miR-874 regulates multiple-drug resistance in gastric cancer by targeting ATG16L1

HAIJIN HUANG1*, JIE TANG2*, LEI ZHANG2*, YANZHI BU3 and XIAOYU ZHANG4

1Department of General Surgery, Hongze District People's Hospital, Huaian, Jiangsu 223100; 2Department of Pediatric Surgery, Children's Hospital of Nanjing Medical University, Nanjing, Jiangsu 210000; 3Department of General Surgery, Lianshui Country People's Hospital, Lianshui Country, Huaian, Jiangsu 223400; 4Department of General Surgery, Huai'an Second People's Hospital and The Affiliated Huai'an Hospital of Xuzhou Medical University, Huaian, Jiangsu 223001, P.R. China

Received May 9, 2018; Accepted August 13, 2018

DOI: 10.3892/ijo.2018.4593

Abstract. Chemotherapy is an important treatment option for gastric cancer (GC); however, chemotherapy usually fails due to drug resistance, particularly multidrug resistance (MDR). In our previous studies, microRNA (miR)-874 was demonstrated to serve an important role in tumour growth, apoptosis and angiogenesis. In the present study, the precise roles and underlying mechanisms of miR-874 in MDR were investigated in GC. The overexpression of miR-874 reversed cancer cell drug resistance in vitro. According to reporter gene and western blot assays, Autophagy-related 16-like 1 (ATG16L1) was identified as a direct target of miR‑874. ATG16L1 was also demonstrated to be positively associated with autophagy. Reducing the expression of ATG16L1 and inhibiting the occurrence of autophagy sensitized GC cells to chemotherapy. Thus, the miR-874/ATG16L1/autophagy regulatory loop was demonstrated to serve an important role in MDR in GC. Furthermore, miR-874 may be used as a prognostic factor in GC. Overall, miR-874 could inhibit autophagy and sensitize GC cells to chemotherapy via the target gene ATG16L1, highlighting the potential clinical application of miR-874 in chemotherapeutic resistance.

Introduction

During the past century, gastric cancer (GC) has remained the fourth most prevalent type of malignant cancer and the second leading cause of cancer-associated mortality worldwide (1). Currently, the majority of patients with GC are diagnosed at the advanced stage of the disease due to the lack of effective diagnostic methods at the early stages. The five-year survival rate of advanced patients with GC is only 5-20%, and the median overall survival is <1 year (2). For advanced-stage patients, chemotherapy is the first-line treatment. Unfortunately, chemotherapy resistance is common, particularly multidrug resistance (MDR), which affects treatment and prognosis (3,4). Although the mechanisms underlying MDR have been extensively explored, the key multiple-drug resistance features of this clinical phenomenon remain unclear.

Micro (mi)RNAs are short (20-24 nt), stable, non-coding RNA molecules that negatively regulate 60% of coding genes by targeting the 3' untranslated regions (3'UTRs) of specific mRNAs to prevent translation and/or promote degradation (5). Accumulating evidence indicates that miRNAs serve important roles in chemoresistance in various types of cancer, including GC (6-14). However, the exact mechanisms underlying the regulation of chemoresistance in GC by miRNAs remain unclear. miR-874 has been reported in numerous types of cancer, including GC, hepatocellular carcinoma, colorectal cancer, breast cancer, non-small-cell lung cancer, and head and neck squamous carcinoma (8,15-18). In our previous studies, miR-874 was demonstrated to serve a potential role in tumour growth, apoptosis and angiogenesis in GC. However, to the best of our knowledge, the association between miR-874 and chemotherapy drug resistance in GC has not been reported. The present study demonstrated that miR-874 enhanced the sensitivity of GC cells to chemotherapy. Furthermore, autophagy-related 16-like 1 (ATG16L1) was identified as a direct and functional target of miR-874. In addition, ATG16L1 expression was revealed to be increased in GC MDR cells, and positively associated with autophagy and chemotherapy resistance. Lastly, it was demonstrated that miR-874 expression was downregulated in chemoresistant...
patients, and was associated with ATG16L1 expression and overall survival in patients with GC.

