear forms of the host genes\(^10\). Some studies have reported that circRNAs regulate cell growth, migration, invasion, and tumorigenesis\(^11,12\). In addition, circRNAs play significant role in autophagy\(^13\). Most of the circRNAs reported so far contain exons and can act as microRNA (miRNA) sponges to regulate gene expression\(^14,15\). However, the characterization and function of circRNAs in human cancer remain largely unknown.

In this study, we demonstrated for the first time that apatinib promoted autophagy activation via upregulation of autophagy-related gene 7 (ATG7) expression, whereas autophagy inhibition enhanced apatinib-induced apoptosis in human GC. miRNA sequencing (miRNA-seq) and circRNA sequencing (circRNA-seq) of the GC tumor xenografts from the control and apatinib groups were performed to further study the mechanism. The downregulated miRNAs of the apatinib group identified by miRNA-seq were further validated by quantitative reverse-transcriptase PCR (qRT-PCR). Our results demonstrated that dysregulated miR-3657 might contribute to apatinib-induced ATG7 upregulation. With circRNA-seq and bioinformatics analyses, we demonstrated that circRACGAP1 might act as an endogenous sponge for miR-3657 to inhibit its activity. Moreover, under apatinib treatment, circRACGAP1 was upregulated and triggered autophagy via decreasing miR-3657 and increasing ATG7 levels in GC cells and xenografts. Furthermore, silencing of circRACGAP1 inhibited autophagy and promoted apatinib-induced apoptosis in vitro and in vivo. These findings provided the first evidence that the circRACGAP1-miR-3657-ATG7 axis mediates a regulatory pathway critical for the regulation of autophagy and apatinib sensitivity in GC. In addition, the correlation analysis among the expression of circRACGAP1, miR-3657, and ATG7 in GC patients verified the in vitro and in vivo results. Thus, specific blockade of circRACGAP1 could be a potential therapeutic target for autophagy inhibition in the context of apatinib use in GC.

**Methods**

**Cell lines and culture**

The human GC cell lines BGC-823 and HGC-27 were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 medium (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were incubated in a humidified atmosphere under 5% CO₂ at 37 °C.

**Drug preparations and reagents**

Apatinib (Selleck Chemicals, Houston, TX, USA) was dissolved in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St Louis, MO, USA) and then diluted with culture medium to the desired concentrations. DMSO added in the treatment group was equal to that in the control group with a final DMSO concentration <0.2% (v/v). Chloroquine were purchased from Sigma-Aldrich (St Louis, MO, USA).

**Plasmids and transfections**

The siRNAs specific for ATG7 and circRACGAP1, miR-3567 mimics, and miR-3567 inhibitors were synthesized by RiboBio (Guangzhou, China). The mRFp-GFP-LC3 plasmid was used to monitor autophagy flux as previously reported\(^16\). ATG7 plasmid and pcDNA3.1 plasmid were purchased from HanBio (Shanghai, China). Transfections were performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) or DharmaFECT 4 (Thermo Scientific, Lafayette, CO, USA), according to the manufacturer’s protocol.

**Clonogenic assay**

BGC-823 cells or HGC-27 cells were seeded in 6-well plates (300 cells per well) and incubated overnight. Then, the cells were treated with apatinib at indicated concentrations for 24 h and further cultured in no-drug medium for 2 weeks. For colony scoring, the cells were stained with crystal violet (Beyotime Biotechnology, Nantong, China).

**Cytotoxicity assay and apoptosis assay**

The cells were seeded at 5000 cells per well in 96-well plates and incubated overnight. After a particular
treatment, the cell viability was determined using Cell Counting Kit-8 (Dojindo, Japan), according to the manufacturer’s instructions. The cell survival rates are expressed as the means ± SD from three independent experiments.

Apoptosis was examined by flow cytometric analysis. The cells were treated with certain concentrations of apatinib for the indicated durations. Both floating and adherent cells were collected, stained with Annexin V–fluorescein isothiocyanate (FITC), and propidium iodide (Dojindo, Kumamoto, Japan), and further analyzed with a flow cytometer (FACScan, BD Biosciences, San Jose, CA, USA) equipped with Cell Quest software (BD Biosciences). Apoptosis was also determined using the TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) apoptotic cell detection kit (Roche, Basel, Switzerland), according to the manufacturer’s instructions. Apoptosis was expressed as the mean ± SD from three independent experiments.

