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WORD COUNT	6798	TIME SUBMITTED	30-MAY-2023 11:00AM
		PAPER ID	100076194

Name of Journal: *World Journal of Gastroenterology*
Manuscript NO: 84682
Manuscript Type: ORIGINAL ARTICLE

Basic Study

18β-glycyrrhetinic acid promotes gastric cancer cell autophagy and inhibits proliferation by regulating miR-328-3p/signal transducer and activator of transcription-3

Yang Y *et al*. 18β-GRA inhibits GC proliferation *via* miR-328-3p/STAT3

Abstract

BACKGROUND

Gastric cancer (GC) is one of the most common cancer types in the world, and its prevention and treatment methods have attracted much attention. As the active ingredient of licorice, 18β-glycyrrhetinic acid (18β-GRA) has a variety of pharmacological effects. To explore the effective target of 18β-GRA in the treatment of GC, in order to provide effective ideas for clinical prevention and treatment of GC.

AIM

To investigate the mechanism of 18β-GRA in inhibiting the cell proliferation and promoting autophagy flux in GC cell.

METHODS

Whole transcriptomic analysis was used to analyze and screen differentially expressed microRNAs in AGS cells after 18β-GRA intervention. Lentivirus transfected AGS cells, CCK-8 was used to detect the cell proliferation ability, and cell colony formation ability was detected by clone formation assay, and flow cytometry was used to detect cell cycle and apoptosis. The nude mouse transplanted tumor model of AGS cells was constructed to verify the effect of overexpression of miR-328-3p on the tumorigenicity of GC cell. Tumor tissue morphology was observed by hematoxylin-eosin staining, and microtubule-associated proteins light chain 3 (LC3) expression was detected by immunohistochemistry. TransmiR, STRING and miRWalk databases were used to predict the relationship between miR-328-3p and signal transducer and activator of transcription 3 (STAT3) related information. Expressions of *STAT3* mRNA and *miR-328-3p* were detected by real-time quantitative polymerase chain reaction (qRT-PCR) and expression levels of STAT3, p-STAT3 and LC3 were detected by western blot. The targeted relationship between *miR-328-3p* and *STAT3* was detected using the dual-luciferase reporter gene system. AGS cells were infected with monomeric Red

Fluorescent Protein-green fluorescent protein-LC3 adenovirus double label, LC3 was labeled and autophagy flow was observed under a confocal laser microscope.

RESULTS

The expression of miR-328-3p was significantly up-regulated after 18β-GRA intervention in AGS cells (P = 4.51E-06). Overexpression of miR-328-3p can inhibits GC cell proliferation, colony formation ability, arrest of the cell cycle in the G0/G1 phase, promotes cell apoptosis, and inhibit the growth of subcutaneous tumors in BALB/c nude mice ($P < 0.01$). No obvious necrosis was observed in the tumor tissue in negative control (no drug intervention or lentivirus transfection) and vector groups (the blank vector for lentivirus transfection), and more cells were loose and necrotic in miR-328-3p group. Bioinformatics tools predict that miR-328-3p has a targeting relationship with STAT3, and STAT3 was closely related to autophagy markers such as p62. After overexpressing miR-328-3p, the expression level of *STAT3* mRNA was significantly decreased ($P < 0.01$), and p-STAT3 were down-regulated ($P < 0.05$). Dual-luciferase reporter gene assay showed that the luciferase activity of miR-328-3p and *STAT3* 3′ untranslated regions (3′UTRs) wild-type reporter vector group was significantly decreased ($P < 0.001$). Overexpressed miR-328-3p combined with Baf A_1 was used to detect the expression of LC3 II, compared with the vector group, the expression level of LC3 II in the overexpressing miR-328-3p group was down-regulated ($P < 0.05$), and compared with Baf A_1 group, the expression level of LC3 II in overexpressed miR-328-3p+Baf A_1 group was up-regulated ($P < 0.01$). The expression of LC3 II was detected after the intervention of 18β-GRA in GC cell, and the results were consistent with the results of overexpression of miR-328-3p ($P < 0.05$). Further studies showed that 18β-GRA promoted autophagy flow by promoting autophagosome synthesis ($P < 0.001$). qRT-PCR showed that the expression of *STAT3* mRNA was down-regulated after drug intervention ($P < 0.05$), Western blot showed that the expression levels of STAT3 and p-STAT3 were significantly down-regulated after drug intervention ($P < 0.05$).

CONCLUSION

18β-GRA promotes the synthesis of autophagosomes and inhibits GC cell proliferation by regulating the miR-328-3p/STAT3 signaling pathway.

Key Words: 18β-Glycyrrhetinic acid; miR-328-3p; Signal transducer and activator of transcription 3; Cell proliferation; Autophagy flow

Yang Y, Nan Y, Du YH, Huang SC, Lu DD, Zhang JF, Li X, Chen Y, Zhang L, Yuan L. 18β-glycyrrhetinic acid promotes gastric cancer cell autophagy and inhibits proliferation by regulating miR-328-3p/signal transducer and activator of transcription-3. *World J Gastroenterol* 2023; In press

Core Tip: 18β-glycyrrhetinic acid (18β-GRA) is an important bioactive component of glycyrrhiza liquorice. Our results showed that 18β-GRA up-regulates the expression of miR-328-3p in gastric cancer (GC) cell. Overexpression of miR-328-3p inhibits GC cell proliferation and colony formation, arrests the cell cycle, promotes apoptosis, inhibits subcutaneous tumor formation, and signal transducer and activator of transcription 3 (STAT3) expression. Dual-luciferase reporter assay showed that miR-328-3p could target the regulation of STAT3. The study of monomeric Red Fluorescent Protein-green fluorescent protein-light chain 3 double-labeled adenovirus and western blot indicated that 18β-GRA promotes autophagy flow by promoting autophagosome synthesis. These data suggest that 18β-GRA promotes cell autophagy and inhibits the proliferation of GC cell by regulating miR-328-3p/STAT3.