Materials and methods

Tissue samples. The acquisition of tissue specimens and the study protocol were performed in strict accordance with the regulations of the Institutional Review Board of Xuzhou Medical University (Xuzhou, China). All patients signed informed consent forms. Human GC specimens were collected between January 2010 and December 2012, and detailed clinicopathological and follow-up information were obtained from the Tissue Sample Centre of the Affiliated Huai'an Hospital at Xuzhou Medical University in China. In all cases, the diagnoses and grading were confirmed by two experienced pathologists and were performed according to the criteria of the American Joint Committee on Cancer (19,20).

Cells and cell culture. The human GC cell lines SGC7901, BGC823 (Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China), AGS (American Type Culture Collection, Manassas, VA, USA), and SGC7901/cisplatin (DDP) (established and maintained in our laboratory) were cultured in RPMI-1640 supplemented with 10% foetal bovine serum (both from Wisent Biotechnology, Nanjing, China) and antibiotics (1% penicillin/streptomycin) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). All cell lines were cultured in a humidified chamber supplemented with 5% CO₂ at 37°C. Cell resistance was induced by gradually increasing the cisplatin concentration in the culture medium. SGC-7901 cells in the logarithmic growth phase were seeded in the culture medium, which contained DDP (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at a low starting concentration of 0.05 µg/ml. After 48 h, the culture medium was discarded, fresh medium was added and culturing was continued. Once normal growth was observed, the cells were continuously treated with 0.05 µg/ml DDP for 48 h after digestion and passage. The cells were cultured and passaged in this manner, and the DDP concentration was gradually increased (the concentration of DDP was increased by 0.05 µg/ml each time; range, 0.05-1 µg/ml) to continuously induce the cells. Finally, a cell line that was tolerant of 1 µg/ml DDP was established.

RNA extraction, cDNA synthesis and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from frozen tissues or cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA purity was assessed using a NanoDrop 2000 (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA) using the standard absorbance ratios of A260/A280 ≥1.8 and A260/A230 ≥1.5. Complementary DNAs were synthesized from 1 µg of total RNA using the TaqMan Reverse Transcription kit (Life Technologies; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The following thermocycling conditions were maintained: 95°C for 10 min; followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. All mRNA and miRNA quantification data were normalized to GAPDH and U6, respectively. All experiments were performed independently in triplicate. The relative expression levels of target genes were normalized to those of the internal control genes using the 2^(-ΔΔCq) cycle quantification method (21). The primers were as follows: GAPDH forward, 5'-ATCTCTGGCTCCCCCTGCTGA-3' and reverse, 5'-GATGACCTTTGCCACAGCCT-3'; miR-874 forward, 5'-GGCCCTAGGAAGAAGAATG-3' and reverse, 5'-TGAAGAAGGAGGCTCATAGC-3'; SGC7901/DDP-NC, SGC7901/DDP-pre, SGC7901/DDP-sh-NC, and ATG16L1 forward, 5'-AGGACAGGCAGGAGCATCCTGAGTTTAA-3'. The sequence of ATG16L1-shRNA was 5'-TTTTGTGTAGTACAA-3' was used as a negative control (sh-NC). The sequence of pre-miR-874 was 5'-TTAGCCCTGCGGCCCACGCACGCCGATGAGATCTCAAT-3'. The sequence of miR-874-inhibitor was 5'-GCCCAGCCAGTCGGCCCTGGCCCGAGGGACCGACTGGCTGGGC-3'. The sequence of miR-NC was 5'-TTTGATACCAAGATGACTGCCATGAAAACACATCATGTATGTTACATACATATATACATTATCTGTACAGAAACATCATATGTATGTTACATACATATATACATTATCTGTTTGTGTAGTACA-3' was used as a negative control (sh-NC). The cells were infected and selected as aforementioned. RT-qPCR and western blotting was then performed to detect the expression of ATG16L1, and confirm the efficiency of transfection.

