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Original article

MicroRNA-143 enhances chemosensitivity of Quercetin through autophagy inhibition via target GABARAPL1 in gastric cancer cells

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ABSTRACT

MicroRNAs have emerged as fundamental regulators in gene expression through silencing gene expression at the post-transcriptional and translational levels. In this study, miR-143 expression and biological functions in AGS/MNK28 cell lines was investigated. Results indicated that the expression of miR-143 was significantly down-regulated in cancer tissues and in gastric cancer (GC) cell lines. Target prediction algorithms (Target Scan and miRanda) showed that GABARAPL1 was a potential target gene of miR-143. GABARAPL1, also regarded as autophagy-related protein 8 (Atg8) is a ubiquitin-like protein required for the formation of autophagosomal membranes. Then, several different assays were conducted to detect autophagy in AGS/MNK28 after transfected with miR-143. In the present study, miR-143 was firstly identified as a autophagy inhibitor in GC cells via targeting GABARAPL1. Quercetin is one of the most prominent dietary antioxidants in human diet and lately it is grabbing some serious attention as a potentially powerful cancer fighter. However, the effect of Quercetin was unexpected decreased in GC cells on account of the appearance of Quercetin-induced autophagy. Therefore, applicable autophagy inhibitors might enhance the chemosensitivity of Quercetin. Furthermore, the therapeutic response of Quercetin in the combination of miR-143 was evaluated by MTT, Hochest and western blot, results suggesting that the chemosensitivity of Quercetin was enhanced when in combination with miR-143 in AGS/MNK28 cells. In conclusion, we determined miR-143 as a potent inhibitor of autophagy via targeting GABARAPL1 and miR-143 could improve the efficacy of Quercetin though autophagy inhibition in GC cell lines, thus representing a novel potential therapeutic target for gastric cancer.

1. Introduction

Gastric cancer (GC) is the second leading cause of cancer-related deaths and approximately 760,000 cases of stomach cancer are diagnosed worldwide. Despite of the available multimodality treatment, no effective therapy for advanced gastric cancer has been developed until now [1]. Intriguingly, intrinsic resistance via cell autophagy resulted in limited efficacy of chemotherapeutics has been certified in a wide variety of cancer cells, including gastric cancer [2–4]. Autophagy is a highly conserved process that entails the degradation of intracellular components to regenerate metabolites for energy and growth through the lysosomal machinery [5]. According to previous studies, autophagy promotes cell survival under stressful conditions such as nutrient and growth factor deprivation [6,7].

Quercetin, a bioflavonoid commonly present and dietary antioxidant present in a variety of fruits and vegetables [8–10]. Recently, substantial evidences implicate that Quercetin exerts potential anti-cancer effects on different malignant cells through induction of growth arrest in G1/G2 [11,12], apoptosis [13] and inhibition of angiogenesis [14]. Prior researches also shown that autophagy usually acted as a protective mechanism in tumor cells to against Quercetin [15]. Therefore, applicable autophagy inhibitors might enhance the chemosensitivity of Quercetin, especially in clinical application.

MicroRNAs (miRNAs) are an abundant class of small, noncoding RNAs that negatively regulate gene expression by inhibiting ribosome function at the post-transcriptional level by degrading the target mRNA [16]. miRNAs usually play critical roles in myriad cellular function and biological processes, including cell growth, cellular differentiation and apoptosis [17,18]. miR-143 have been certified as a tumor suppressor gene and lost expression in several cancer types [19–23]. However, the relevant effect of miR-143 in gastric cancer remains largely unknown. In the present study, we

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examined miR-143 effect on the cell proliferation and cell apoptosis in human gastric cancer cell lines AGS and MNK28. Results indicated that the expression of miR-143 was significantly down-regulated in the tissues of GC tissues and miR-143 was firstly identified as a autophagy inhibitor in gastric cancer cells via targeting GABARAPL1(Atg8), which is a ubiquitin-like protein required for the formation of autophagosomal membranes. Furthermore, to clarify the underlying mechanisms of miR-143 related to autophagy, we determined whether miR-143 could enhanced the chemosensitivity of Quercetin through autophagy inhibition in GC cells. In this study, We confirmed that miR-143 as a new autophagy inhibitor in human GC cells and the information

