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Basic Study

18β-glycyrrhetinic acid regulates Mitochondrial Ribosomal Protein L35**-associated apoptosis signaling pathways to inhibit the proliferation of gastric carcinoma cells**

18β-GRA inhibits GC proliferation *via* MRPL35-related-signaling

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Abstract

BACKGROUND

Gastric carcinoma (GC) is a common gastrointestinal malignancy worldwide. Based on the cancer-related mortality, the current prevention and treatment strategies for GC still show poor clinical results. Therefore, it is important to find effective drug treatment targets.

AIM

To explore the mechanism by which 18β-glycyrrhetinic acid (18β-GRA) regulates Mitochondrial Ribosomal Protein L35 (MRPL35) related signal proteins to inhibit the proliferation of GC cells.

METHODS

Cell Counting Kit-8 (CCK-8) assay was used to detect the effects of 18β-GRA on the survival rate of human normal gastric mucosal cell line GES-1 and the proliferation of GC cell lines MGC80-3 and BGC-823. The apoptosis and cell cycle were assessed by flow cytometry. Cell invasion and migration were evaluated by Transwell assay, and cell scratch test was used to detect cell migration. Furthermore, the tumor model was established by hypodermic injection of 2.5×106 BGC-823 cells at the selected positions of BALB/c nude mice to determine the effect of 18β-GRA on GC cell proliferation, Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to detect *MRPL35* expression in the engrafted tumors in mice. We used the term tandem mass tag (TMT) labeling combined with liquid chromatography–tandem mass spectrometry (LC-MS/MS) to screen for differentially expressed of proteins extracted from GC cells and control cells after 18β-GRA intervention. A detailed bioinformatics analysis of these differentially expressed proteins (DEPs) was performed, including Gene Ontology (GO) annotation and enrichment analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis and so on. Moreover, STRING database (https://string-db.org/) was used to predict protein-protein interaction (PPI)

relationships and western blotting was used to detect the expression of proteins of interest in GC cells.

RESULTS

The results indicated that 18β-GRA could inhibit the proliferation of GC cells in dose and time-dependent manner. It could induce GC cell apoptosis and arrest the cell cycle in G0/G1 phase. The proportion of cells arrested at S phase decreased with the increase of 18-GRA dose, the migration and invasiveness of GC cells were inhibited. The results of animal experiments showed that 18β-GRA could inhibit tumor formation in BALB/c nude mice, and qRT-PCR results showed that *MRPL35* expression level was significantly reduced in the engrafted tumors in mice. Using TMT technology, we observed 609 DEPs, among which 335 proteins were up-regulated and 274 proteins were down-regulated, were identified in 18β-GRA intervention compared with control. We found that the intervention of 18β-GRA in GC cells involved many important biological processes and signaling pathways, such as cellular process, biological regular and TP53 signaling pathway. Notably, after the drug intervention, MRPL35 expression was significantly down-regulated (p=0.000247), TP53 expression was up-regulated (p=0.02676) and BCL2L1 was down-regulated (p=0.01699). Combined with the Retrieval of Interacting Genes/Proteins database, we analyzed the relationship between MRPL35, TP53 and BCL2L1 signaling proteins, and we found that COPS5, BAX, and BAD proteins can form a PPI network with MRPL35, TP53 and BCL2L1. Western blotting confirmed the intervention effect of 18β-GRA on GC cells, MRPL35, TP53 and BCL2L1 showed dose-dependent up/down-regulation, the expression of COPS5, BAX and BAD also increased/decreased with the change of 18β -GRA concentration.

CONCLUSION

18β-GRA can inhibit the proliferation of GC cells by regulating MRPL35, COPS5, TP53, BCL2L1, BAX and BAD.

Key Words: Gastric carcinoma, 18β-glycyrrhetinic acid, Mitochondrial ribosomal protein L35, Proliferation, Invasion, Apoptosis

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Core Tip: 18β-glycyrrhetinic acid (18β-GRA) is a pentacyclic triterpene derivative extracted from the natural medicine licorice. Our results showed that 18β-GRA could inhibit Gastric carcinoma (GC) cell proliferation, cell migration, invasion, and tumor formation, induce GC cell apoptosis and arrest the cell cycle. TMT analysis found that the expression of Mitochondrial ribosomal protein L35 (MRPL35) was significantly decreased after 18β-GRA intervention in GC cells, which was confirmed by Western blotting results. These data indicate that 18β-GRA inhibits proliferation/migration and promotes apoptosis of GC cells by down-regulating MRPL35 expression, suggesting that MRPL35 is a therapeutic target for GC.