Xenografts in mice
Female nude mice (6 weeks old) were purchased from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China) and maintained under specific pathogen-free conditions. The tumor xenograft models were conducted in nude mice bearing BGC-823 cells (model 1) or BGC-823 cells stably transfected with circRACGAP1 short hairpin RNA (shRNA) or control shRNA lentivirus (ViGene Biosciences, Rockville, MD, USA) (model 2). In total, 4 × 10⁶ BGC-823 cells were subcutaneously injected into the right axilla of nude mice. When palpable tumors formed, the mice were separately randomized into each group. Then the mice were orally administered control vehicle or 50 mg/kg apatinib daily. When palpable tumors formed, the mice were separately randomized into each group. Then the mice were washed with phosphate-buffered saline (PBS) and left in the dark before fluorescence in situ hybridization analysis. Confocal images of cells were sequentially acquired with Zeiss AIM software on a Zeiss LSM 700 confocal microscope system (Carl Zeiss Jena, Oberkochen, Germany). The autophagosomes are shown as yellow dots (RFP+GFP+), whereas the autolysosomes are shown as red dots (RFP−GFP−).

Quantitative reverse-transcriptase PCR
Total RNA was extracted from cell cultures by using the Trizol reagent (Gibco Life Technologies, Grand Island, NY, USA), according to the manufacturer’s instructions. The cDNA was amplified with the primers listed in Supplementary Table S1. For miRNA quantification, Bulge-loopTM miRNA qRT-PCR Primer Sets (one RT primer and a pair of qPCR primers for each set) specific for miR-3657 and U6 were designed by RiboBio. Divergent primers were used for circRNAs to detect backsplice junctions and convergent primers were used for linear mRNAs. The qRT-PCR analysis was performed using AceQ qPCR SYBR Green Master Mix or miRNA Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Piscataway, NJ, USA) in an ABI Prism 7900 Sequence detection system (Applied Biosystems, Canada). The following thermal cycling conditions were used: 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s and 60 °C for 1 min. Before calculation using the △△Ct method, the levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and 18S RNA were used to normalize the relative expression levels of mRNA and circRNA, and the levels of small nuclear U6 were used to normalize the miRNA expression levels. Student’s t-test was performed to assess the differences between different groups.

Western blotting, immunohistochemical staining, and antibodies
Western blotting and immunohistochemical staining were performed as previously described. The antibodies used were as follows: monoclonal anti-GAPDH (loading control) (1:1000, Beyotime, Haimen, Jiangsu, China) and monoclonal anti-LC3B, SQSTM1, ATG7, and VEGFR-2 (1: 1000, Cell Signaling Technology, Danvers, MA, USA).

Transmission electron microscopy
For the electron microscopy observation, the tumor xenograft tissues or tumor cells were fixed in 2.5% glutaraldehyde immediately after removal. Samples were fixed using 1% osmium tetroxide, followed by dehydration.
with an increasing concentration gradient of ethanol and propylene oxide. Samples were then embedded in TAAB Epon (Marivac Canada, Inc., St Laurent, Canada), cut into 50 nm sections, and stained with 3% uranyl acetate and lead citrate. Images were acquired using a JEM-1010 electron microscope (JEOL, Tokyo, Japan).

miRNA-seq and circRNA-seq

Following RNA isolation, the RNA was quantified (Qubit RNA Assay Kit in Qubit® 3.0 Fluorometer; Life Technologies, CA, USA) and the quality was assessed (RNA Nano 6000 Assay Kit of the BioAnalyzer 2100 system; Agilent Technologies, CA, USA). Next, 3 μg total RNA (miRNA-seq) and 1 μg qualified RNA (circRNA-seq) per sample were used as input material for each library preparation. The library preparations were sequenced on an Illumina Hiseq 2500 platform with a 50 bp single-end module (miRNA-seq) and an Illumina Hiseq X Ten platform with a 150 bp paired-end module (circRNA-seq). Then, the raw reads were filtered for subsequent analysis. CircRNA prediction was performed with circRNA Finder33 (https://github.com/bioxfu/circRNAFinder). The miRNA target genes and the circRNA–miRNA interactions were predicted using TargetScan34 (http://www.targetscan.org/) and miRanda35 (http://www.microrna.org/), and the intersection or union of target genes were treated as the final target genes.

Sanger sequencing

Sanger sequencing was applied to determine the full length of the amplification products. The divergent primers (Sangon Biotech, Shanghai, China) were designed to confirm the backsplice junction of circRACGAP1. The distinct product of the expected size was amplified by outward-facing primers of circRACGAP1 and was confirmed by Sanger sequencing (Tsingke, Nanjing, China).

RNA immunoprecipitation

The MagnaRIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, MA, USA) was used for RNA immunoprecipitation (RIP) assay, according to the manufacturer’s instructions. The cell lysates were incubated with beads coated with 5 μg antibody against Argonaute-2 (AGO2) (Abcam, MA, USA) or control rabbit IgG with rotation at 4 °C overnight. Then, total RNA was retrieved and the expression of circRNAs and miRNAs was detected by qRT-PCR analysis.