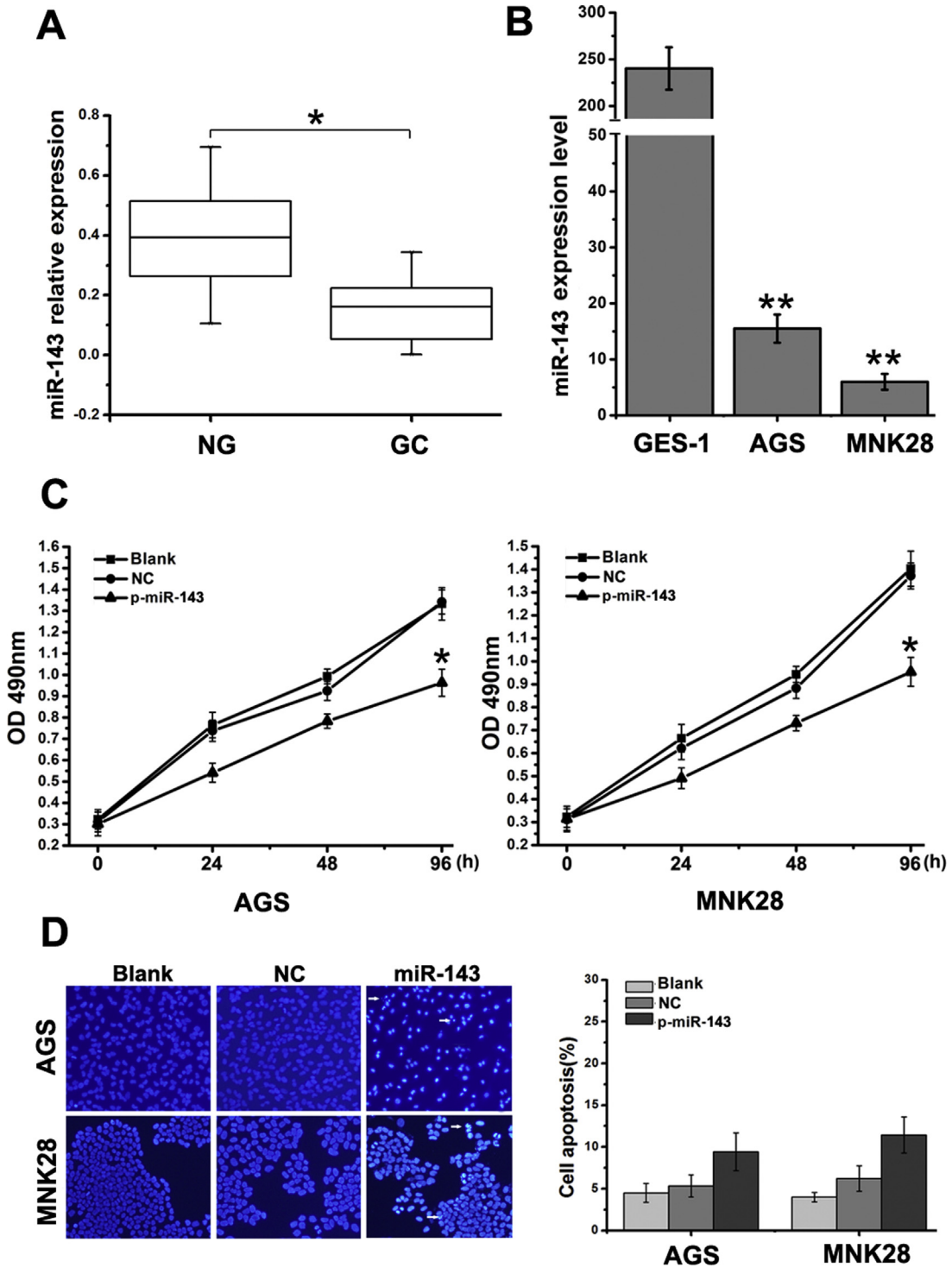


Fig. 1. miR-143 expression is down-regulated in gastric cancer and over expression of miR-143 inhibits cell proliferation and promotes cell apoptosis in human gastric cancer cells. (A) Fifty-two pairs of GC tissues and paired normal gastric mucosal tissues from patients were assessed by qRT-PCR analysis ($^{*}P < 0.05$). The values were standardized to the U6 expression level. (B) The expression levels of miR-143 in GES-1 cells and GC cell lines AGS and MNK28 were measured by qRT-PCR. The values represent the mean ± standard deviation of triplicates ($^{**}P < 0.01$). (C) MTT assays were performed to detect the cell viability of AGS and MNK28 after transfected with miR-143 or negative control (NC) ($^{*}P < 0.05$). (D) Hochest assays were performed to detect cell apoptosis after transfected with miR-143 and negative control. (GC, gastric cancer; qRT-PCR, real-time quantitative polymerase chain reaction).

provided in this article would be meaningful for further studies of cancer therapeutic treatment related to autophagy or miRNAs.

2. Materials and methods

2.1. Cell lines and tissue specimens

Human gastric cancer cell lines AGS and MKN28 cells were purchased from American Type Culture Collection (ATCC) and non-malignant gastric epithelial cell line GES-1 were obtained from Shanghai Institute of Digestive Surgery. All cell lines were cultured with the complete medium DMEM-F12 medium (Invitrogen) with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 100 units/ml of penicillin G sodium and 100 μg/ml streptomycin sulfate (Sigma), All cells were incubated at 5% CO^2 at 37 °C. 52 Gastric carcinoma (GC) and paired normal gastric mucosal tissues were randomly obtained from Department of gastroenterology. The histologic diagnosis of tumors was made and agreed upon by two senior pathologists based on World Health Organization (WHO) criteria. This study was approved by the Institutional Review Board and Human Ethics Committee Second People's Hospital of Liaocheng. Tissue samples were flash frozen and stored at liquid nitrogen until used.

2.2. Regents and antibodies

Reagents used were as follows: Quercetin (Sigma, Q4951), MTT (Sigma, M5655), Hoechst 33,342 (Sigma, B2261), acridine orange (AVO, Sigma, A6014), Quercetin was dissolved in DMSO, while MTT, Hoechst 33342, acridine orange were dissolved in phosphate-buffered saline (PBS). Antibodies were obtained from the following sources: LC3I/II (Abcam, ab58610), GABARAPL1 (Abcam, ab77586), caspase3 (Abcam, ab2171), β-actin (Abcam, ab6276).