Vector constructs, lentivirus production and cell transfection. The lentiviral vectors has-miR-874-pre-microRNA (pre-miR-874) and has-miR-874-pre-inhibitor (miR-874 inhibitor) were purchased from Shanghai GenePharma Co., Ltd. A scrambled lentiviral construct (miR-NC) was used as a negative control (NC). The sequence of pre-miR-874 was 5'-TTAGCCCTGCGGCCCACGCACGCCGATGAGATCTCAAT-3'. The sequence of miR-874-inhibitor was 5'-GCCCAGCCAGTCGGCCCTGGCCCGAGGGACCGACTGGCTGGGC-3'. The sequence of miR-NC was 5'-TTTGATACCAAGATGACTGCCATGAAAACACATCATGTATGTTACATACATATATACATTATCTGTACAGAAACATCATATGTATGTTACATACATATATACATTATCTGTTTGTGTAGTACA-3' was used as a negative control (sh-NC). The cells were infected and selected as aforementioned. RT-qPCR and western blotting was then performed to detect the expression of ATG16L1, and confirm the efficiency of transfection.

In vitro and in vivo drug sensitivity assays. Each well of a 96-well plate was seeded with 5x10³ cells (SGC7901, SGC7901-NC, SGC7901-inhibitor, SGC7901/DDP, SGC7901/DDP-NC, SGC7901/DDP-pre, SGC7901/DDP-sh-NC, SGC7901/DDP-sh-ATG16L1 or SGC7901/DDP+CQ). After 24 h, culture media containing different concentrations of the chemotherapeutic drugs DDP (0-25 µg/ml), 5-Fluorouracil (5FU) (0-25 µg/ml) and vincristine (VCR) (0-50 µg/ml) (both from Sigma-Aldrich; Merck KGaA) were added to each well. After the plates were incubated for 48 h, a Cell Counting Kit 8 (CCK8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) assay was performed. The half maximal inhibitory concentration (IC₅₀) of each drug was calculated. For the in vivo experiments, ~2.0x10⁶ cells stably transfected with pre-miR-874 were maintained: 95°C for 10 min; followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. All mRNA and miRNA quantification data were normalized to GAPDH and U6, respectively. All experiments were performed independently in triplicate. The relative expression levels of target genes were normalized to those of the internal control genes using the 2^(-ΔΔCq) cycle quantification method (21). The primers were as follows: GAPDH forward, 5'-ATCTCTGGCTCCCCCTGCTGA-3' and reverse, 5'-GATGACCTTTGCCACAGCCT-3'; miR-874 forward, 5'-GGCCCTAGGAAGAAGAATG-3' and reverse, 5'-TGAAGAAGGAGGCTCATAGC-3'; SGC7901/DDP-NC, SGC7901/DDP-pre, SGC7901/DDP-sh-NC, and ATG16L1 forward, 5'-AGGACAGGCAGGAGCATCCTGAGTTTAA-3'. The sequence of ATG16L1-shRNA was 5'-TTTTGTGTAGTACAA-3' was used as a negative control (sh-NC). The sequence of pre-miR-874 was 5'-TTAGCCCTGCGGCCCACGCACGCCGATGAGATCTCAAT-3'. The sequence of miR-874-inhibitor was 5'-GCCCAGCCAGTCGGCCCTGGCCCGAGGGACCGACTGGCTGGGC-3'. The sequence of miR-NC was 5'-TTTGATACCAAGATGACTGCCATGAAAACACATCATGTATGTTACATACATATATACATTATCTGTACAGAAACATCATATGTATGTTACATACATATATACATTATCTGTTTGTGTAGTACA-3' was used as a negative control (sh-NC). The cells were infected and selected as aforementioned. RT-qPCR and western blotting was then performed to detect the expression of ATG16L1, and confirm the efficiency of transfection.
or NC were inoculated subcutaneously into both flanks of nude mice. After 2 weeks, the mice were intraperitoneally injected with PBS containing 5-FU or DDP (10 mg/kg) once weekly. The tumour size on their skin surfaces was measured once weekly. The mice were humanely euthanized on day 28, and the tumours were measured and images. A total of six female nude mice (BALB/c nude mice, 5-weeks old, 15-18 g) were purchased from Shanghai Experimental Animal Centre (Shanghai, China) and housed under specific pathogen-free conditions. All animal experiments were conducted according to the recommendations of the Xuzhou Medical University Institutional Animal Care and Use Committee (approval no. IACUC-201601012).