Dual-luciferase reporter assay

The luciferase reporter plasmids encoding wild-type genes (ATG7 3’UTR-Wt, circRACGAP1 Wt) and mutated ones (ATG7 3’UTR-Mut, circRACGAP1 Mut) were produced by Geneseed Biotech (Guangzhou, China). The binding sites of ATG7 and circRACGAP1 were inserted into the Xhol and NotI sites of the psiCHECK2.0 plasmid (Promega, Madison, WI, USA) in the dual-luciferase reporter assay. First, cells were plated on 24-well plates. Then, 500 ng luciferase reporter plasmid, 50 nM miR-3657 mimics and negative control were transfected into cells by applying Lipofectamine 3000. Cells were collected and analyzed following the manufacturer’s instructions by using the dual-luciferase assay (Promega, Madison, WI, USA) after 48 h of transfection. All experiments were repeated three times independently.

Tissue collection

We obtained ten GC tissues from patients who were diagnosed with GC based on histopathological evaluation and underwent surgery at the First Affiliated Hospital of Nanjing Medical University. The study was approved by the Ethics Committee on Human Research of the First Affiliated Hospital of Nanjing Medical University and written informed consent was obtained from all patients.

Statistical analysis

Data are expressed as the means ± SD. The statistical significance of the differences between the cell lines was analyzed by the parametric unpaired Student’s t-test (two-sided). The correlation of the expressions of circRACGAP1, miR-3657, and ATG7 were established by Pearson’s correlation analysis. Differences were considered statistically significant when P < 0.05.

Results

Apatinib inhibits the growth and promotes the apoptosis of GC cells in vitro and in vivo

Colony-formation assays of BGC-823 cells and HGC-27 cells were conducted after 24 h of apatinib treatment (Fig. 1a, b). The results demonstrated that apatinib inhibited the proliferation of the two cell lines in a concentration-dependent manner. Flow cytometry assays confirmed the apoptosis induced by apatinib in BGC-823 cells and HGC-27 cells (Fig. 1c, d).

To evaluate the effectiveness of apatinib in vivo, we established tumor xenograft models in nude mice using BGC-823 cells. The mice were orally treated with control vehicle or 50 mg/kg apatinib daily for 14 days. The results showed that apatinib reduced the tumor volumes in the GC xenograft model (Fig. 1e–g). More necrosis was found in tumor sections of apatinib-treated mice compared with those of vehicle-treated mice by histological examination. In addition, immunohistochemical staining showed increased caspase 3 expression together with decreased Ki67 expression in the tumor xenograft tissues of the apatinib group compared with the expression in the tissues of the control vehicle group (Fig. 1g). Collectively, these results indicated that apatinib inhibits the growth and promotes the apoptosis of GC cells in vitro and in vivo.
Fig. 1 Apatinib inhibits the growth and promotes the apoptosis of GC cells in vitro and in vivo. a, b Colony-formation assay of BGC-823 cells and HGC-27 cells after apatinib treatment for 24 h. Quantification of numbers of colonies in BGC-823 cells and HGC-27 cells; each colony containing >50 cells was counted. c, d BGC-823 cells and HGC-27 cells were incubated with apatinib at various concentrations for 48 h. Then, apoptosis induced by apatinib was detected by Annexin V–FITC/PI staining and analyzed using flow cytometry. e–g Effects of apatinib on the growth of GC in vivo. Statistical analyses of tumor volumes (e) and body weights (f) in the different groups. Photograph of tumors, hematoxylin–eosin (HE) staining and immunohistochemical staining of tumor tissues (g) in mice treated with control vehicle or apatinib (n = 6 per subgroup). Bar scale, 100 μm. *P < 0.05; **P < 0.01; ***P < 0.001.
Apatinib promotes GC autophagy activation in vitro and in vivo

The mechanisms by which apatinib promotes GC cell apoptosis have been reported in some studies. However, the relationship between autophagy and apatinib and the mechanisms are not clear. To examine the potential relationship between autophagy and apatinib, we detected the expression of autophagy genes in vitro and in vivo. First, qRT-PCR analysis of the ATG genes were conducted in BGC-823 cells after apatinib treatment (Supplementary Fig. S1B). Apatinib increased the expression of multiple autophagy genes, with the change in the expression of the ATG7 gene being the most significant. Next, we confirmed that apatinib affected the ATG7 mRNA levels in the tumor tissues of BGC-823 cell-mediated xenografts (Fig. 2a) and in GC cell lines (Fig. 2b, c). These data suggested that apatinib might drive autophagy activation in GC. To examine this possibility, we performed autophagy functional assays in GC cells co-cultured with apatinib. Apatinib increased ATG7 protein expression and the LC3-II protein expression of multiple autophagy genes, with the change in the expression of the ATG7 gene being the most significant. Next, we confirmed that apatinib affected the ATG7 mRNA levels in the tumor tissues of BGC-823 cell-mediated xenografts (Fig. 2a). Apatinib increased the expression of multiple autophagy genes, with the change in the expression of the ATG7 gene being the most significant. Next, we confirmed that apatinib affected the ATG7 mRNA levels in the tumor tissues of BGC-823 cell-mediated xenografts (Fig. 2a).