2.3. Quantitative real-time PCR analysis

Total RNA for Quantitative real-time PCR (qRT-PCR) analysis was extracted using 74,104 Rneasy Mini Kit (Qiagen) and reverse-transcribed into cDNA with the Reverse Transcriptase MMLV (Takara) according to the manufacturer's protocol. For analysis of miR-143 expression by qRT-PCR, reverse transcription and PCR were carried out using Bulge-Loop miRNA qPCR Primer Set for hsa-miR-143 and U6 snRNA (RiboBio, China) according to the manufacturer's instructions. Following parameters: 95 °C for 20 s followed by 50 cycles of 95 °C for 10 s and 55 °C for 20 s. The relative expression level of miR-143 was normalized to that of internal control U6 by using $2^{-\Delta\Delta Ct}$ cycle threshold method.

2.4. Cell transfection

pre-miR-143 precursors(p-miR-143) and negative control (NC) were purchased from Ambion® life Technologies (pre-miR™ miRNA Precursor system) and transfected with Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol.

2.5. Cell proliferation assay and cell apoptosis assay

Cells were seeded in 96-well culture plates and were evaluated by MTT assays after transfected with or without miR-143. Similar method description of the prior study [24].

2.6. Luciferase reporter assay

The 3'untranslated region (UTR) of the human GABARAPL1 gene that was predicted to interact with miR-143 was synthesized and inserted into pMIR-REPORT (Ambion), yielding pMIR-REPORT GABARAPL1. Mutations within potential miR-143 binding sites were generated by nucleotide replacement of wild-type sequence to inhibit miR-143 binding. AGS cells were plated into 96-well plates and were co-transfected with 0.4 mg of the reporter construct, 0.2 mg of control vector, and miR-143 negative controls (NC) after 24-h incubation. Luciferase values were determined using the Dual-Luciferase Reporter Assay System. All transfection experiments were performed in triplicate and reproduced at 3 times.

2.7. Detection of acidic vesicular organelles (AVOs)

Cells were maintained on coverslips in 6-well plates and after transfection with or without miR-143 for 24 h, cells were treated with 1 μg/ml acridine orange in PBS for 10 min, washed with PBS and examined under fluorescence microscope (Olympus Optical Co, Germany).

2.8. Detection of GFP-LC3 autophagic dots

The ANG and MNK28 cells which stably expressed high level of GFP-LC3 protein were established by pcDNA3.1-GFP-LC3 transfected and were selected by G418. Stable expression of GFP-LC3 cells was confirmed by fluorescence microscope and used for next experiment. The ANG-GFP-LC3 and MNK28-GFP-LC3 cells were maintained on coverslips in 6-well plates and after transfection with or without miR-143 for 24 h, the percentage of GFP-LC3 punctate-positive cells was quantified and analysis by automated image acquisition using a threshold of $\geq$5 dots/cell. Data were shown as the mean ± s.d. and was representative of three independent experiments.

2.9. Western blot

Similar to the method description of the prior study [25].

2.10. Statistical analysis

All statistical analyses were performed using SPSS 13.0 software. The data were expressed as mean ± s.d., one-way ANOVA and an unpaired Student's *t*-test were used to determine the significant differences of results. The level of significance was set at $p < 0.05$.

3. Result

3.1. miR-143 expression is down-regulated in gastric cancer

52 Gastric carcinoma (GC) and paired normal gastric mucosal tissues were obtained and qPCR was used to analysis miR-143 expression in patient's tissues. Result showed that the expression of miR-143 in Gastric carcinoma tissues (41/52,78.9%) was significantly down-regulated compared with the their normal counterparts (Fig. 1A). Besides, miR-143 expressions in gastric cancer cell lines (AGS and MNK28) were lower than those in non-malignant gastric epithelial cell line GES-1 (Fig. 1B). Subsequent investigate showed that the expression levels of miR-143 were not associated with age, sex, TNM stage and lymphatic metastasis (Supplement Fig. 1A). To investigate the biological function of miR-143 in gastric cancer, the expression of miR-143 restored by transfection of p-miR-143 when compared with the negative control (NC) group (Supplement Fig. 1B). Then, cell proliferation and cell apoptosis assays were performed after AGS and MNK28 transfected with p-miR-143 or NC. We found that cell viability of AGS and MNK28 were inhibited after transfected with p-miR-143 (Fig. 1C). Meanwhile, emerging cell apoptosis were

observed in the p-miR-143 group, while, in contrast, NC group almost did not appear cell apoptosis (Fig. 1D). Data above indicated that miR-143 could inhibit cell proliferation and promote cell apoptosis in human gastric cancer cells.