3' UTR luciferase constructs and assay. The 3' UTR of ATG16L1 mRNA containing either the putative or mutated miR-874 binding site was synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The cells were co-transfected with plasmids (Shanghai GenePharma Co., Ltd.) expressing wild-type ATG16L1 or mutant ATG16L1 in addition to cells stably transfected with pre-miR-874 or miR-NC. After 36 h of transfection, firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter assay kit (Promega Corporation, Madison, WI, USA).

Transmission electron microscopy (TEM). Cells were collected and centrifuged at 150 x g, 4˚C for 10 min and then fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) overnight at 4˚C. Next, the cells were fixed in 1% OsO4 for 1 h at room temperature. Subsequently, the samples were dehydrated using an increasing concentration gradient of ethanol (50-100%) and the cells were then embedded in Epon. The samples were cut into ultrathin (50 nm) sections and counterstained with 0.3% lead citrate at room temperature for 20 min. Images were generated using a JEM-1010 electron microscope at a magnification of x10,000 and x40,000.

Western blotting. Protein extract was obtained using RIPA Lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) containing 1% phenylmethylsulfonyl fluoride (Sigma-Aldrich; Merck KGaA). The protein concentration of cell lysate was determined using an Enhanced BCA Protein assay kit (Beyotime Institute of Biotechnology, Haimen, CA, USA). The membranes were blocked with 5% non-fat milk in Tris-buffered saline for 2 h at room temperature, and then incubated with specific antibodies at 4˚C overnight. Following washing with TBS-Tween-20 for 15 min at room temperature, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at room temperature. The proteins were visualized using a SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Fisher Scientific, Inc.). The software Image-Pro Plus (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA) was used to quantify protein expression. Mouse anti-human GAPDH primary antibodies (cat. no. SC-365062; dilution 1:1,000) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Goat anti-human ATG16L1 primary antibodies (cat. no. ab223238; dilution 1:1,000) were purchased from Abcam (Cambridge, UK) and rabbit anti-human LC3/II primary antibodies (cat. no. 4108; dilution 1:1,000) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The HRP-conjugated second antibodies used were as follows: Donkey anti-goat IgG (cat. no. A0181; dilution 1:1,000), goat anti-rabbit IgG (cat. no. A0208; dilution 1:1,000) and goat anti-mouse IgG (cat. no. A0216; dilution 1:1,000) (all from Beyotime Institute of Biotechnology).

Immunohistochemistry (IHC). All specimens were fixed in 4% formalin at temperature for 24 h and embedded in paraffin prior to performing the IHC analysis, as described in detail in our previous report (18). The specimens were examined in a blinded manner. Five fields were selected for examination, and the percentage of positive tumour cells and the cell-staining intensity were determined at the magnification of x200 and x400 with a Nikon Eclipse 90i digital microscope (Nikon Corporation, Tokyo, Japan).

Bioinformatics. TargetScan (http://www.targetscan.org), miRanda (http://www.microrna.org/microrna/home.do), miRBase (http://www.mirbase.org), miRDB (http://www.mirdb.org) and CLIPdb (http://clipdb.cnrnalab.org) were used to predict the genes targeted by miR-874.