INTRODUCTION

Gastric cancer (GC) is the fifth most common type of cancer and the fourth leading cause of cancer-related death worldwide[1]. GC is a local disease, and the main causes of GC are diet, environment, genetic factors and Helicobacter pylori infection[2]. The current standard treatment for GC is surgical excision of operable tumor tissue,

combined with local radiotherapy or chemotherapy and conventional anticancer drugs. However, many GC cell will appear to have significant invasion and metastasis after surgery. Although chemotherapy is an important means to prevent invasion and metastasis, the quality of life of patients is sharply decreased due to serious toxic and side effects (hair loss, bone marrow transplantation, gastrointestinal reactions, *etc.*) and drug resistance[3,4]. Therefore, there is an urgent need to find natural, effective and low-toxic anti-GC drugs.

Glycyrrhiza uralensis Fisch. is a traditional Chinese medicine, and 18β-glycyrrhetinic acid (18β-GRA, Figure 1A), as an important bioactive component of licorice, has been shown to have many pharmacological effects, including anti-inflammatory, liver protection, antiviral, anti-allergic, anti-tumor, antioxidant and immunomodulatory effects[5-8]. In recent years, it has been found that 18β-GRA has excellent antitumor activity against human malignant tumors such as breast cancer, glioma, lung cancer, and prostate cancer[8-12]. In GC, 18β-GRA regulates migration of SGC-7901 cells by regulating the reactive oxygen species (ROS)/protein kinase C-α/extracellular regulated protein kinases signaling pathway and dose-dependently inhibits the activities of matrix metalloproteinases 2 (MMP2) and MMP9[13]. It has also been discovered that the expression of the anti-oncogene potassium-transporting ATPase alpha chain 1 (ATP4a) is downregulated in GC, and 18-GRA can activate ATP4a, inhibiting the occurrence of GC[14]. In transgenic mice, 18β-GRA can improve the severity of gastritis and inhibit the occurrence of GC, and further studies showed that 18β-GRA can improve the tumor inflammatory microenvironment by down-regulating the expression of cyclooxygenase-2 and inhibit the expression of the direct target Wnt-1 by up-regulating the anti-oncogene miR-149-3p, thus inhibiting the occurrence and development of GC[15]. Cao *et al*[16] found that 18β-GRA regulates toll-like receptor 2 (TLR2) expression through methylation and inhibits the proliferation, energy metabolism and carcinogenesis of TLR2-activated GC cell. Our previous study found that 18β-GRA can inhibit GC cell proliferation by regulating mitochondrial ribosome

protein L35[17]. These results suggest that 18β-GRA may be an effective drug for the prevention and treatment of GC.

MicroRNA (miRNA) is a novel endogenous, highly conserved non-coding RNA molecule with a length of 18-25 nucleotides[18]. miRNAs play an important role in cancer, and so far, many human miRNA sequences have been identified to participate in cancer pathogenesis and regulate protein expression levels in a complementary manner to their target messenger RNAs (mRNAs). Regulates and participates in the regulation of tumorigenesis, cellular processes and pathways, such as proliferation, differentiation, and apoptosis[19]. miRNA expression profiles also differ between cancerous and non-cancerous tissues, between different types and subtypes of cancer, and between early and advanced cancers, as well as depending on the aggressiveness of the disease[20-22]. Current studies have found that some miRNAs can not only exist as oncogenes but also inhibit the occurrence of tumors. For example, miR-381 is up-regulated in glioma, osteosarcoma and other cancers, while overexpression of miR-381 in GC and colon cancer can inhibit cell migration and invasion, and promote cell apoptosis[23-26]. miRNA also plays a dual role in inducing or inhibiting tumorigenesis in GC. Studies have shown that the expression level of miR-148a in the plasma of GC patients is lower than that of healthy individuals, and miR-148a overexpression can inhibit proliferation, migration, cell invasion and epithelial-mesenchymal transition[27]. Li *et al*[28] found that low expression of miRNA-149 and overexpression of forkhead box C1 (FOXC1) were found in GC tissues and cell. Overexpression of miRNA-149 inhibits GC cell proliferation and migration, arrests cell cycle and promotes cell apoptosis. The biological functions of AGS and MKN28 cells in GC were inhibited by silencing FOXC1[28]. Overexpression of miR-140-3p can inhibit the migration and invasion ability of GC cell and directly down-regulate the expression of BCL2, which has the ability to inhibit cell apoptosis. Down-regulation of BCL2 further activates autophagy induced by Beclin-1, a marker of autophagy, and inhibits epithelial mesenchymal transformation[29]. More and more GC-associated miRNAs were found to play a role in the formation and development of GC.