INTRODUCTION

GC is among the most common cancers as well as one of the most common causes of cancer-related mortality worldwide. Based on the Global Cancer Observatory (GLOBOCAN) database, GC ranks fifth in the incidence and fourth in the mortality among various types of cancers worldwide, after lung cancer, colorectal cancer and liver cancer, and there would be more than one million new cases per year since 2020 by the World Health Organization (WHO) predicting[1]. The current treatment of GC is mainly based on surgery, chemotherapy and radiotherapy [2]. Most patients with early and advanced GC can be treated by surgery, but the treatment effect is very poor for patients with advanced GC and metastasis [3]. Although chemotherapy and radiotherapy are effective, they have severe side effects, such as hair loss and

gastrointestinal reactions, leading to low compliance. Therefore, it is critical to find an effective therapeutic target for GC.

18β-GRA is a pentacyclic triterpene derivative extracted from the natural medicine licorice (Figure 1). It has significant anti-inflammatory [4], anti- oxidant, anti-bacterial and anti-proliferative effects [5]. Recent studies have shown that 18β-GRA has reliable anticancer effects on human malignant tumors, such as lung cancer [6], breast cancer [7], colon cancer and GC. Huang *et al*. found that 18β-GRA inhibited the extracellular signal-regulated kinase (ERK)/Cyclic Adenosine Monophosphate Response Element Binding Protein (CREB) pathway by inhibiting Thromboxane Synthase (TxAS), thereby reducing the cell proliferation of non-small cell lung cancer cells [6]. 18β-GRA showed potent inhibitory effects on human breast carcinoma MCF-7 proliferation in a concentration and time-dependent manner, without affecting immortalized normal mammary epithelial cell line. 18β-GRA induced apoptosis in MCF-7 cells *via* caspase activation and modulation of Protein kinase B (Akt)/Phosphorylated Forkhead Box O3 (FOXO3a) pathway [7]. *In vivo* and *in vitro*, 18β-GRA inhibited the proliferation, invasion and migration of colorectal cancer cells, and had no obvious inhibitory effect on normal cells. It prolonged the survival of tumor-bearing mice by promoting apoptosis of colorectal cancer cells. Moreover, 18β-GRA inhibited the initiation and progression of gastric tumors by ameliorating the inflammatory microenvironment through down-regulation of cyclooxygenase-2 (COX-2) expression and by inhibiting Wnt-1 expression through the up-regulation of tumor suppressor miR-149-3p [8]. 18β-GRA inhibited the migration and invasion of GC SGC-7901 cells *via* the Reactive Oxygen Species (ROS)/Protein Kinase C-α (PKC-α)/ERK signaling pathway, and suppressed MMP-2 and 9 activities on SGC-7901 cells in a dose-dependent manner [9]. Toll-like receptor 2 (TLR2) mRNA and protein expression levels were elevated in the GC cell lines and human GC tissues. Over-expression of TLR2 was correlated with high histological grade. However, the expression of TLR2 was found to be down-regulated by 18β-GRA through a dynamic process of DNA methylation regulation. GRA pretreatment inhibited TLR2-activated GC cell proliferation, energy metabolism and carcinogenesis

[10]. In general, 18β-GRA showed excellent inhibitory effect on GC, and its pharmacological mechanism is still worth exploring.