Chloroquine (CQ). The autophagy inhibitor CQ (Sigma-Aldrich; Merck KGaA) was used to examine whether autophagy affected chemoresistance in GC cells. Cells were treated with or without CQ (20 µmol/l) for 24 h prior to the other experiments.

Statistical analysis. SPSS software (version 18.0; SPSS, Inc., Chicago, IL, USA) was used to perform statistical analyses. Student's t-test (two-tailed) or one-way analysis of variance followed by the Student-Newman-Keuls post-hoc test was performed to analyse the in vitro and in vivo data. The clinicopathological factors were compared by performing unpaired t-tests or Pearson's χ2 tests. The quantitative data are presented as the mean ± standard deviation. The Kaplan-Meier method was used to estimate the survival curve. The differences in the survival distributions were determined by performing log-rank tests. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-874 regulates the sensitivity of GC cells to chemotherapeutic drugs in vitro. First, RT-qPCR analysis was performed to detect miR-874 expression in SGC7901 and SGC7901/DDP cells. miR-874 expression was significantly downregulated in SGC7901/DDP cells compared with SGC7901 cells (Fig. 1A). To investigate the effect of miR-874 on chemotherapeutic resistance, miR-874 overexpression and knockdown cell lines were established. SGC7901 and SGC7901/DDP cells were transfected with mi-R-874 inhibitors or pre-miR-874. miR-874 inhibitor transfection resulted in miR-874 being knocked down by ~80% in SGC7901 cells compared with the NC control group (Fig. 1B). Furthermore, miR-874 was significantly increased by ~30-fold in SGC7901/DDP cells following transfection with pre-miR-974 compared with the NC control (Fig. 1C). CCK8 assays were then performed to generate
cell growth curves and calculate the IC_{50} values. Overexpression of miR-874 significantly enhanced the sensitivity of SGC7901/DDP cells to DDP, VCR and 5-FU compared with the blank control and NC groups. Conversely, transfection of SGC7901 cells with inhibitors of miR-874 significantly increased the IC_{50} values of these three chemotherapeutic agents compared with the two control groups (Fig. 1D and E).

ATG16L1 is a direct target of miR-874. ATG16L1 was identified as a potential target gene of miR-874 through bioinformatic algorithms. RT-qPCR and western blot assays were performed, which revealed that ATG16L1 was significantly upregulated in SGC7901/DDP cells compared with SGC7901 cells (Fig. 2A-C). Furthermore, the overexpression of miR-874 reduced the expression of ATG16L1 at the mRNA and protein levels in SGC7901/DDP cells (Fig. 2D and E), whereas the downregulation of miR-874 led to the opposite effect (Fig. 2E and F). A putative miR-874 binding site was identified within the 3′UTR of ATG16L1 (Fig. 2G). A dual-luciferase reporter system was used to validate whether ATG16L1 is a direct target of miR-874. Wild-type (Wt) and mutant (Mu) versions of the ATG16L1 3′UTR were cloned into the reporter plasmids. Forced expression of miR-874 significantly suppressed luciferase activity from the wild-type reporter, but did not affect the mutant reporter (Fig. 2H). Thus, ATG16L1 is likely a direct target of miR-874. To further explore the function of ATG16L1 in MDR in GC, a shRNA targeting ATG16L1 was constructed and introduced using lentiviral gene transfer, and its inhibitory effect was confirmed by RT-qPCR and western blot assays (Fig. 3A-C). Remarkably, the silencing of ATG16L1 sensitized SGC7901/DDP cells to chemotherapeutic agents and significantly decreased the IC_{50} values of these drugs (Fig. 3D). Thus, ATG16L1 likely serves an important role in GC as a direct functional target gene of miR-874.