Autophagy is essential in many physiological and pathological processes, such as development, metabolism, inflammation and cancer[30]. According to the mode of transport to lysosomes, autophagy can be divided into micro-autophagy, macro-autophagy and chaperone-mediated autophagy, and autophagy is closely related to the diagnosis, treatment and prognosis of GC. Through Oncomine analysis, Wang *et al*[31] found that there were 10 autophagy-related (*ATG*) genes with significant differences in expression between GC and para-cancer tissue. It was also found that genes such as *ATG14* were significantly associated with TNM stage, and the expression of these genes was significantly lower in early T, suggesting that autophagy may play a role in early GC progression[31]. The expression of miR-183 was significantly decreased in GC tissues compared with para-cancerous tissues, while mechanistic target of rapamycin (mTOR) was significantly increased. By targeting the expression of mTOR, miR-183 inhibited cell proliferation, promoted cell apoptosis and autophagy[32]. Sec62 is an endoplasmic reticulum (ER) membrane protein, which is highly expressed in GC. Overexpression of Sec62 could increase the expression of eukaryotic translation initiation factor 2-alpha kinase 3/cyclic AMP-dependent transcription factor ATF-4, binds to light chain 3 II (LC3 II), and activates the FIP200/Beclin-1/ATG5 pathway to activate autophagy. Therefore, dual inhibition of Sec62 and autophagy may be a therapeutic strategy for GC metastasis[33]. Thus, autophagy is a "double-edged sword" to promote and inhibit cell survival[34].

Therefore, on the basis of previous studies, this study further analyzed the differential expression of miRNA after the intervention of 18β-GRA. Cell function experiment and animal experiment were used to detect the cell phenotype and subcutaneous tumor formation in nude mice. The targeting relationship between miRNA and signal transducer and activator of transcription 3 (STAT3) and the changes of autophagy flow rate were detected. An important finding is that 18β-GRA can affect autophagy flow and inhibit GC cell proliferation by regulating the miR-328-3p/STAT3 signaling pathway, which may contribute to the clinical development of effective therapies.

MATERIALS AND METHODS

Cell culture

Human AGC cell lines were purchased from GeneChem (Cat. No. GCC-ST0003RT, Shanghai, China). The cell lines were cultured in DMEM/F12 medium (Cat. No. SH30023.01, Hyclone, United States) containing 100 mL/L fetal bovine serum (FBS, Cat. No. 35-081-CV, Corning, United States) and 10 mL/L penicillin and streptomycin (Cat. No. P1400, Solarbio, China). The culture flasks were placed in an incubator at a constant temperature of 37 °C, with 50 mL/L CO_2 and saturated humidity.

Total transcriptomic analysis

Human gastric adenocarcinoma AGS cells were cultured and treated with 18β-GRA (purity > 97%; Cat. No. G10105-10G, Sigma, United States) for 24 h. The cells of negative control (NC) group and 18β-GRA group were collected and total RNA was extracted. Agarose gel electrophoresis was used to analyze the degradation degree of RNA and whether there was contamination. Nano Drop 2000 spectrophotometer and Qubit 2.0 were used to determine the purity and concentration of total RNA, and Agilent 2100 biological analyzer was used to accurately detect the integrity of RNA. After the samples were tested as qualified, a library was constructed using small RNA sample pre kit. Using the 3'-end hydroxyl structure of small RNA and the complete 5'-end phosphoric acid group, the total RNA was taken as the starting sample, and the two ends of small RNA were added with joint, and then reverse transcription was performed to synthesize cDNA. After polymerase chain reaction (PCR) amplification, PAGE gel electrophoresis was used to isolate the target DNA fragment, and the cDNA library was recovered.

After the library was successfully constructed, Qubit2.0 was used for preliminary quantification, and Agilent 2100 was used to determine the size of the library insert. Once the insert size reaches the expected value, use quantitative PCR to accurately quantify the effective library concentration to ensure library quality (effective

concentration of the library > 2 nmol/L). After the library is approved, pool different libraries into Illumina SE50 sequencing as needed.

Bioinformatics analysis

TransmiR v2.0 database (http://www.cuilab.cn/transmir) was used to predict miR-328-3p related proteins, and then STRING database (https://cn.string-db.org/) was used to predict the relationship between miR-328-3p related proteins and autophagy related proteins. Finally, the binding sites of related proteins to miRNA were searched on the miRWalk website (http://129.206.7.150/).

Real-time quantitative PCR

Trizol reagent was used for total RNA (Cat. No. DP424, TIANGEN Biochemical Technology, China). cDNA reverse transcription was used by PrimeScript™ RT reagent Kit with gDNA Eraser (Cat. No. RR04B, TaKaRa, Japan). BlazeTaq SYBR Green qPCR Mix 2.0 (Cat. No. P031-S, GeneCopoeia, United States)

The primer sequences: *miR-328-3p*: forward: ATATCTGGCCCTCTCTGCCCTTC, reverse: AGTGCAGGGTCCGAGGTATT; *U6*: forward: CTCGCTTCGGCAGCACA, reverse: AACGCTTCACGAATTTGCGT; *STAT3*: forward: ACCAGCAGTATAGCCGCTTC, reverse: GCCACAATCCGGGCAATCT; *GAPDH*: forward: CACCCACTCCTCCACCTTTGA, reverse: TCTCTCTTCCTCTTGTGCTCTTGC. All experimental groups repeated 3 samples.

Lentivirus transfection

AGS cells were cultured in the T25 cell breathable culture flask. After 24 h, GC cells were transfected with green fluorescent protein (GFP)-labeled overexpressed miR-328-3p lentivirus (GeneChem, China). The virus volume = [multiplicity of infection (MOI) × number of cells]/virus titer. 8 h after transfection, fresh complete medium was replaced and observed under fluorescence microscope. The transfection efficiency of lentivirus was determined according to the cells with GFP fluorescence in bright and GFP fields.

Follow-up experiments were performed when the transfection efficiency reached about 90%.