The human *mitochondrial ribosomal protein* (*MRP*) gene family consists of 30 members encoding small mitochondrial ribosomal subunits and 50 members encoding large subunits. All *MRPs* encoded by nuclear genes are important for the mitochondrial function and protein synthesis, which are involved in ribosomal protein transcription and translation, and mitochondrial oxidative phosphorylation. *MRPs* can regulate cellular functions outside mitochondria, such as cell proliferation, apoptosis, protein biosynthesis and signal transduction. In non-small cell lung cancer (NSCLC), over-expression of *MRPL15* was associated with poor prognosis [11]. *MRPL15* plays a role in ovarian cancer through cell cycle, DNA repair and mTOR1 signaling pathway, suggesting that *MRPL15* may be a prognostic indicator and therapeutic target for ovarian cancer [12]. Hao *et al* found that *MRPL42* expression was tested to be up-regulated in glioma tissues compared with normal tissues. The proliferation of glioma cells was largely blunted by silencing *MRPL42*. The results suggested that MRPL42 silencing resulted in increased distribution of cell cycle in G and G/M phases, while the S-phase decreased. Taken together, *MRPL42* is a novel oncogene in glioma [13]. In conclusion, these findings suggest that *MRPs* should play a crucial role in regulating the development of various malignant tumors.

MRPL35 is a 25ku protein encoded by the *MRPL35* gene located on chromosome 2p11.2, which is a mitotic component of the central protuberance (CP) of the mitochondria. It is of the member of the large subunit family of MRPs [14] and plays an important role in the assembly of cytochrome C oxidase complex (COX) as the specific component of mitochondrial ribosomes [15]. The expression of MRPL35 rises in the tissues of colorectal carcinoma (CRC), compared with the matched cancer-adjacent tissues. *In vitro*, down-regulation of MRPL35 resulted in increased production of ROS along with DNA damage, reduction of cell proliferation, G/M arrest, apoptosis and autophagy induction [16]. Silencing MRPL35 suppresses the proliferation of TE-1 cells of esophageal cancer, down-regulates MMP-2 and promotes cell apoptosis, after

transfection of human esophageal cancer cells with lentivirus [17]. Our previous study found that the expression of MRPL35 was significantly up-regulated in GC, and the survival analysis showed that the up-regulated MRPL35 was associated with poor survival rate in GC. Knockdown of MRPL35 was showed to inhibit proliferation of GC cells by regulating BCL-XL, PICK1 and AGR2 which are apoptosis-related proteins [18].

In this study, we focused to examine the effects of 18β-GRA intervention on GC cell function *in vitro* and tumor formation in nude mice *in vivo*. TMT analysis and STRING database prediction were used to investigate the molecular mechanism, and western blotting was used to verify the expression changes of related proteins. An important conclusion is that 18β-GRA inhibits GC cell proliferation/migration and promotes apoptosis by interfering with the expression of MRPL35-related signaling proteins, which may contribute to the development of effective therapies. These results suggest that MRPL35 may be a potential therapeutic target for the inhibition of GC of 18β-GRA.

MATERIALS AND METHODS

Cell culture and animals

Human gastric mucosal epithelial cells GES-1 (Cat.No.BNCC353464, BNCC, China) were cultured in DMEM (Cat.No.C11995500BT, Gibco, United States) medium containing 100 mL/L fetal bovine serum (FBS, Cat.No.SH30256.01, Gibco, United States) and penicillin and streptomycin (Cat.No.p1400-100, Gibco, United States). GC cell lines BGC-823 (Cat.No.GCC-ST0008CS, GeneChem, China) and MGC80-3 (Cat.No.GCC-ST0004CS, GeneChem, China) were cultured in RPMI-1640 (Cat.No.C11875500BT, Gibco, United States) medium containing 100 mL/L FBS and penicillin and streptomycin. The culture flasks were placed in an incubator at a constant temperature of 37℃, with 50 mL/L CO_2 and saturated humidity. BALB/c nude mice (male, weighing 18-22g) of Specific Pathogen-Free (SPF) grade were purchased from the Animal Experiment Center of Ningxia Medical University. All animals were fed with standard laboratory feed and water in 12 h light/dark cycle environment. The animal

protocols (IACUC-NYLAC-2019-083) were approved by the Institutional Animal Care and Use Committee of Ningxia Medical University.