3.2. miR-143 direct GABARAPL1 in gastric cancer cells

GABARAPL1 was predicted to be target gene of miR-410 (Target prediction algorithms, Target Scan) and the putative binding sites of miR-143 in the 3'UTR of GABARAPL1 mRNA was predicted. GABARAPL1,also regarded as ATG8, is a ubiquitin-like protein and a component of large protein complex essential for autophagosomes formation. Recently, autophagy is generally considered a prosurvival mechanism that preserves cell viability in cancer therapy but the mechanism under the treatment remains large unknown. Here, luciferase reporter vector containing wild type (WT) or mutant (MT) 3′UTR of GABARAPL1 (Fig. 2A) was constructed and luciferase assay showed that miR-143 inhibited the luciferase intensity in GABARAPL1 3′UTR but the inhibitory effect was abolished when the binding sites of GABARAPL1 3′UTR were mutated (Fig. 2B). To validate whether GABARAPL1 expression have direct influence on autophagy or not, protein interaction of GABARAPL1 and LC3 was proceed. Transform processing of full-length LC3I to LC3II is regarded as an important symbol of autophagy activation and LC3II recruited to autophagosomal membranes reflected autophagic activity directly. Immunoprecipitation and immunoblotting assays shown there is a direct interaction between GABARAPL1 and LC3 (Fig. 2C). Further study indicated the GABARAPL1-LC3 binding protein was accumulated under the starvation treatment when compared to the nutrition-rich group (Fig. 2D). The results suggested that miR-143 may work as a anti-cancer gene which relate to autophagy inhibition.

3.3. miR-143 inhibit autophagy effectively in gastric cancer

To validate the repressive effect of miR-143 overexpression on autophagy in gastric cancer, several independent methods were employed. Firstly, acridine orange staining(AVO) was used to analyze the formation of acidic vesicular organelles (AVOs), a main feature of autophagy [26,27]. The starvation treatment

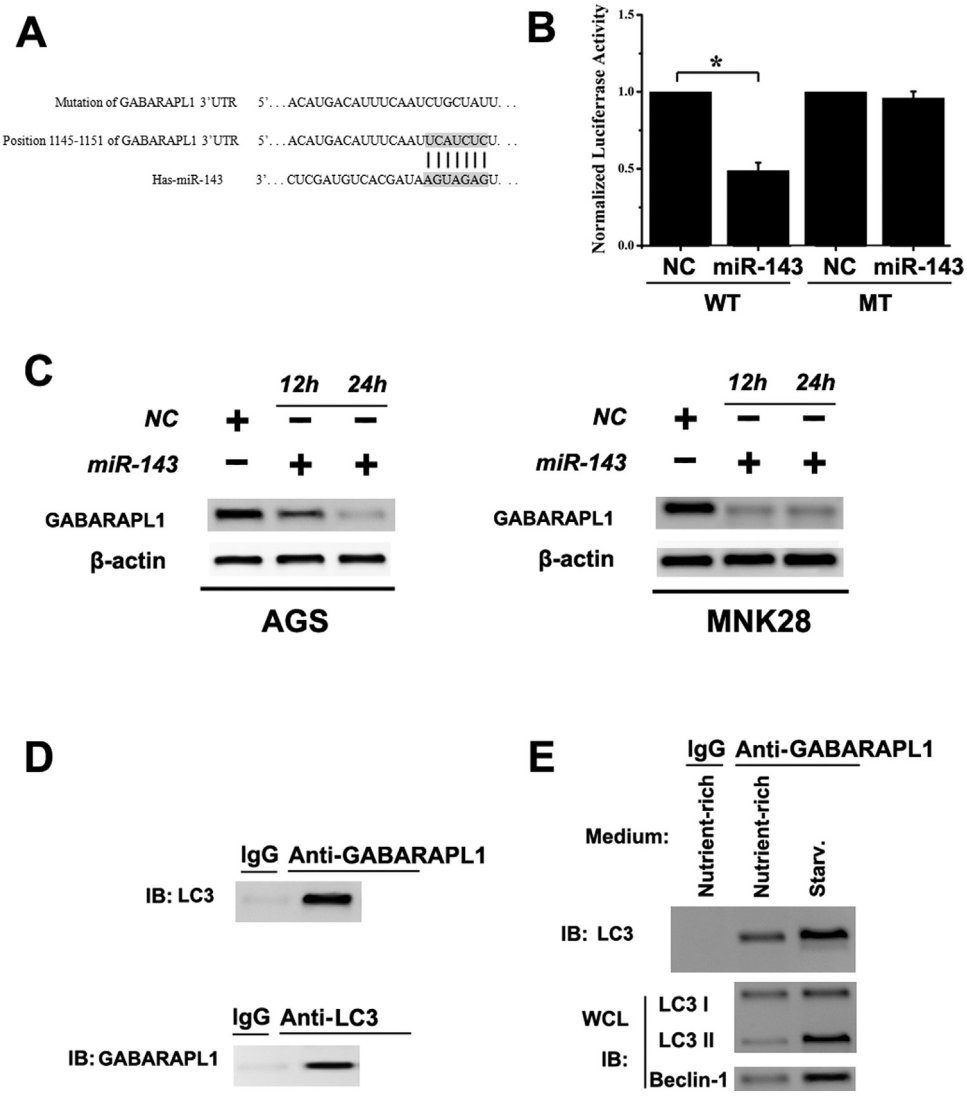


Fig. 2. miR-143 directly target GABARAPL1 in gastric cancer cell lines. (A) miR-143/GABARAPL1 alignment by miRanda analysis and the schematic diagram of the pMIR-GABARAPL1/pMIR- GABARAPL1^{mut} paired sequences for miR-143. (B) Normalized luciferase activity of pMIR- GABARAPL1 /pMIR- GABARAPL1^{mut} reporter in 293T cells transfected with miR-143 mimics or the negative control (NC). (C) Immunoblotting was used to detect the expression of GABARAPL1 after ANS and MNK28 transfected with miR-143 or the negative control (NC) in 24 h. (D) GABARAPL1-LC3 interaction in vivo in ANS. IB, immunoblotting; IP, immunoprecipitation. (E) GABARAPL1-LC3 interaction under the Medium with serum (nutrient-rich) or serum deprivation (starv.) after ANS transfected with miR-143 or the negative control (NC); WCL, whole-cell lysate.