CCK-8

Lentivirus-transfected AGS cells and normal AGS cells were inoculated into 96-well plates, and cultured for 24 h, 48 h and 72 h, respectively. Then, CCK-8 reagent (Cat. No. CK04, DOJINDO, Japan) was added to each well, and absorbance (OD) was measured at 450 nm with an enzyme marker. Cell viability = $(OD_{experiment} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100\%$.

Clone formation

The AGS cells were inoculated into a 6-well plate with 600 cells per well and 3 multiple wells in each group for further culture, and the cells were observed under the microscope. When the number of clones of most single cells was greater than 50 and the diameter was 0.3-1.0 mm, the experiment was terminated, 4 mL/L paraformaldehyde (Cat. No. D16013, Saint-Bio, China) was fixed for 15 min, crystal indigo water solution was dyed for 4-5 min, the cells were washed with normal saline, left to dry, and then photographed. Finally, visual counting. Rate of clone formation = amount of clones/number of inoculated cells ×100%.

Flow cytometry

The AGS cells transfected with lentivirus were inoculated in 6-well plates for 24 h and digested, centrifuged at 3000 r/min for 5 min and washed once with PBS. The cell concentration was adjusted to 1×10^6/mL and 0.5 mL cold ethanol was added to fix overnight. 16 h later, the cells were washed with PBS once, and then 500 μL was added into the staining solution prepared according to Rnase A:PI = 1:9 (Cat. No. KGA511, KeyGEN, China), and incubated for 30 min. Flow cytometry is used to measure the cell cycle.

The AGS cells were collected, the concentration was adjusted to 4×10^5/mL, 1 mL cell suspension was centrifuged, 500 mL Binding Buffer was added, and then 5 μL Annexin V-APC was added. The mixing process should not be too forceful. Then, 5 μL 7-AAD staining solution (Cat. No. KGA1023, KeyGEN, China) was added, mixed and reacted for 20 min. Apoptosis was detected by machine.

Tumor formation experiment

The BABL/c nude mice were kept in polypropylene cages with humidity of 50% ± 5% and temperature of 22 ± 1 °C for a day and night cycle of 12 h/12 h, ad libitum access to food and water. After 2 wk of adaptive feeding, random grouping. The AGS cells and lentivirus transfected GC cell were collected, and the cell concentration of each group was adjusted to 4×10^6/200 μL. The 200 μL cell suspension was absorbed by a microinjection and injected slowly into the subcutaneous area of the right back of nude mice. The subcutaneous tumor bearing conditions of nude mice were observed daily. Measure the long diameter (L) and short diameter (W) of the tumor with an electronic vernier caliper, the formula for calculating the tumor volume (V) is: $V = (W^2 \times L)/2$. When the tumor volume of nude mice grew to a certain size, the experiment was over, and all animals were euthanized by 1000 mL/L CO_2, and the tumor was exfoliated and photographed. The animal protocols were approved by the Institutional Animal Care and Use Committee of the Ningxia Medical University (IACUC-NYLAC-2022-108).

Hematoxylin-eosin staining

Tissue samples fixed with 4 mL/L paraformaldehyde were dehydrated in 75 mL/L, 85 mL/L, 90 mL/L ethanol and anhydrous ethanol and then embedded in paraffin. Paraffin sections 3-5 μm thick were taken from the microtome, baked at 65 °C for 1 h, and then quickly sliced into xylene and ethanol for dewaxing treatment. Double steaming water for 3 min, hematoxylin dye (Cat. No. BA-4041, BaSO, China) for 5 min, double steaming water for 3 min, acid alcohol fast differentiation solution (Cat. No. C0163M, Beyotime, China) for 5 s, eosin dye solution (Cat. No. BA-4024, BaSO, China)

for 5 min, wash for 5 min. The slices were dehydrated in 85 mL/L, 95 mL/L ethanol solution and anhydrous ethanol. The dehydrated paraffin sections were put into xylene transparent for 10 min and sealed with neutral gum (Cat. No. Zli-9555, ZSGB-Bio, China). The cell structure and morphology were observed by optical microscope (BX43, OLYMPUS, Japan).

Immunohistochemical staining

Paraffin sections were routinely dewaxed, and the sections were repaired with citric acid repair solution (pH = 6.8) under high pressure for 10 min and washed with distilled water for 5 min. Incubate with 3 mL/L hydrogen peroxide for 20 min, wash with water for 5 min, and soak with PBS for 1 min. The primary antibody [LC3 (Cat. No. 12741T, CST, 1:50); STAT3 (Cat. No. AF6294, Affinity, 1:90); p-STAT3 (Cat. No. AF3293, Affinity, 1:100)] was added at 4 °C overnight, and washed with PBS buffer solution for 3 times, 5 min each time. The second antibody was added and incubated at 37 °C for 20 min. Wash with PBS buffer solution. DAB (Cat. No. ZLI-9018, ZSGB-Bio, China) color development, wash with tap water. Hematoxylin was used for retaining, gradient alcohol was used for dehydration, and neutral gum was used for sealing. The images were observed and acquired at OLYMPUS imaging System (UC90, Japan).

Western blot

The protein lysis buffer consisted of 10 μL phosphatase inhibitor, 1 μL protease inhibitor and 5 μL 100 mmol/L protease inhibitor (Cat. No. KGP2100, KeyGEN). The cells were collected and the protein lysate was added to extract the whole protein. The protein content was determined by BCA (Cat. No. KGPBCA, KeyGEN). Appropriate protein loading buffer (Cat. No. DL101, TransGen Biotech) was added and boiled at 100 °C for 10 min. According to the experimental design, the sample was added to the prefabricated glue (Cat. No. M00659, GenScript), and the constant pressure electrophoresis was carried out at 120 v until the marker strip with the lowest molecular weight appeared. After electrophoresis, transmembrane was performed. Then the

PVDF membrane (Cat. No. ISEQ00010, Millipore, United States) was closed and immersed in the diluent of primary antibody, and incubated overnight in the refrigerator at 4 °C. Then add the secondary antibody and incubate for 1 h. The luminescent solution (Cat. No. KF005, Affinity) was prepared according to the reagent instructions, and the PVDF membrane was soaked in it for 2-3 min, and then placed in the intelligent chemiluminescence imaging system (iBright 1500, Invitrogen, United States) to obtain protein bands. Protein bands were analyzed using image J.