Chemoresistant GC cells exhibit increased autophagy. ATG16L1 is a member of a large protein complex that is necessary for autophagy (22). As the expression of ATG16L1 was demonstrated to be increased at the mRNA and protein levels, we hypothesized that autophagy may be enhanced in SGC7901/DDP cells. To test this hypothesis, TEM was performed to evaluate autophagosomes. Compared with SGC7901 cells, autophagosomes markedly accumulated in the cytoplasm of SGC7901/DDP cells (Fig. 4A). The processing of the LC3-I protein to LC3-II, which is a hallmark of autophagy, was also evaluated by performing western blot analysis. A
marked increased expression of LC3-II in SGC7901/DDP cells was observed compared with that in SGC7901 cells (Fig. 4B). The results suggested that the chemoresistant GC cells exhibited increased autophagy.

**ATG16L1 inhibits chemosensitivity in chemoresistant GC cells by promoting autophagy.** The silencing of ATG16L1 increased the sensitivity of SGC7901/DDP cells to chemotherapeutic agents. Next, whether ATG16L1 regulated autophagy was investigated. According to western blot analysis, autophagy was markedly decreased in SGC7901/DDP cells in which ATG16L1 was silenced compared with the NC-transfected group (Fig. 4C). The expression of LC3-II was markedly increased in SGC7901/DDP cells following CQ treatment for 24 h compared with the untreated control group (Fig. 4D). Additionally, according to the CCK8 assays, CQ treatment significantly increased the sensitivity of SGC7901/DDP cells to chemotherapeutic drugs (Fig. 4E). In summary, ATG16L1, autophagy-related 16-like 1.
miR-874 inhibits autophagy by regulating ATG16L1. The aforementioned results suggested that ATG16L1 may be a target gene of miR-874 and that ATG16L1 regulated autophagy. Next, we hypothesized that the suppression of miR-874...
contributes to increased autophagy. To examine this hypothesis, SGC7901/DDP or SGC7901 cells were transfected with pre-miR-874 or inhibitors. Following transfection, autophagy was detected in the aforementioned cells. Downregulation of miR-874 markedly increased the expression of LC3-II in SGC7901 cells compared with the blank and NC groups. In contrast, overexpression of miR-874 led to the opposite effect in SGC7901/DDP cells (Fig. 5A). Furthermore, according to the RT-qPCR and western blot analyses, following treatment of the cells with a low concentration of 5-FU for 24 h, the expression of miR-874 in AGS and BGC823 cells was significantly reduced, whereas the expression levels of ATG16L1 and LC3-II were markedly upregulated (Fig. 5B-D). SGC7901 cells were then co-transfected with miR-874-inhibitors and shRNA-ATG16L1. ATG16L1 was then evaluated by western blot analysis. (E) The IC\textsubscript{50} values were detected when sh-ATG16L1 or NC was co-transfected with miR-874-inhibitors. *P<0.05 and **P<0.01. DDP, cisplatin; VCR, vincristine; 5-FU, 5-fluorouracil; sh, short hairpin RNA; NC, negative control; ATG16L1, Autophagy-related 16-like 1; miR, microRNA.

Restoration of miR-874 increases chemosensitivity in GC cells in vivo. To determine whether miR-874 affected the chemosensitivity of GC cells in vivo, we constructed SGC7901/DDP cells that stably overexpressed miR-874. SGC7901/DDP-NC or SGC7901/DDP-pre-miR-874 cells were then transplanted into nude mice. The volumes of the pre-miR-874-transfected tumours were significantly decreased following chemotherapy compared with the SGC7901/DDP-NC group, indicating that ectopic miR-874 expression reverted chemoresistance (Fig. 6).

miR-874 expression is downregulated in chemoresistant patients and is associated with overall survival in patients with GC. RT-qPCR and immunohistochemistry were performed to detect miR-874 and its targets, respectively, in clinical samples. A total of 50 clinical samples (25 chemoresistant samples and 25 chemosensitive samples) were obtained from patients who received neoadjuvant chemotherapy prior to surgery between 2014 and 2016 at the Affiliated Huai’an Hospital, Xuzhou
Medical University. Chemosensitivity or resistance was the result of clinical multidisciplinary discussions. In drug-resistant patients, the expression of miR-874 was significantly decreased and the expression of ATG16L1 was markedly upregulated compared with drug-sensitive patients (Fig. 7A and B). The association between miR-874 expression and clinicopathological parameters from 200 cases of gastric malignant tissues were analysed from Tissue Sample Center, which demonstrated that miR-874 expression was significantly associated with distant metastasis, whereas no significant association was observed between miR-874 expression and patient age, sex, tumour differentiation, tumour depth, nodal metastasis or tumour node metastasis stage (Table I). In addition, patients with low miR-874 expression had a significantly poorer prognosis compared with those with high expression (Fig. 7C).