(serum-free) induced pronounced formation of orange AVOs in gastric cancer cells, while cells transfected with p-miR-143 exhibit green fluorescence, which indicating the lack of AVOs (Fig. 3A). Then, we constructed GFP-LC3 stable expressing cell strains (AGS -GFP-LC3 and MNK28-GFP-LC3) after AGS and MNK28 were transfected with p-GFP-LC3. AGS-GFP-LC3 and MNK28-GFP-LC3 which stably expressed high level of GFP-LC3 protein were used to quantify accumulation of autophagosomes via GFP-LC3 translocation. Further, the percentage of GFP-LC3 puncta-positive cells was significantly reduced in cells overexpression miR-143 (Fig. 3B and C). Western blot also showed that over-expression of miR-143 in AGS and MNK28 cells resulted in the decreased expression of GABARAPL1 and LC3I/LC3II (Fig. 3D). Therefore, we demonstrated that miR-143 functioned as a negative regulator of autophagy in GC cells. In addition, we hypothesised that the anti-tumor effect of miR-143 was seem to be enhanced under starvation treatment which stimulated autophagy in above experiments.

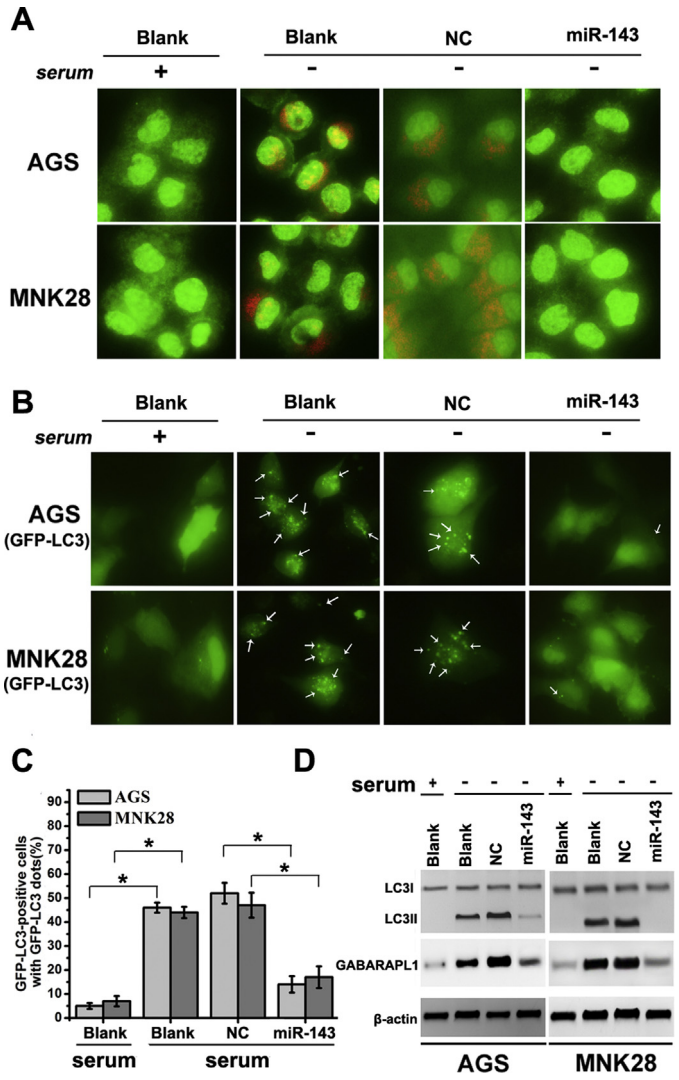


Fig. 3. miR-143 inhibit autophagy effectively in gastric cancer cell lines. (A) Acridine orange staining of AGS and MKN28 cells treated with NC, p-miR-143 for 24 h. (B)ANG-GFP-LC3/ MNK28-GFP-LC3 cells which stable expressing GFP-LC3 were used to defect autophagy and autophagy was activated by the serum deprivation(starvation treatment). (C) The percentage of GFP-LC3 punctate-positive cells was quantified and analysis by automated image acquisition using a threshold of ≥5 dots/cell after 24 h starvation treatment. Data were shown as the mean ± s.d. and was representative of three independent experiments. (*$P < 0.05$). (D) Western Blot analysis of LC3I/II, and GABARAPL1 expression from lysates of AGS and MKN28 cells treated with NC, miR-143 or serum deprivation for 24 h. β-actin was used as the internal control.