Silencing of ATG7 promotes apatinib-induced apoptosis in GC cells

We next examined the potential role of the autophagy pathway in apatinib sensitivity in GC cells. An autophagy lysosomal inhibitor, chloroquine, was used with or without apatinib to treat HGC-27 cells and BGC-823 cells. Combination of chloroquine and apatinib significantly inhibited cancer cells proliferation (Supplementary Fig. S2A and Fig. 3a, b). Moreover, the cell proliferation inhibition induced by apatinib was promoted by silencing of ATG7 in BGC-823 cells (Supplementary Fig. S2B). Knockdown of ATG7 decreased the LC3-II expression, increased SQSTM1 expression (Fig. 3c, d and Supplementary Fig. S2C, D), and promoted the BGC-823 cell and HGC-27 cell apoptosis induced by apatinib (Supplementary Fig. S2E and Fig. 3e, f). Collectively, the data strongly suggested that apatinib activates the autophagy pathway, whereas downregulation of the expression of the ATG7 promotes apatinib sensitivity in GC cells.

Apatinib activates ATG7 via downregulation of miR-3657

miRNAs often play significant gene-regulatory roles by pairing with target mRNAs and repressing their expression. We hypothesized that dysregulated miRNAs might contribute to the increase in ATG7 expression. To test this hypothesis, miRNA-seq was performed in six tumor xenograft tissues from the BGC-823 cell-mediated xenograft model in Fig. 1g. Figure 4a shows the criteria we used to pick out the top ten most differentially downregulated miRNAs that might target ATG7. A qRT-PCR assay was conducted to confirm the expression of the ten downregulated miRNAs and miR-3657 was the most differentially expressed (Supplementary Fig. S3A). Bioinformatics analyses with TargetScan (Fig. 4b) and miRanda (Supplementary Fig. S3B) revealed that miR-3657 most likely targeted the 3′-untranslated region (3′-UTR) regions of the ATG7 gene. The intersection of sequences obtained by the two miRNA target prediction softwares was proposed to be a potential binding site. Then, wild-type and mutant ATG7 reporter plasmids targeting the identified sequence were designed (Fig. 4b). Luciferase reporter assays demonstrated that miR-3657 suppressed luciferase activity in BGC-823 cells transfected with wild-type ATG7 reporter plasmid but not in those transfected with the mutant one (Fig. 4c). MiR-3657 expression was decreased in apatinib-treated GC xenograft tissues and GC cells compared with that in the control conditions (Fig. 4d–f). In addition, qRT-PCR showed that miR-3657 mimics decreased the mRNA levels of ATG7 and ATG7 mRNA expression was rescued by miR-3657 inhibitors in BGC-823 cells and HGC-27 cells (Fig. 4g, h). Also, western blotting confirmed the protein levels of LC3-II regulated by miR-3657 (Fig. 4i, j). Confocal microscopy analysis demonstrated that miR-3657 inhibitors induced the autophagic flux, whereas miR-3657 mimics decreased the number of autophagosomes in BGC-823 cells and HGC-27 cells transiently transfected with mRFP-GFP-LC3 plasmid (Fig. 4k). Thus, these data indicated that apatinib increases ATG7 expression via a selective loss of miR-3657.

CircRNA-seq of the tumor xenograft tissues from the apatinib group and control group

We next investigated the mechanisms by which apatinib upregulates ATG7 and downregulates miR-3657. CircRNAs have been reported to act as competing endogenous RNA (ceRNA) sponges, interacting with...
Fig. 2 Apatinib promotes GC autophagy activation in vitro and in vivo. a–c qRT-PCR was performed in vivo (a) and in BGC-823 cells (b) and HGC-27 cells (c) cultured with apatinib. *P < 0.05; **P < 0.001. d–e Western blotting was performed on autophagy proteins expressed in BGC-823 cells (d) and HGC-27 cells (e) co-cultured with apatinib at various concentrations for 48 h. f, g BGC-823 cells (f) and HGC-27 cells (g) were transfected with mRFP-GFP-LC3 plasmid, followed by incubation with apatinib. Confocal microscopy analysis is shown. Bar scale, 10 μm. h, i The numbers of GFP+ or RFP+ dots per cell are presented as the means ± SD of three independent experiments. **P < 0.01; ***P < 0.001 compared with the corresponding control group. # P < 0.05 compared with the GFP group treated with apatinib. j Immunohistochemical staining of LC3-II, SQSTM1, and ATG7 in the tumor xenograft tissues from the apatinib and control groups. Bar scale, 100 μm. k Autophagosomes were observed by TEM in tumor xenografts from the apatinib group. Bar scale, 500 nm (right, enlarged).
miRNAs and influencing their activity. Thus, we proposed the following hypothesis: certain circRNAs might function as a miR-3657 sponge and further regulate ATG7 expression. Hence, we performed circRNA-seq analysis to identify differentially expressed circRNAs between the control group and apatinib group in the BGC-823 cell-mediated xenograft model. In all samples, 19,904 distinct circRNA candidates were found, of which 3798 circRNAs have been found in circBase (Supplementary Fig. S4A). The Venn diagrams illustrate the relationships among the six sequencing samples of the circRNAs (Supplementary Fig. S4B, C). Considering the circRNA characteristics and ease of validation in future studies, circRNAs that met the following criteria were included in this study: exonic circRNAs expressed in every sequencing sample with a length <1000 nucleotides. The differentially expressed circRNAs are shown in the heat map (Fig. 5a).