**Discussion**

GC is among the most common causes of cancer-associated mortality. Currently, the rates of early detection and diagnosis among patients with GC are low, and chemotherapy remains a primary treatment method. However, due to drug resistance, the effectiveness of chemotherapy is limited. The 5-year survival rate of patients with GC remains low (3,23). Although numerous mechanisms contribute to chemoresistance, including increased drug efflux, inactivation of detoxification enzymes, alterations...
in drug metabolism, mutations of drug targets, dysfunction of pro-apoptotic proteins and enhancement of DNA repair activity, the mechanisms involved in cancer chemoresistance remain poorly understood (24-28). Evidence suggests that miRNAs are involved in chemoresistance in various types of cancer, including GC (6,14). However, relatively few studies investigating MDR in GC have been performed.

The dysregulation of miR-874 has been reported in various cancer types, as aforementioned. In the present study, the underlying mechanisms through which miR-874 regulates MDR in GC were studied. miR-874 expression was demonstrated to be decreased in MDR GC cells compared with parental GC cells, indicating that miR-874 is involved in chemoresistance.

miRNAs usually have multiple target genes. Therefore, a search for potential target genes of miR-874 in GC using several computational algorithms was performed. Among the potential targets, ATG16L1 was focused on as its function is associated with chemoresistance in cancer (29). The results of the present study revealed that the expression of ATG16L1 was increased in drug-resistant GC cells. Furthermore, miR-874 reduced ATG16L1 expression at the mRNA and protein levels. Knockdown of ATG16L1 expression by shRNA increased the chemosensitivity of chemoresistant GC cells, suggesting that ATG16L1 is associated with chemosensitivity in GC.

Autophagy is increased in various cancer types and contributes to drug resistance. ATG16L1 is a member of a large protein complex that is necessary for autophagy, which is the major process by which intracellular components are targeted to lysosomes for degradation (30,31). The expression of ATG16L1 was increased in MDR GC cells, suggesting that autophagy may be involved in MDR. To examine this hypothesis, the level of autophagy was evaluated by performing TEM and western blot analyses. The results demonstrated that MDR cells exhibited increased autophagy, which functioned as a mechanism of chemoresistance. The level of autophagy decreased following the knockdown of ATG16L1 or treating MDR cells with CQ, both of which resulted in increased sensitivity to chemotherapy agents. These results suggest that autophagy in MDR GC cells may be a mechanism that promotes chemotherapy resistance. The inhibition of autophagy by interfering with ATG16L1 may be a novel approach to improving chemotherapeutic efficacy.

miRNAs serve an important role in regulating autophagy (32,33). The expression of miR-874 was modified