Dual-luciferase reporter assay system

Retrieval of hsa-miR-328-3p sequence from NCBI Nucleotide website: UGGCCCUCUCUGCCCUUCCGU, the binding sites of miR-328-3p and STAT3 were retrieved from the miRWalk website, and the binding sites with high binding energy were screened based on the scores.

AGS cells were cultured in 24-well plates with 2×10^4 cells per well, and the degree of cell confluence reached about 40%. The experimental group is: Luc-STAT3-3′ untranslated region (3′UTR) Mutant (Mut)-NC + miR-328-3p-NC, Luc-STAT3-3′UTR Mut-NC + miR-328-3p, Luc-STAT3-3′UTR Wild Type (WT) + miR-328-3p-NC, Luc-STAT3-3′UTR WT + miR-328-3p, Luc-STAT3-3′UTR Mut + miR-328-3p-NC, and Luc-STAT3-3′UTR Mut + miR-328-3p. The plasmid and transfection reagent (Cat. No. IV1216075, Invigentech) were mixed 1:1 to prepare the complex, then the complex was added and mixed, and the culture was continued for 24 h, then the liquid was changed, and then the culture was continued.

After 48 h of co-transfection with plasmids, the 24-well plates were removed and balanced to room temperature. Extraction 250 μL medium was removed from each well and Passive lysis buffer 100 μL was added to each well and shaken for 15 min for lysis. The lysate was added into 1.5 mL EP tubes, and then into black 96-well plates with 20 μL per well and 3 multiple Wells per group. Then Luciferase Assay Reagent II 100 μL was added. Fluorescence values were detected at 560 nm with multifunctional reagent reagent II. Then 100 μL of Stop & Glo® reagent (Cat. No. E1910, Promega) was added to

each well, and the fluorescence value was detected at 465 nm. Luciferase activity = $OD_{firefly\ luciferase}/OD_{renilla\ luciferase}$.

Transfection with adenovirus

AGS cells with a concentration of 4×10^4 cells/mL were inoculated in a 15 mm confocal dish (Biosharp Biotechnology, United States) and cultured in the incubator. When the cells were fused to 40%, the fresh medium volume was changed to 1 mL. Monomeric Red Fluorescent Protein (mRFP)-GFP-LC3 tandem fluorescent protein adenovirus (Cat. No. P21071905, HanBio) was added, and the volume of virus = MOI × cell number/virus titer × 10^3, and then 1 mL culture medium was added and placed in the incubator for 4 h. After 16 h of infection, the culture medium was changed into a fresh medium for further culture. At 24 h and 48 h, the cell state and infection situation were observed under fluorescence microscope. The infection efficiency could be determined by observing the fluorescence expression level of GFP.

Laser confocal

The AGS cells infected with adenovirus were treated synchronously for 6 h, and then given corresponding drug treatment (the NC group was replaced with fresh medium), then placed in an incubator for 24 h, fixed with 4 mL/L paraformaldehyde for 20 min, and observed with laser confocal microscope. Cells infected with adenovirus using mRFP-GFP-LC3 tandem fluorescent protein can be identified by observing the fluorescence of mRFP and GFP after microscopic imaging.

Statistical analysis

The statistical methods of this study were reviewed by Li-Qun Wang from Department of Epidemiology, Department of Medical Statistics, Institute of Public Health and Management, Ningxia Medical University. All analyses in this study were performed using GraphPad Prism 7.0. And all data are presented as mean ± SD. Statistical

significance was analyzed using one-way ANOVA. $P < 0.05$ means the difference is statistically significant.

RESULTS

18β-GRA promotes the expression of miR-328-3p in AGS cells

Whole transcriptomics was used to analyze and screen the differentially expressed miRNAs in AGS cells after 18β-GRA intervention. Through the selection criteria of log2 |fold change| > 1, $P < 0.05$, 283 differentially expressed miRNAs were obtained, of which 120 were up-regulated and 163 were down-regulated. Notably, the expression of miR-328-3p was up-regulated after drug treatment, log2(fold change) = 3.04871, P value was 4.51E-06 (Figures 1B and 1C).

Real-time quantitative PCR (qRT-PCR) was used to verify whether the intervention process of 18β-GRA on AGS cells was related to miR-328-3p. The results showed that the expression of miR-328-3p in AGS cells treated with 18β-GRA was significantly up-regulated compared with the NC group($P < 0.01$) (Figure 1D).

Overexpression of miR-328-3p inhibited the survival rate of GC cell

The expression level of miR-328-3p after transfection was detected by qRT-PCR. miR-328-3p in the overexpression group was significantly higher than that in the vector group (Figure 1E). Cell status and transfection efficiency were observed under Cytation 5 microscope (Figure 1F), and cell survival rate was detected. The results showed that, compared with the vector group, the survival rate of cells in the overexpressed miR-328-3p group decreased significantly ($P < 0.01$) (Figure 1G).