3.4. The anti-tumor effect of miR-143 was enhanced through autophagy inhibition

In order to verify our hypothesis, MTT and Hochest assays were employed to characterise the cell viability and cell apoptosis respectively. As shown in Fig. 4A and B, cell viability in AGS and MNK28 were significantly decreased, and cell apoptosis were increased sharply. Furthermore, the expression of autophagy marker protein LC3I/LC3II and cell apoptosis protein cleaved-caspase3 were measured by Western blot. Results suggested that autophagy marker protein LC3I/LC3II expression was increased under starvation treatment (serum-free), while miR-143 inhibited the expression of LC3I/LC3II through autophagy inhibition. Moreover, compared with the serum (+) groups transfected with miR-143, serum (−) groups transfected with miR-143 emerged more apoptosis protein cleaved-caspase3 expression (Fig. 4C). Thus, these results indicated that autophagy acted as a protective mechanism when cells was exposed to nutrient and growth factor deprivation (serum-free). Besides, it suggested that autophagy inhibition via miR-143 improve the efficacy of miR-143 therapy in GC cell lines.

3.5. Quercetin exhibited effective anti-tumor activity under the combination of miR-143

For the first time, miR-143 was verified a potential autophagy inhibitor in GC cells in our study. Besides, autophagy was identified as a protective mechanism when GC cells was treated with Quercetin [2]. We characterized whether or not miR-143 could enhances chemosensitivity of Quercetin through autophagy inhibition in GC. To validate the repressive effect of miR-143 in combination with Quercetin, several different assays were conducted. Firstly, 50% inhibitive concentration (IC_{50}) of Quercetin was examined by MTT assay. We found that the IC_{50} values of Quercetin against AGS and MKN28 cells were 40 μM and 150 μM. Meanwhile, the IC_{50} values of Quercetin deceased obviously with the presence of miR-143 pre-treated (Fig. 5A and B). Cell apoptosis was also emerging accelerated (Fig. 5C), while autophagy was significantly decreased in the combination group (Fig. 5D). It suggested that miR-143 sensitized AGS and MKN28 to Quercetin in a synergistic fashion, and the combination therapy could improve therapeutic response of Quercetin via autophagy inhibition. Finally, we found that the expression of autophagy marker proteins, LC3I/II and the target of miR-143, GABARAPL1, both increased when cells were treated with Quercetin, while, in contrast, decreased in the combination therapy group and after cell autophagy inhibition via miR-143 treatment alone. Furthermore, the cleaved-caspase3 was observed increased sharply (Fig. 5E). As a result, these data indicated that miR-143 could enhance chemosensitivity of Quercetin through autophagy inhibition in GC cells (Fig. 5F).

4. Discussion

Autophagy is a lysosomal degradation pathway that is essential for survival, differentiation, development, and homeostasis [28]. The process of autophagy is to sequester the cytoplasmic materials to form a double membrane vesicle and then fuse with lysosomes. The degradation products, including amino acids, carbohydrates and lipids, are released into the cytosol to synthesize new proteins and organelles for recycling or decomposed to provide energy for cells [29]. In most chemotherapeutic strategies, after the microenvironment of cancer cells is disturbed by pharmaceuticals,