Then we confirmed the circRNA-seq results of these circRNAs from Fig. 5a in the tumor xenograft tissues by qRT-PCR analysis (Fig. 5b). This analysis confirmed one of the most differentially expressed circRNAs (hsa_circRNA004582, chr12: 49991998–49992655). The corresponding parent gene of hsa_circRNA004582 is RACGAP1 (Rac GTPase activating protein 1). Thus, we termed this circRNA “circRACGAP1”. A qRT-PCR assay demonstrated that circRACGAP1 mRNA expression was upregulated in apatinib-treated GC cells (Fig. 5c, d). In addition, we...
Novel miRNAs (n = 7)

Known miRNAs (n = 18)

Top ten most differentially expressed miRNAs (n = 10)

1. Downregulated miRNAs
2. Fold change \( \leq -2 \)
3. Potentially target ATG7 3' UTR by bioinformatics prediction

miRNA-seq

Six xenograft tumor tissues from control/apatinib group

miRNAs picked out (n = 25)

Novel miRNAs (n = 7)

Known miRNAs (n = 18)

Top ten most differentially expressed miRNAs (n = 10)

qRT-PCR analysis validation

ATG7

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examined the mRNA level of RACGAP1 in GC cells treated with apatinib. The results showed no difference in the mRNA expression of RACGAP1 between control and apatinib-treated GC cells (Fig. 5e, f). Therefore, the results excluded the hypothesis that miR-3657 might bind the corresponding parent gene of circRACGAP1.

Figure 5g shows the circular junction of circRACGAP1 confirmed by Sanger sequencing. RT-PCR with divergent primers detected circRACGAP1 in cDNA but not in genomic DNA (Supplementary Fig. S4D). The fluorescence in situ hybridization (FISH) indicated that circRACGAP1 mainly resided in the cytoplasm in BGC-823 cells (Fig. 5h). Taken together, these results confirmed the most differentially expressed circRNA-circRACGAP1 identified by circRNA-seq.

CircRACGAP1 acts as an endogenous sponge for miR-3657

Next, we constructed a circRNA–miRNA network (Supplementary Fig. S5) to visualize their interactions based on our circRNA-seq data and miRNA-seq data using bioinformatics analyses. A schematic model shows the possible binding sites of circRACGAP1 on miRNAs (fold change ≥ 2 or ≤ –2) from the miRNA-seq analysis by miRanda, and the possible binding site of circRACGAP1 on miR-3657 is shown in red (Fig. 5i). To examine whether miR-3657 could bind to circRACGAP1, RIP for AGO2 in BGC-823 cells was performed, indicating that circRACGAP1 and miR-3657 were substantially accumulated in the AGO2 pellet (Fig. 5j). Bioinformatics analyses with bioinformatics tools TargetScan (Fig. 5k) and miRanda (Supplementary Fig. S4E) predicted that miR-3657 might target the 3′-UTR of circRACGAP1. Furthermore, luciferase reporter assays demonstrated that miR-3657 suppressed the luciferase activity in BGC-823 cells transfected with wild-type circRACGAP1 reporter plasmid but not in those transfected with the mutant one (Fig. 5l). The immunofluorescence experiment confirmed the co-localization of circRACGAP1 and miR-3657 (Supplementary Fig. S4F). Thus, these results indicated that miR-3657 binds circRACGAP1.

CircRACGAP1 modulates apatinib-induced autophagy via targeting miR-3657 and ATG7

With miRNA-seq and circRNA-seq, we found that circRACGAP1 might function as an endogenous sponge for miR-3657 and ATG7. A qRT-PCR assay demonstrated that silencing of circRACGAP1 increased miR-3657 expression and decreased ATG7 mRNA levels in BGC-823 cells (Fig. 6a–c) and HGC-27 cells (Fig. 6d–f). Moreover, the TEM assay showed that transfection with circRACGAP1 siRNA reduced the number of autophagosomes in BGC-823 and HGC-27 cells (Fig. 6g).