Effect of overexpression of miR-328-3p on cloning formation of AGS cells

The results of the cloning formation experiment showed that, compared with the vector group, the crystal violet staining positive of overexpressing miR-328-3p group was significantly decreased (Figure 2A), and the cloning formation rate of AGS cells was significantly reduced, with statistical significance ($P < 0.01$) (Figure 2B). These results

indicated that overexpression of miR-328-3p could inhibit the colony formation of GC cell.

Overexpression of miR-328-3p arrested cell cycle and promotes apoptosis

The cell cycle results showed that the average percentage of G0/G1 phase, S phase and G2/M phase cells in vector group was 49.23%, 29.41% and 21.37%, respectively. The average percentage of G0/G1 phase, S phase and G2/M phase cells in the overexpressed miR-328-3p group was 63.73%, 20.19% and 16.08%, respectively. Compared with the vector group, the percentage of G0/G1 phase cells in the overexpressing miR-328-3p group was significantly higher ($P < 0.001$) (Figures 2C and 2D).

The results of the cell apoptosis experiment showed that, the cell fragments in the vector group were increased, indicating that lentivirus transfection had a certain damage effect on AGS cells. And the early apoptosis rate of cells overexpressing miR-328-3p was significantly higher than that of the vector group ($P < 0.001$) (Figures 2E and 2F).

Overexpression of miR-328-3p inhibited subcutaneous tumor in nude mice

The results of the subcutaneous tumor experiment with nude mice showed that the subcutaneous tumor volume in the back of nude mice overexpressed with miR-328-3p was significantly smaller than that in the NC and vector groups ($n = 6$, Figures 3A and 3B). The tumor growth curve also showed that the tumor growth rate of the overexpressed miR-328-3p group was significantly lower than that of the vector group and the NC group, with statistical significance ($P < 0.01$) (Figure 3C). During the whole experiment, there was no significant difference in body weight among the groups.

The histopathological observation of transplanted tumor and the expression of STAT3, p-STAT3 and LC3 proteins

hematoxylin-eosin (HE) staining showed that tumor cells in the NC and vector groups grew strongly, with different sizes and certain atypia, larger nuclei, and no obvious necrosis in the tumor tissue. In the overexpress group, the cell arrangement was loose, the tissue structure was irregular, and there were more necrotic cells (Figure 3E).

Immunohistochemical staining results showed that, compared with the vector and NC groups, the expression of p-STAT3 and LC3 in miR-328-3p group was significantly decreased, with statistical significance ($P < 0.05$), and there was no significant difference in the positive rate of STAT3 expression in transplanted tumor tissues among all groups (Figures 3E and 3F).

miR-328-3p targeted STAT3 mRNA and was closely related to autophagy-related proteins

TransmiR v2.0 database predicted miR-328-3p related proteins and found that miR-328 was associated with STAT3 (Figure 4A). Then, the relationship between STAT3 and autophagy-related proteins LC3, p62, BECN1 and ATG13 was predicted in the STRING database (Figure 4B). The above prediction results suggest that STAT3 is a target gene of miR-328-3p.

STAT3 is targeted by miR-328-3p

qRT-PCR was used to detect the expression level of STAT3 mRNA in transfected lentivirus in GC cell. The results showed that the expression level of STAT3 mRNA in the overexpressed miR-328-3p group was significantly lower than that in the vector group ($P < 0.01$) (Figure 4C).

We also detected STAT3 and p-STAT3 in AGS cells overexpressed with miR-328-3p by western blot (Figure 4D). The experimental results showed that the expression level of STAT3 in the miR-328-3p group was not significantly changed, and the expression of p-STAT3 was down-regulated ($P < 0.05$). After the ratio of p-STAT3 to STAT3, the p-STAT3/STAT3 ratio in the over-expressing miR-328-3p group was significantly down-regulated ($P < 0.01$) (Figure 4E).

Dual luciferase reporter system verified the targeting relationship between miR-328-3p and STAT3

The miRWalk online tool was used to find the binding site of miR-328-3p and STAT3, suggesting that there is a targeting relationship between miR-328-3p and STAT3 (Figure 4F). Dual-luciferase reporter assay was used to detect the relationship between miR-328-3p and STAT3 in AGS cells (Figure 4G). The results showed that the luciferase activity of STAT3 WT + miR-328-3p NC group was lower than that of STAT3 WT + miR-328-3p group, and the difference was statistically significant($P < 0.001$) (Figure 4H). The results showed that miR-328-3p could bind specifically to the 3'UTR of STAT3.

miR-328-3p can regulate STAT3 and promote autophagy flow in GC cell

In order to further investigate the effect of miR-328-3p targeted regulation of STAT3 on the autophagy flow of AGS cells, western blot was used in combination with 0.5 μmoL/L Baf A_1 to detect the expression of LC3 II in GC cells after overexpression of miR-328-3p (Figure 5A). The experimental results showed that, compared with the vector group, the expression level of LC3 II in the overexpressing miR-328-3p group was significantly down-regulated, and the difference was statistically significant ($P < 0.05$). Compared with Baf A_1 group, the expression level of LC3 II in miR-328-3p combined with Baf A_1 group was significantly up-regulated ($P < 0.01$) (Figure 5B).

18β-GRA promotes autophagosome synthesis in GC cell

To further investigate the effect of 18β-GRA on autophagic flow in AGS cells, we treated AGS cells with different drugs. The results showed that compared with NC group, LC3 II expression level in 18β-GRA group was significantly down-regulated ($P < 0.05$). Compared with Baf A_1 group, LC3 II in 18β-GRA + Baf A_1 group was significantly up-regulated ($P < 0.05$) (Figures 5C and 5D).