Cell proliferation assessed by a CCK-8 assay

Normal gastric mucosal epithelial GSE-1 cells were cultured in an incubator at 37°C and 50 mL/L CO_2, collected and inoculated in a 96-well plate at the concentration of 4×10^4 cells/mL. On the next day, the cells were treated with different concentrations of 18β-GRA (purity >97%; Cat.No.G10105-10G, Sigma, United States) for 24 h, 48 h and 72 h. Thereafter, 10 μL CCK-8 (Cat.No.KGA317, KeyGEN, China) was added to each well and incubated for 2 h. Optical density (OD) value was detected at 450 nm. All experiments were repeated three times.

MGC80-3 and BGC-823 cells were cultured in an incubator at 37°Cand 50 mL/L CO_2. Cells in the logarithmic growth phase were inoculated in 96-well plates at an adjusted concentration of 4×10^4 cells/mL. After 24 h, cells were treated with different concentrations of 18β-GRA for 24 h, 48 h and 72 h. Thereafter, 10 μL CCK-8 was added to each well and incubated for 2 h. OD was detected at 450 nm. All experiments were independently repeated at least three times.

Cell cycle and apoptosis *via* flow cytometry analysis

MGC80-3 and BGC-823 cells were inoculated in 6-well plates. The cells were treated with 18-GRA for 48 h on the second day. Then the cells were collected and centrifuged at 3000 r/min for 5 min. Annexin V-FITC apoptosis detection kit (Cat.No.KGA107, KeyGEN, China) was used for staining and apoptosis was detected by flow cytometry after 15 min at room temperature in dark. Cells from each group were collected, washed with pre-cooled PBS, and fixed overnight in 750 mL/L ethanol. The cells were stained with cell cycle kit and incubated at room temperature for 30-60 min in the dark. The cell cycle was detected by flow cytometry. All experiments were repeated three times.

Transwell assays

First, 8 μmol/L pore size Transwell (Cat.No.Costar.3422, Corning, United States) with and without Matrigel were placed into a 24-well plate. MGC80-3 and BGC-823 cells were inoculated into the upper chambers at 2×10^5 cells/mL. After adding 750 μL complete medium in the lower chambers and culturing at 37°Cfor 24 h, the medium was replaced with different concentrations of 18β-GRA and cultured for 48 h. The cells were gently wiped off with cotton swabs and fixed with 4% paraformaldehyde for 5 min, stained with crystal Violet for 5 min and washed with PBS. Thereafter, the cells were observed with an inverted microscope and photographed to count and compare the differences in the cell invasion between groups. The number of migrated cells was counted, and the average value was used for statistical analysis. All experiments were repeated three times.

Wound-scratch assays

GC cells MGC80-3 and BGC-823 were inoculated on 6-well plates. After the cells were attached, a scratch was made with a 200 μL pipette tip. The cells were washed with PBS. Then medium containing 18-GRA was added, cultured in a 37°Ç 50 mL/L CO_2 incubator for 0 h and 48 h, and photographed with a microscope to calculate the cell migration rate of each group. All experiments were repeated three times.

Tumor formation experiment in BALB/c nude mice

Four-week-old male BALB/c nude mice (18-22g) of SPF grade were purchased from the Animal Experiment Center of Ningxia Medical University. All animals were housed in polypropylene cages with a temperature of 22±1°Ç 50±5% humidity, in a 12:12 h light/dark cycle, and provided free access to standard laboratory chow and water. There were 6 BALB/c nude mice in each group. A microsyringe was used to extract 200 μL of BGC-823 cell suspension at logarithmic growth phase at a concentration of 2.5×10^6 cells/mL, which was subcutaneously injected into the right back of each nude mouse. The 18β-GRA group was intragastrically administered 50 mg/kg [10, 19] every other day, and the control group was similarly administered equal volume of normal saline.