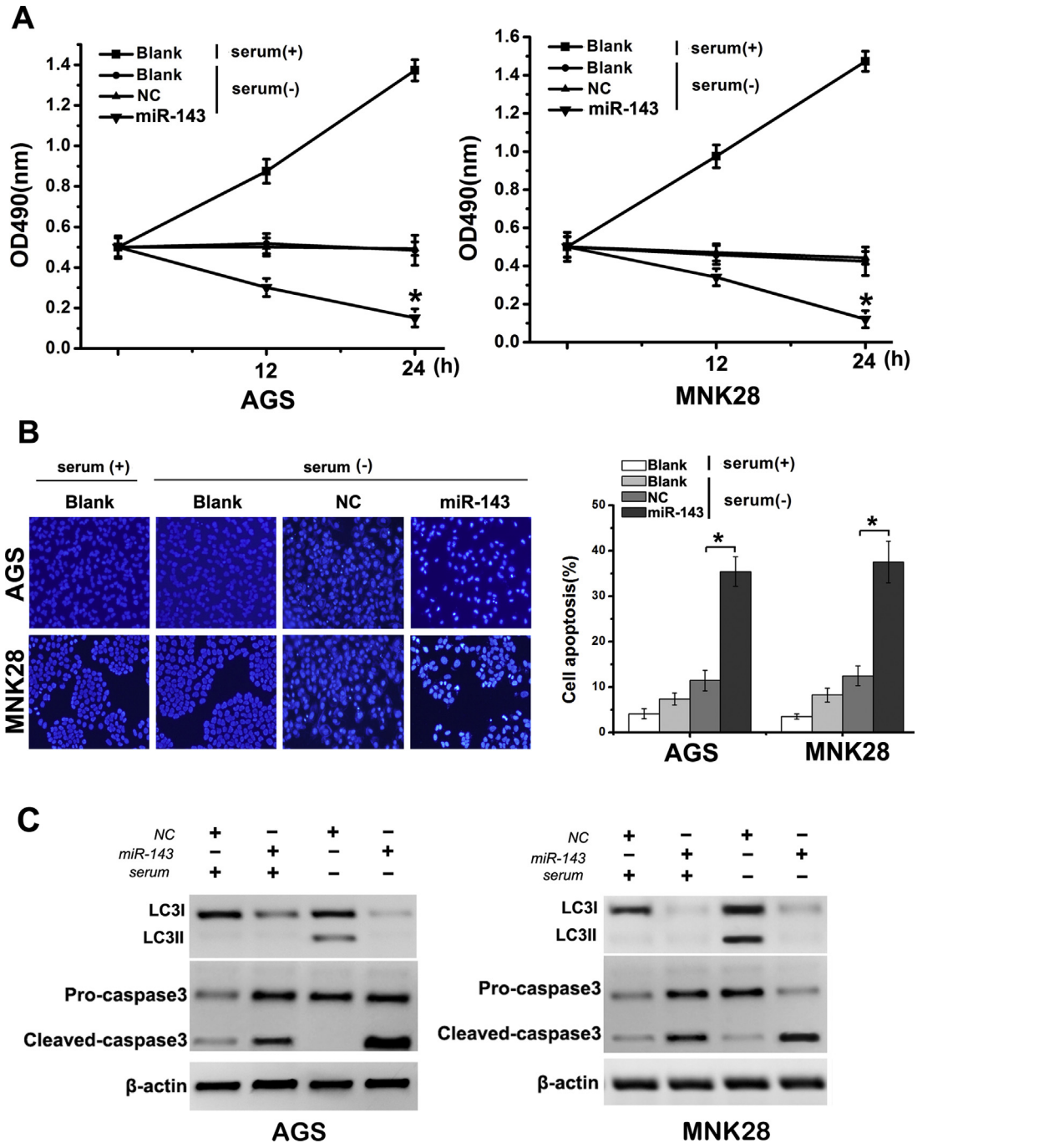
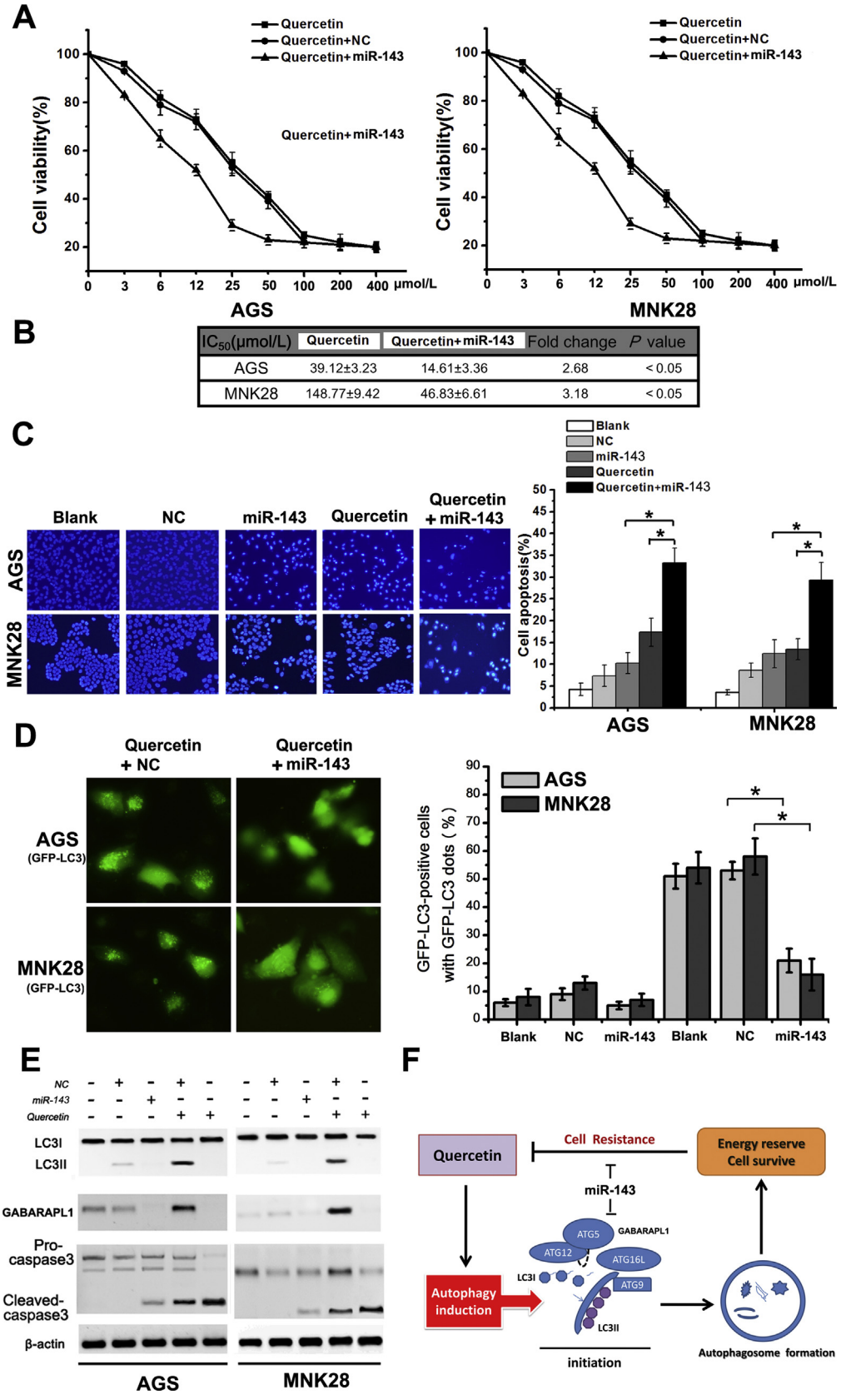


Fig. 4. The anti-tumor effect of miR-143 was enhanced through autophagy inhibition. (A) MTT assays were used to detect the cell viability of AGS and MNK28 after transfected with miR-143 or NC under the serum deprivation (*$P < 0.05$). (B) Hochest assays were performed to detect cell apoptosis after transfected with miR-143 or NC under the serum deprivation or not. (C) Western Blot analysis of LC3I/II and cleaved-caspase3 expression from lysates of AGS and MKN28 cells treated with miR-143 or NC under serum deprivation-induced autophagy in 24 h. β-actin was used as the internal control.

autophagy usually functions as a protective mechanism that degrade the abnormal proteins and organelles and then reuse these as energy resources to promote cell survival [30–33].