Apatinib-induced activation of circRACGAP1, which functions as a ceRNA sponge for miR-3657 and ATG7, led us to hypothesize that circRACGAP1 might regulate autophagy-mediated apatinib resistance. To test this hypothesis, BGC-823 cells and HGC-27 cells were cotransfected with circRACGAP1 siRNA or control siRNA, miR-3657 inhibitors or control inhibitors, and mRFP-GFP-LC3 plasmid, and followed by incubation with apatinib. The apatinib-induced autophagosomes and autolysosomes were reduced in circRACGAP1 knockdown BGC-823 cells and HGC-27 cells (Fig. 6h, i and Supplementary Fig. S6A, B), but rescued by transfection with miR-3657 inhibitors. In addition, the TEM assay confirmed that transfection with circRACGAP1 siRNA reduced the number of autophagic vacuoles in apatinib-treated BGC-823 cells, but rescued by transfection with miR-3657 inhibitors (Fig. 6j, k). Therefore, the data demonstrated that circRACGAP1 modulates apatinib-induced autophagy via targeting miR-3657 and ATG7.

Silencing of circRACGAP1 sensitizes GC cells to apatinib in vitro and in vivo via modulating autophagy

Next, we demonstrated that silencing of circRACGAP1 expression promoted apatinib-induced apoptosis, but rescued by transfection with ATG7 plasmid in GC cells (Fig. 7a, b). In addition, we established a tumor xenograft model in nude mice bearing BGC-823 cells stably transfected with circRACGAP1 shRNA lentivirus or control shRNA lentivirus. The transfection effect of circRACGAP1

(see figure on previous page)

Fig. 4 Apatinib activates ATG7 via downregulation of miR-3657. a The flowchart shows the inclusion criteria for the downregulated miRNAs potentially targeting ATG7 from the miRNA-seq analysis. b The predicted binding sequences for miR-3657 within the human ATG7 3′-UTR by TargetScan. The mutation sequences of ATG7 Mut are shown in red. c Luciferase activity was measured in BGC-823 cells transfected with miR-3657 mimics or control miRNA and ATG7 3′-UTR-Wt or ATG7 3′-UTR-Mut. Luciferase activity was normalized to that in cells transfected with control miRNA. d-f qRT-PCR was performed in vivo (d) and in BGC-823 cells (e) and HGC-27 cells (f) incubated with apatinib, to detect the expression of miR-3657. g-h qRT-PCR was performed in BGC-823 cells (g) and HGC-27 cells (h), to detect the expression of ATG7 after transfection with miR-3657 mimics or inhibitors. i-j The protein level of LC3-II was detected by western blotting in BGC-823 cells (i) and HGC-27 cells (j) transfected with miR-3657 mimics or inhibitors. The LC3-II protein level was quantified by densitometry and normalized to GAPDH, to reflect the LC3-II/GAPDH expression ratio. *P < 0.05, **P < 0.1, ***P < 0.001 compared with the corresponding control group. #P < 0.05 compared with the GFP group transfected with miR-3657 inhibitors.
Fig. 5 (See legend on next page.)
shRNA lentivirus was confirmed in Supplementary Fig. S7. When palpable tumors formed, the mice were separately randomized into the control group or apatinib group. The results showed that silencing of circRACGAP1 promoted the antitumor effect of apatinib (Fig. 7c–e). TEM assay demonstrated that silencing of circRACGAP1 reduced the number of autophagic vacuoles of the xenograft tissues in the apatinib group (Fig. 7f). In addition, immunohistochemical staining of the xenograft tissues showed that transfection with circRACGAP1 shRNA lentivirus in the apatinib group decreased the expression of ATG7 and LC3-II, and rescued SQSTM1 expression compared with the expression in the tissues of the apatinib group transfected with control shRNA lentivirus (Fig. 8a). Collectively, these results demonstrated that silencing of circRACGAP1 sensitizes GC cells to apatinib via autophagy inhibition.

Moreover, we examined the circRACGAP1, miR-3657, and ATG7 expression in ten GC tissues (Fig. 8b). Positive correlation was observed between circRACGAP1 and ATG7 \((P = 0.0072)\). Negative correlations were observed between ATG7 and miR-3657 \((P = 0.0343)\), and between circRACGAP1 and miR-3657 in GC tissue \((P = 0.0150)\). Thus, the results of circRACGAP1-miR-3657-ATG7 axis in GC patients were in accordance with the previous conclusions from GC cell lines and animal experiments.