<table>
<thead>
<tr>
<th>Variables</th>
<th>No. of cases</th>
<th>Expression of miR-874</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low (%)</td>
</tr>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥50</td>
<td>151</td>
<td>53 (35.1)</td>
</tr>
<tr>
<td>&lt;50</td>
<td>49</td>
<td>20 (40.8)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>111</td>
<td>31 (27.9)</td>
</tr>
<tr>
<td>Female</td>
<td>89</td>
<td>32 (36.0)</td>
</tr>
<tr>
<td>Degree of differentiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well and moderately differentiated</td>
<td>171</td>
<td>62 (36.3)</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>29</td>
<td>13 (44.8)</td>
</tr>
<tr>
<td>T classification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>14</td>
<td>6 (42.9)</td>
</tr>
<tr>
<td>T2</td>
<td>58</td>
<td>15 (25.9)</td>
</tr>
<tr>
<td>T3</td>
<td>116</td>
<td>43 (37.1)</td>
</tr>
<tr>
<td>T4</td>
<td>12</td>
<td>7 (58.3)</td>
</tr>
<tr>
<td>N classification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>119</td>
<td>49 (41.2)</td>
</tr>
<tr>
<td>N1</td>
<td>81</td>
<td>31 (38.3)</td>
</tr>
<tr>
<td>M classification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>166</td>
<td>52 (31.3)</td>
</tr>
<tr>
<td>M1</td>
<td>34</td>
<td>21 (61.8)</td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I/II</td>
<td>121</td>
<td>39 (32.2)</td>
</tr>
<tr>
<td>Stage III/IV</td>
<td>79</td>
<td>34 (43.0)</td>
</tr>
</tbody>
</table>

TNM, tumour node metastasis; miR, microRNA.
by transfecting GC cells with pre-miR-874 or inhibitors to ascertain whether miR-874 regulated autophagy. The upregulation of miR-874 significantly inhibited autophagy in MDR cells, while the downregulation of miR-874 had the opposite effect. To verify whether these effects were mediated by ATG16L1, SGC7901 cells were co-transfected with miR-874 inhibitors and shRNAs targeting ATG16L1. The downregulation of ATG16L1 by shRNAs reversed the effect of miR-874 inhibition on autophagy. Taken together, these findings indicated that miR-874 inhibits autophagy by targeting ATG16L1 in MDR cells, highlighting the potential of miR-874 as a target in human GC therapy.

To determine the effects of miR-874 in the regulation of drug resistance in vivo, a xenograft tumour model and clinical GC specimens were used. The results confirmed that miR-874 also regulates drug resistance in vivo. In addition, miR-874 was not associated with any clinicopathological parameters except distant metastasis. Furthermore, according to a Kaplan Meier analysis, the prognosis of patients with GC was associated with the expression level of miR-874. Thus, miR-874 may serve as an MDR marker in GC to guide chemotherapy and may be used as a predictor of overall survival.

miR-874 was also demonstrated to be significantly downregulated in chemoresistant cells and GC tissue samples. To further explore whether chemotherapeutic agents influenced the expression of miR-874, two typical gastric adenocarcinoma cell lines, AGS and BGC823, were treated with 5-FU, which is widely used in clinical settings as a chemotherapeutic agent. The results revealed that 5-FU treatment significantly decreased the expression of miR-874. Further studies are required to determine the exact effects of chemotherapy on the expression of miR-874.

In summary, the results of the current study suggest that miR-874 is a novel miRNA, which regulates MDR in GC. miR-874 inhibits autophagy by targeting ATG16L1 in MDR cells, leading to increased chemotherapeutic sensitivity. These findings reveal a novel miR-874/ATG16L1/autophagy/chemosensitivity regulatory axis. Furthermore, this study has clinical relevance as miR-874 as a target in human GC therapy.

Acknowledgements
Not applicable.

Funding
This study was funded by a Huai’an International Science and Technology Cooperation Research Project (grant no. HAC201709), the 333 High-Level Talents Training Project of Jiangsu Province (grant no. BRA2017247), and a Jiangsu Province Young Medical Talent Project (grant no. QNRC2016423).

Availability of data and materials
The datasets used and/or analysed in the current study are available from the corresponding author on reasonable request.

Authors’ contributions
HH, JT and LZ designed and performed the experiments and contributed to the data analysis; YB enrolled the patients, measured the RNA levels in the clinical samples and analysed the relevant data; XZ initiated the work and wrote the manuscript; and all authors read and approved the final version of the manuscript.

Ethics approval and consent to participate
The use of human tissues was approved by the Ethics Committee of the Affiliated Huai’an Hospital of Xu Zhou Medical University, and patient consent was obtained.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References