Quercetin have many potential medicinal values and the anti-tumor activity is one of the research hot spot [34–36]. In human gastric cancer, substantial evidence implicates a functional role of Quercetin as a powerful cancer fighter [37–39]. However, Wang et al. found that Quercetin induces protective autophagy in gastric cancer cells and the autophagy inhibitor-Chloroquine enhanced Quercetin-induced suppression of gastric cancer cell growth through AKT/HIF-1α signalling [2]. Thus, autophagy inhibitors seems to be a potential choice in combination with Quercetin to treat gastric cancer. However, classical autophagy inhibitors, such as 3-methyladenine [40] and Chloroquine [41], have different defects in clinical application [42]. For example, 3-methyladenine represents a dual role in modulation of autophagy via different temporal patterns of inhibition on class I and III phosphoinositide 3-kinase and sort of chemical toxicity to ordinary cells [43–46]. Chloroquine was utilized to disrupt lysosomal function and prevent completion of autophagy. However, Recently it has been proved acidic extracellular pH would neutralize the autophagy-inhibiting activity of chloroquine and chloroquine sensitivity to



IC_{50}(μmol/L)	Quercetin	Quercetin+miR-143	Fold change	*P* value
AGS	39.12±3.23	14.61±3.36	2.68	< 0.05
MNK28	148.77±9.42	46.83±6.61	3.18	< 0.05

Fig. 5. Quercetin exhibited effective anti-tumor activity in gastric cancer cells under the pre-treatment of miR-143. (A) AGS and MNK28 cells transfected with miR-143 were treated with different concentration of Quercetin and Cell viability was measured using the MTT assay. (B) The IC_{50} for different treatment was calculated and is shown in the table (*$P < 0.05$). (C) Hochest assay was used to detect cell apoptosis after treated with Quercetin (40 μM for AGS cells and 150 μM for MKN28 cells) in the absence or presence of miR-143 in 24 h. (D) The percentage of GFP-LC3 positive cells was quantified and analysis by automated image acquisition using a threshold of ≥5 dots/cell. Data were shown as the mean ± s.d. and was representative of three independent experiments. (*$P < 0.05$). (E) Immunoblot analysis of LC3I/II, GABARAPL1 and Cleaved-caspase3 expression from lysates of AGS and MKN28 cells treated with certain concentrations of quercetin for 24 h. β-actin was used as the internal control. (F) miR-143 enhances chemosensitivity of Quercetin through autophagy inhibition.

autophagy is context-dependent [47–49]. To find a new kind of autophagy inhibitor which characterise a specific and effective inhibition on autophagy and improve therapeutic response of Quercetin has been the focus of the study. Lisa Frankel et al. found that miR-101-mediated inhibition of autophagy via targeting ATG4D sensitized MCF-7 cells to 4-hydroxy-tamoxifen (4-OHT)-mediated cell death [50]. However, the function role of other miRNAs related to autophagy in tumors are still largely unknown.

In this research, we found that the expression of miR-143 was significantly down-regulated when compared with the paired normal gastric mucosal tissues in most Gastric carcinoma cases (41/52,78.9%). Further results indicated that transfection of miR-143 inhibited autophagy significantly which reflected in the decreasing formation of orange AVOs and GFP-LC3 autophagic dots. However, the most interesting was that we actually took notice of higher anti-cancer efficacy of miR-143 under the starvation treatment

which simulated autophagy in GC cell lines. Autophagy has been identified as a protective mechanism when gastric cancer cells was treated with Quercetin [2] and the inhibition of autophagy might improve the efficacy of Quercetin in gastric cancer cells. As shown in the result, cell viability was significantly decreased and cell apoptosis was accumulated under the combination therapy treatment. Emerging cell apoptosis were also observed in the combination group of Quercetin and miR-143, which suggested that miR-143 could enhance the chemosensitivity of Quercetin through autophagy inhibition in gastric cancer cells. Moreover, limited cell cytotoxicity of miR-143 was proved by MTT assay in gastric cancer cells when compared with the conventional autophagy inhibitor 3-methyladenine and chloroquine.

In summary, autophagy inhibitor in combination with cytotoxic chemotherapy would be a promising therapeutic strategy for gastric cancer. In this study, miR-143 was firstly confirmed as a potential autophagy inhibitor in gastric cancer cells and sensitized gastric cancer cells to Quercetin through autophagy inhibition. Information provided in this article would be useful for further studies of quercetin or miR-143 in cancer therapeutic treatment.

Conflict of interest statement

There are no conflicts of interest.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biopha.2015.08.005.

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