**Discussion**

In this translationally relevant study, we reported for the first time that specific blockade of circRACGAP1 may be a potential therapeutic strategy to enhance the antitumor effect of apatinib in GC. The results demonstrated that apatinib promotes autophagy activation via upregulation of ATG7 expression and autophagy inhibition promotes apatinib-induced apoptosis in human GC. With miRNA-seq and circRNA-seq analyses, our study provided new insights into the function of circRACGAP1. The results showed that circRACGAP1 acts as an endogenous sponge for miR-3657 to inhibit its activity and further upregulate the miR-3657-targeted gene ATG7. Silencing of circRACGAP1 inhibited apatinib-induced autophagy by downregulation of ATG7, which was rescued by miR-3657. Moreover, knockdown of circRACGAP1 sensitizes GC cells to apatinib via autophagy inhibition in vitro and in vivo. These findings provided the first evidence that the circRACGAP1-miR-3657-ATG7 axis mediates a regulatory pathway critical for the regulation of autophagy and apatinib sensitivity in GC (Fig. 8c).

To improve sensitivity to kinase inhibitors, current strategies attempt to target the same kinase in a different way or target a parallel signaling pathway, whereas the strategy of inhibiting autophagy may represent an entirely independent process of reversing drug resistance. Under most circumstances, autophagy constitutes a stress adaptation that suppresses apoptosis and promotes cell survival. However, autophagy can also kill cells by type II or autophagic cell death, which is a form of non-apoptotic programmed cell death. Interestingly, similar stimuli can induce apoptosis or autophagy and sometimes a mixed phenotype of autophagy and apoptosis. The mechanisms for these different phenotypes are related to the degradation of different pro-apoptotic or anti-apoptotic regulators by autophagy. A role of autophagy in protecting cells from undergoing programmed cell death was found in some completed and ongoing phase I/II clinical studies concerning chloroquine and hydroxychloroquine. In addition, inhibition of autophagy by chloroquine or hydroxychloroquine combined with chemotherapy, kinase inhibitors, or radiation therapy has been shown to improve antitumor activity. Apatinib was reported to promote apoptosis in osteosarcoma, hepatocellular carcinoma, and intrahepatic cholangiocarcinoma. However, the relationship between autophagy and apoptosis, and the mechanisms had not yet been reported in GC. Moreover, whether inhibiting autophagy might reverse apatinib resistance in GC and the related mechanisms still remained to be resolved. In this study, we demonstrated that apatinib promoted autophagy activation in GC. Our study is the first to report that autophagy inhibition promoted apatinib-induced apoptosis via downregulation of ATG7 level in GC. The ATG7 gene plays a complex role in cancer therapy. Strohecker et al. reported that ATG7...
Fig. 6 (See legend on next page.)
initially showed antitumor efficacy at earlier stages of BRAt-driven lung tumorigenesis via decreasing oxidative stress, whereas ATG7 promoted tumorigenesis by preserving mitochondrial function at later stages of tumorigenesis. Acute ATG7 ablation in mice with pre-existing non-small cell lung cancer suppressed tumor growth via shutting off the mammalian target of rapamycin and mitogen-activated protein kinase signaling. These studies provided the rationale for targeting ATG7 in cancer therapy.

Furthermore, we report a novel autophagy regulatory mechanism in mediating tumor cell fate and tumor progression by a circRNA in response to apatinib treatment. CircRNAs are novel biomarkers for human GC, hepatocellular carcinoma development, and oral cancer. Moreover, circRNAs participate in autophagy regulatory networks and mediate the transcriptional and post-transcriptional regulation of ATG5, ATG7, and ATG12. Huang et al. reported that circHIPK2 regulated astrocyte activation via the regulation of autophagy and endoplasmic reticulum stress by targeting MIR124-2HG and SIGMAR1. Hansen et al. reported that circR-7 acted as a sponge for miR-7 and increased the expression levels of miR-7 target genes. Herein, with miRNA-seq and circRNA-seq technology, we demonstrated that apatinib induces expression of circRACGAP1, which interacts with miR-3657 and as a result, miR-3657 repression of ATG7 mRNA is released, eliciting increased autophagy. In addition, silencing of circRACGAP1 inhibited autophagy and promoted apatinib-induced apoptosis via sponging of miR-3657 and further decreasing ATG7 expression in vitro and in vivo. Collectively, the circRACGAP1-miR-3657-ATG7 axis mediates a novel regulatory pathway critical for the apatinib sensitivity via regulation of autophagy in GC.