We used also mRFP-GFP-LC3 tandem fluorescent protein adenovirus to infect AGS cells and locally tag LC3 to detect autophagy flow. First, we explored the MOI of mRFP-

GFP-LC3 tandem fluorescent protein adenovirus infected AGS cells. The NC, MOI = 30, MOI = 50 and MOI = 80 groups were set up separately. Corresponding virus volumes were given for transfection, and photographs were taken under the fluorescence microscope at 24 h and 48 h. The results showed that, compared with MOI = 50, the virus transfection efficiency was lower when MOI = 30, while the cell state was poorer when MOI = 80. When MIO = 50 and the transfection time was 48 h, both transfection efficiency and cell state were good. Therefore, a MOI of 50 was determined as the MOI for subsequent experimental infection (Figures 5E and 5F).

We used mRFP-GFP-LC3 tandem fluorescent protein adenovirus (MOI = 50) to infect GC cell for 48 h, and gave corresponding drug treatment. The experimental results showed that, compared with NC group, the number of yellow spots (GFP/mRFP) in 18β-GRA group increased significantly ($P < 0.001$). Separate red signals were also significantly increased ($P < 0.01$) (Figure 5G), suggesting that 18β-GRA could increase the number of early autophagosomes and late autolysosome. Compared with Baf A_1 group, the number of yellow spots in 18β-GRA + Baf A_1 group increased significantly ($P < 0.001$), and the single red signal showed no significant change, indicating that the late autophagy inhibitor caused the blocking of late autophagic lysosome degradation. 18β-GRA promotes autophagosome synthesis and results in an increase in the number of yellow spots (Figure 5H).

18β-GRA promotes autophagy flow and inhibits proliferation of GC cell by regulating miR-328-3p/STAT3 signaling pathway

To investigate whether 18β-GRA promotes autophagy flow through the miR-328-3p/STAT3 signaling pathway, we first used qRT-PCR to collect 18β-GRA intervention AGS cells to detect the expression level of STAT3 mRNA. The results showed that, compared with the NC group, STAT3 mRNA expression was significantly down-regulated in AGS cells after 18β-GRA intervention ($P < 0.05$) (Figure 5I). Then, western blot results showed that the expression levels of STAT3 and p-STAT3 in AGS cells after

18β-GRA intervention were down-regulated, and the p-STAT3/STAT3 ratio was also significantly down-regulated ($P < 0.05$) (Figures 5J and 5K).

DISCUSSION

In this study, we found that 18β-GRA could regulate the expression of miR-328-3p, inhibit the proliferation of GC cell, arrest cell cycle and promote cell apoptosis. Dual-luciferase reporter gene experiment confirmed that miR-328-3p targeted the regulation of STAT3 and played a role in promoting autophagy. Our results suggest that 18β-GRA promoted the synthesis of autophagosomes and inhibited cell proliferation by regulating the miR-328-3p/STAT3 signaling pathway.

miR-328-3p expression has been found to be significantly inhibited by palmitate-induced ER stress in liver cell lines[35]. In tumors, overexpression of miR-328-3p can inhibit the proliferation and invasion of hepatocellular carcinoma cells, promote cell apoptosis, and have an excellent targeting relationship with ER metalloproteinases[36]. Overexpression of miR-328-3p inhibited colorectal cancer cells proliferation, migration and invasion, and inactivated the phosphatidylinositol 3 kinase/protein kinase B (Akt) signaling pathway[37]. Xu *et al*[38] analyzed the miRNA expression profiles of 389 GC patients, using forward and reverse variable selection and multiple Cox regression analysis models, and found that miR-328-3p exists in GC patients as a protective miRNA. However, Xiao *et al*[39] defined miR-328-3p as an oncogene for the first time and found that miR-328-3p promotes the progression of GC by targeting the kelch-like ECH-associated protein 1/nuclear factor erythroid 2-related factor 2 axis. Although there is some controversy about the effect of miR-328-3p expression on GC progression, it also suggests that miR-328-3p may be a target for GC drug therapy.

Autophagy ("self-eating") usually refers to the process in which cells wrap their own pathological organelles into double-layer vesicles to form autophagosomes when they resist stress such as hunger, and then deliver them to lysosomes and combine with them to form autolysosome to degrade the contents and produce energy for recycling and utilization to maintain cell survival[40]. During autophagy, the autophagy containing

misfolded proteins and damaged organelles is degraded in the lysosome, which has a limiting membrane to prevent the leakage of its degrading enzymes, and the autophagy process is fast and frequent and involves relatively complex membrane dynamics[41]. It is generally believed that the conversion of LC3 I to LC3 II detected in western blot, or the increased expression level of LC3 II, represents the activation of autophagy flow, and after the autophagy flow is blocked, the expression level of LC3 II was decreased (the conversion of LC3I to LC3 II was blocked) or LC3 II was excessively degraded by autolysosome[42]. In this study, autophagy marker protein LC3 was detected by combining with autophagy inhibitor Baf A_1 to explore the effects of drugs and miR-328-3p on the autophagy of GC cell, and the results showed that both drugs and miR-328-3p could promote the occurrence of autophagy flow.

To further explore whether drugs promote the synthesis of autophagosomes or inhibit the degradation of autophagosomes, we infected GC cell with mRFP-GFP-LC3 autophagic double-labeled adenovirus, and the mRFP and GFP are used to label LC3. Because GFP fluorescent protein is sensitive to acidic environments, after fusion of autophagosome and lysosome, green fluorescence will be quenched, and only mRFP fluorescence can be detected. The yellow signal composed of GFP and mRFP fluorescence signals co-located in the cell represents the early autophagosome, while the mRFP fluorescence signal alone represents the late autophagosome. The increased yellow and red signals indicate an increased autophagic flow level[43]. Using a dual verification approach, we found that drugs promoted autophagy flow by promoting autophagy synthesis at the causal level.