Intragastric gavage administration was carried out in conscious animals. The tumor volume was measured daily using the formula $V= W^2xL/2$ (V, volume; W, width; L, length). All animals were euthanized by exposure to 1000 mL/L CO_2 for 5 min for tissue collection. The animal protocols were approved by the Institutional Animal Care and Use Committee of Ningxia Medical University.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA of the Transplanted tumor tissue from mice was extracted using Trizol reagent (Cat.No. DP424, Tiagen Biochemical Technology, China), and PrimeScript™ RT reagent Kit with gDNA Eraser was used to synthesize first-strand cDNA from total RNA. qRT-PCR was used to detect by using BlazeTaq™SYBR® Green qPCR Mix 2.0 (Cat.No. QP031-S, GeneCopoeia, United States) according to the manufacturer's instructions. The primers used are as follows:

MRPL35: Forward, 5′-TTGGCATCTTCAACCTACCGC-3′ and reverse, 5′-GGAGGAAACAACTGGTGTCTGA-3′;

GAPDH: Forward, 5′-TGACTTCAACAGCGACACCCA-3′ and reverse, 5′-CACCCTGTTGCTGTAGCCAAA-3′.

All experiments were repeated three times.

Protein extraction, trypsin digestion, and TMT proteomic labeling

SDT buffer was added into the control and 18β -GRA-treated GC cells. After being boiled for 15 min, the protein content was quantified with the BCA Protein Assay Kit (Cat.No.P0012, Beyotime, China), 20μg proteins were taken and 6X loading buffer(Cat.No. P0015F, Beyotime, China) was added, respectively. After being boiled for 5 min, the proteins were separated on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein bands were visualized by Coomassie Blue R-250 staining.

The method of filter-aided sample preparation (FASP) digestion is performed by adding dithiothreitol(DTT, Cat.No.43819-5G, Sigma, United States) to a 200μg protein

solution to a final concentration of 100 mmol/L, boiling reduced protein lysate for 5min, Then alkylation with 100μL iodoacetamide buffer（IAA）for 15 min at room temperature protected from light. Then 100μL 0.1M tetraethyl ammonium bromide(TEAB, Cat.No.SE252676/90114, Thermo Fisher, United States) solution was added, centrifuged for 15min, 40μL Trypsin buffer (4μg Trypsin in 40μL 0.1M TEAB solution) was added, and the filtrate was collected overnight at 37°Cto obtain peptide segments. Taking 100 μg peptide mixture was labeled using TMT 6plex Isobaric Mass Tag Labeling kit (Thermo Fisher, United States) according to the manufacturer's instructions.

HPLC fractionation, LC-MS/MS analysis, and data analysis

The labeled peptides of the two groups were fractionated by Agilent 1260 Infinity HPLC system. The peptide mixture was diluted with buffer A (10 mmol/L HCOONH4, 5%/85% ACN, pH 10.0). LC-MS/MS analysis was performed on a Q Exactive Plus mass spectrometer (Thermo Fisher, United States), and peptides were detected in MS at a resolution of 70,000 with a scan range of 350-1800 m/z and with automatic gain control (AGC) of 5e4. All the original data files obtained were processed by Proteome Discoverer 2.2 (Thermo Fisher, United States) software. Proteins that met the expression fold change>1.2 and p value (Student's t test) <0.05 were considered to be DEPs.

Bioinformatics analysis

Blast2GO and InterProScan software are used to analyze the cellular processes of DEPs, the biological processes involved and the corresponding molecular functions. The Fisher's exact test was employed to test GO annotation/KEGG pathway/InterPro domain enrichment analysis. Fisher's exact test was used to test enrichment pathways; InterPro was used to determine enrichment domains. We used WoLFPSOR to locate and predict DEPs. Matplotlib was used to classify samples and protein expression levels at the same time to form hierarchical cluster heat maps. A PPI network incorporating

identified target proteins was prepared with the STRING database, using a score >0.4 as the significance threshold for identified interactions.

Western blotting

The cells were collected after drug intervention. Whole cell lysates were obtained by gentle lysis (Cat.No.KGP250, KeyGEN, China). Protein quantitation was performed by the BCA method (Cat.No.KGPBCA, KeyGEN, China). The protein was separated by SDS-PAGE, and the protein was transferred to PVDF membrane by wet-transfer method. Thereafter, the membrane was incubated with 50 mL/L skim milk powder at room temperature for 1 h. Later on, the membranes were incubated with the primary antibodies, including anti-MRPL35 (Cat.No.YT5669; Immunoway, 1: 2000), anti-TP53 (Cat.No.YM3052; Immunoway, 1: 2000), anti-BCL2L1 (Cat.No.YT0477; Immunoway, 1: 2000), anti-BAX (Cat.No.YM3619; Immunoway, 1: 500), anti-COPS5 (Cat.No.ab124720; Abcam, 1: 2000), anti-BAD (Cat.No.9292; Cell Signaling Technology, 1: 1000), and anti-β-tubulin (Cat.No.YM3030; Immunoway, 1: 10000). Then, the membranes were washed with TBST and incubated with anti-mouse/rabbit IgG antibody (Cat.No.S001/ S004; TDYBio, 1: 10000) at 37°Cfor 1h. After washing, the proteins were detected with an ECL detection system. ImageJ software was used to measure the band intensity.