In this study, we proposed that knockdown of circRACGAP1 may be a potential intervention strategy to reduce the toxicities and promote the antitumor effect of apatinib via autophagy inhibition in GC patients. As circRNAs are stable in blood plasma and exosomes, further study of monitoring the changes of plasma circRNAs levels and exosomal circRNAs levels may help distinguish patients with different curative effects. CircRNA biogenesis and expression has been reported to be regulated by different mechanisms: the core spliceosomal machinery, cis-elements, and RNA-binding proteins. Our results showed that mRNA levels of RACGAP1 were not significantly different between the control group and apatinib group (Fig. 5e, f). Moreover, we detected the expression of some reported genes involved in circRNA biogenesis. We found that SFA32 (splicing factor 3A2) was downregulated in apatinib-treated tumor xenograft tissues compared with that without apatinib treatment (Supplementary Fig. S8A), and silencing of SFA32 increased circRACGAP1 expression (Supplementary Fig. S8B). In the follow-up study, we will provide more results of how apatinib affect circRACGAP1 expression and more systematic/in-depth molecular mechanisms.

Our previous study reported that JWA (adenosine diphosphate-ribosylation-like factor 6 interacting protein 5, ARL6ip5) reversed cisplatin resistance in human GC, whereas lower expression of JWA sensitized cisplatin-resistant GC cells to lapatinib-induced apoptosis. Collectively, these three studies have led to the proposals for some significant therapy regimens in GC patients: higher expression of JWA may be used as a biomarker for cisplatin treatment, lower expression of JWA may be utilized to identify patients that would benefit from HER2-targeted therapy; in terms of the anti-angiogenesis therapy, silencing of circRACGAP1 may be a potential intervention strategy to reduce the toxicities and promote the antitumor effect of apatinib. These proposals may help select effective therapy regimens for specific patients and mitigate drug toxicities. Further clinical studies are needed to demonstrate the results for better personalized therapy regimens in GC patients.

In conclusion, we report for the first time that the circRACGAP1-miR-3657-ATG7 axis mediates a regulatory pathway critical for the regulation of autophagy and apatinib sensitivity in GC. Specific blockade of circRACGAP1 can be utilized as a potential therapeutic target for autophagy inhibition in GC patients undergoing apatinib treatment. Furthermore, we postulate...
that circRACGAP1 might contribute to the further development of personalized therapeutics by combining its inhibitors with apatinib against human GC. These findings might provide new opportunities for prospective multi-institutional trials testing clinical applications of circRACGAP1 as a potential intervention target to promote the antitumor efficacy of apatinib in advanced GC patients.

Fig. 7 Silencing of circRACGAP1 sensitizes GC cells to apatinib in vitro and in vivo via modulating autophagy. a, b Apoptosis was detected in BGC-823 and HGC-27 cells cotransfected with circRACGAP1 siRNA or control siRNA, and ATG7 plasmid or pcDNA3.1 plasmid by the TUNEL assay. Then, the cells were co-cultured with or without 40 µM apatinib for 30 h. **P < 0.01; ***P < 0.001 compared with the control group without apatinib treatment. ^P < 0.05; ^^P < 0.01 compared with the apatinib group cotransfected with pcDNA3.1 plasmid and si control. #P < 0.05; ##P < 0.01 compared with the apatinib group cotransfected with pcDNA3.1 plasmid and si circRACGAP1-1 or si circRACGAP1-2. c–e Tumor xenograft model in nude mice bearing BGC-823 cells stably transfected with circRACGAP1 shRNA lentivirus or control shRNA lentivirus. The mice were randomly divided into control group and apatinib group. Tumor volumes (c) and tumor weights (e) in the different groups (n = 5 per subgroup). Photograph of tumors (d) in mice were taken. f Autophagosomes were observed by TEM in tumor xenografts. Bar scale, 500 nm (enlarged). *P < 0.05; **P < 0.01; ***P < 0.001.
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**Fig. 8 Role of circRACGAP1 in mediating autophagy and apatinib sensitivity.** a Immunohistochemical staining of ATG7, LC3-II, and SQSTM1 in the tumor xenograft tissues from the apatinib and control groups. Bar scale, 100 μm. b Correlation analysis of relative ATG7 expression and relative circRACGAP1 expression (left), relative ATG7 expression and relative miR-3657 expression (middle), and relative circRACGAP1 expression and relative miR-3657 expression (right) in ten gastric cancer tissues. c Schematic diagram of the relationship among apatinib, the circRACGAP1-miR-3657-ATG7 axis, autophagy and apoptosis in GC. Left: apatinib induces autophagy via the circRACGAP1-miR-3657-ATG7 axis. Right: knockdown of circRACGAP1 promotes the antitumor activity of apatinib via inhibition of autophagy.
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Data availability
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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

Ethics approval
All experiments were carried out according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication Number 80–23) revised in 1996. The animal studies were reviewed and approved by the Ethics Committee for Animal Studies at Nanjing Medical University. The study was approved by the Ethics Committee on Human Research of the First Affiliated Hospital of Nanjing Medical University and written informed consent was obtained from all patients.

Consent for publication
Not applicable.

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