A number of studies have found a close relationship between STAT3 and autophagy[44]. In this study, the targeting relationship between miR-328-3p and STAT3 was validated by dual-luciferase reporter gene experiments. In conclusion, the regulation of the miR-328-3p/STAT3 signaling pathway promotes the synthesis of autophagosomes, and 18β-GRA inhibits the proliferation of GC cell. Typically, STAT3 monomers in the cytoplasm are phosphorylated to form STAT3 dimers, which are then shuttled into the nucleus and bind to specific DNA elements to transcriptionally

activate or inhibit the expression of target genes, thereby inhibiting cell proliferation, arresting cell cycle and promoting autophagy[45]. At the same time, STAT3 in the unphosphorylated cytoplasm activates some autophagy-related genes, such as ULK2 and ATG12, *via* FOXO1 and FOXO3 transcription, among others[46]. STAT3 monomer can also be transferred to mitochondria and interact with complex I/II of mitochondria to inhibit the production of reactive oxygen ROS[47] (Figure 6).

This study showed that 18-GRA can inhibit GC cell proliferation, arrest cell cycle in G0/G1, promote apoptosis, and promote autophagosome synthesis of GC cells by regulating the miR-328-3p/STAT3 signaling pathway. Our future research program will be divided into three phases: (1) Electrophoretic Mobility Shift Assay was used to detect the binding ability of the STAT3 promoter regulatory region and miR-328-3p, to explore whether the transcriptional regulation of miR-328-3p on STAT3 is directly binding or indirectly binding; (2) The interaction mechanism between miR-328-3p and STAT3 proteins will be verified, and Chromatin Immuno-precipitation technology will be considered to further verify the binding regulation of the promoter region of transcription factor STAT3 and miR-328-3p; (3) Using Cre mice, adeno-Associated Virus was used for genome editing *in vitro*, and conditional gene targeting of miR-328-3p was performed in gastric mucosal epithelial cells to explore the overall phenotype and physiological status of Cre mice. This study mainly provides scientific basis for clinical treatment of GC, and also seeks effective drug targets for prevention and treatment of GC.

CONCLUSION

18β-GRA can promote the synthesis of autophagosomes in GC cell, inhibit cell proliferation, arrest cell cycle and promote cell apoptosis by regulating miR-328-3p/STAT3 signaling pathway.

ARTICLE HIGHLIGHTS

Research background

Gastric cancer (GC) is one of the most serious gastrointestinal malignancies with high morbidity and mortality. In recent years, more and more evidence has shown that natural products can prevent and inhibit the development of GC by regulating microRNA (miRNA) and other genes, showing great therapeutic potential.

Research motivation

Targeting miRNA and other genes in GC with natural drugs is a promising strategy, which provides a reference for further elucidate the molecular mechanism of natural products in the treatment of GC.

Research objectives

The purpose of this study was to investigate the molecular mechanism of 18β-glycyrrhetinic acid (18β-GRA) regulating the miR-328-3p/signal transducer and activator of transcription 3 (STAT3) signaling pathway, promoting the autophagy flow of GC cells and inhibiting the cell proliferation.

Research methods

The differentially expressed miRNAs were screened by full transcriptomic analysis. The cells were transfected with lentivirus, and the functional indices of the cells were determined by CCK-8, clone forming method and flow cytometry. The effect of overexpression of miR-328-3p on tumorigenicity of GC cells was detected in the transplanted tumor model of nude mouse. Hematoxylin-eosin staining (HE) and immunohistochemistry were used to observe tumor tissue morphology and detect protein expression, respectively. Bioinformatics analysis was performed using TransmiR, STRING and miRWalk databases. Real-time quantitative polymerase chain reaction and western blot were used to detect mRNA and protein expression levels. Dual luciferase reporter system verifies the targeting relationship between genes. The autophagy flow of monomeric Red Fluorescent Protein (mRFP)-green fluorescent

protein (GFP)-light chain 3 (LC3) adenovirus double-labeled infected cells was observed under confocal laser microscopy.

Research results

18β-GRA could up-regulate the expression of miR-328-3p in AGS cells. Overexpression of miR-328-3p can inhibit the cell proliferation and colony formation ability, arrest the cell cycle in G0/G1 phase, promote apoptosis, inhibit the growth of subcutaneous tumors, and lead to GC tissue cell necrosis increase. miR-328-3p has the function of targeting and regulating STAT3. 18β-GRA intervention in GC cells and overexpression of miR-328-3p both had the ability to down-regulate the expression level of *STAT3* mRNA and p-STAT3. Compared with the vector group, the expression level of LC3 II was down-regulated in the overexpressed miR-328-3p + Baf A_1 group, and up-regulated in the overexpressed miR-328-3p + Baf A_1 group. The number of yellow spots (GFP/mRFP) and separate red signals in 18β-GRA group was significantly increased compared with negative control group, and the number of yellow spots in 18β-GRA + Baf A_1 group was significantly increased compared with Baf A_1 group.

Research conclusions

18β-GRA promotes GC cells autophagosome synthesis and inhibits cell proliferation by regulating miR-328-3p/STAT3 signaling pathway.

Research perspectives

miR-328-3p/STAT3 can be regulated by 18β-GRA, and can be used as an effective drug target for the prevention and treatment of GC, providing a scientific basis for the clinical treatment of GC.

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