Statistical analysis

The statistical methods of this study were reviewed by Wang LQ from Department of Epidemiology, Department of Medical Statistics, Institute of Public Health and Management, Ningxia Medical University. All analyses were performed using GraphPad Prism 7.0. All the data are expressed as the mean ± SE. Statistical significance was analyzed using One-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

18β-GRA inhibits GC cell proliferation

CCK-8 assay was used to evaluate the effect of 18-GRA on the survival rate of normal gastric mucosal GES-1 cells. The results showed that 12.5-150 μmol/L of 18β-GRA had little effect on the survival rate of GES-1 cells at 24h and 48h. However, when the concentration of 18β-GRA was greater than 150μmol/L, the survival rate of GES-1 cells was significantly decreased (Figure 2A).

CCK-8 results showed that 6.25-200 μmol/L of 18β-GRA inhibited the proliferation of BGC-823 and MGC80-3 cells in a time and dose-dependent manner (Figure 2B and C). After the intervention of 18-GRA in BGC-823 and MGC80-3 cells for 24 h, 48 h and 72 h, the 50% inhibitory concentration (IC50) of cells was calculated and shown in Figure 2D. Based on the above results, 25 μmol/L(low), 70 μmol/L (medium) and 150 μmol/L (high) were determined for BGC-823 cells, and 50 μmol/L (low), 100 μmol/L (medium) and 150 μmol/L (high) were determined for MGC80-3 cells. Compared with the 0μmol/L (control), the different concentrations of 18-GRA inhibited the proliferation of BGC-823 and MGC80-3 cells ($p < 0.01$) (Figure 2E and F).

18β-GRA arrested GC cell cycle

In order to study the effect of 18β-GRA on GC cell cycle, BGC-823 and MGC80-3 cells were treated with different concentrations of 18β-GRA, and the cell cycle of BGC-823 was arrested in G0/G1 phase, which was 81.84% (150μmol/L), 71.34% (70μmol/L) and 61.66% (25μmol/L) (Figure 3A and B). The cell cycle of MGC80-3 cells was arrested at G0/G1 phase, which was 78.01% (150μmol/L) and 73.37% (100μmol/L) and higher than 60.57% (control). And with the increase of 18-GRA concentration, the proportion of cells in S-phase decreased (Figure 3C and D).

18β-GRA promoted GC cell apoptosis

Flow cytometry was used to detect the effects of different concentrations of 18β-GRA on GC cell apoptosis. After intervention with different concentrations of 18β-GRA in BGC-823 and MGC80-3 cells for 48 h, the apoptosis rate of BGC-823 cells was 14.27% (70μmol/L) and 47.47% (150μmol/L), which was higher than 6.53% (control) (Figure 4A

and B). The apoptosis rates of MGC80-3 cells were 8.87% (50μmol/L), 15.03% (100μmol/L) and 41.8% (150μmol/L), which were all higher than 5.8% (control) (Figure 4C and D).

18β-GRA inhibited GC cell invasion ability

BGC-823 and MGC80-3 cells were treated with different concentrations of 18β-GRA for 48 h, and the cell invasion ability was detected. The results of the Transwell are shown in Figure 5. Compared with control, the different concentrations of 18β-GRA inhibited the invasion of BGC-823 and MGC80-3 cells ($p<0.05$).

18β-GRA inhibited the migration ability of GC cells

The effect of 18β-GRA on GC cell migration was evaluated in Transwell chambers without matrigel. The migration of BGC-823 and MGC80-3 cells were inhibited after 48 h of treatment with different concentrations of 18-GRA (Figure 6).

We also evaluated the effect of 18β-GRA on GC cell migration by wound-scratch assays. The migration of MGC80-3 and BGC-823 cells was inhibited by different concentrations of 18β-GRA for 48 h (Figure 7). With the increase of 18β-GRA concentration, the inhibitory effect was gradually enhanced ($p<0.05$).

18β-GRA inhibited tumor formation in nude mice

The results of tumor formation experiments in nude mice showed that after 2 wk of continuous intragastric administration, the tumor volume in the 18β-GRA group was significantly smaller than that in the control (Figure 8A and B). The tumor volume was recorded within 2 wk, and the tumor growth curve was drawn (Figure 8C). The tumor growth rate of the 18β-GRA group was lower than that of the control ($p<0.01$).

qRT-PCR was used to detect the expression level of *MRPL35* in the transplanted tumor tissues of mice. The results showed that the expression level of *MRPL35* in the transplanted tumor tissues of mice in the 18β-GRA group was significantly lower than that in the control ($p<0.001$) (Figure 8D).

18β-GRA inhibit GC cell proliferation by regulating MRPL35- related apoptosis signaling pathways

Proteomic analysis based on TMT labeling-based was performed on control and GC cells treated with 18β-GRA. A total of 6275 proteins were identified by proteomic analysis, and 609 DEPs were screened using the expression difference ratio>1.2 and p<0.05 as the standard, among which 335 were up-regulated and 274 were down-regulated. To better visualize the DEPs, we drew the volcano plot. The red dots indicate significantly different expressions of up-regulated proteins and blue dots indicate significantly different expressions of down-regulated proteins (Figure 9A). Notably, after 18β-GRA intervention, MRPL35 expression was significantly down-regulated (p=0.000247), TP53 expression was up-regulated (p=0.02676) and BCL2L1 was down-regulated (p=0.01699). The GO annotation and enrichment analysis was used to analyze the DEPs and to determine their biological processes (BP), molecular functions (MF), and cellular components (CC) (Figure 9B and C). In the BP analysis, DEPs were mainly involved in cellular processes, biological regulation and molecular metabolism. It is mainly enriched in complement activation, alternative pathway and classical pathway, and mitochondrial translational termination. With respect to MF, the DEPs involved in the binding, catalytic activity, molecular function regular and so on. It is mainly enriched in the binding of collagen and fibronectin, serine-type endopeptidase inhibitor activity. In the CC analysis, we found that the DEPs were highly localized in the cell, organelle and protein-containing complex part. Those DEPs mainly enriched in extracellular space, collagen-containing extracellular matrix, membrane attack complex and mitochondrial large ribosomal subunit. The DEPs were classified by KEGG pathway enrichment analysis (Figure 9D). KEGG pathway enrichment analyses revealed these DEPs to be enriched in complement and coagulation cascades, ECM-receptor interaction, and cell adhesion molecules. It's worth noting that these DEPs are also involved in the TP53 signaling pathway.

Wolfpsort was used to predict the subcellular localization of these DEPs, which were primarily localized to the nucleus (28.1%), cytosol (23.2%), extracellular (18.4%), mitochondria (13.6%) and so on (Figure 10A). Further protein domain enrichment analysis showed that DEPs were significantly enriched in the immunoglobulin-like fold domain, thrombospondin type-1(TSP1) repeat and superfamily, fibronectin type III and superfamily protein domain (Figure 10B). Cluster analysis was performed on DEPs and drawn as a heat map (Figure 10C). Each row in the figure represented a DEP, red represented up-regulated DEP expression and green for down-regulated DEP expression, and the darker the color was, the more significant the difference was.

Finally, in order to further explore the function of MRPL35, TP53 and BCL2L1, the STRING database was used to construct a PPI network, and the results are shown in Figure 10D, And we found that COPS5 was the protein connecting MRPL35 and TP53, in addition, we also found that TP53 and BCL2L1 are closely related to the pro-apoptotic proteins BAX and BAD.

Effect of 18β-GRA on the expression of MRPL35-related apoptosis proteins

Western blotting was used to detect the effect of 18β-GRA on the expression of MRPL35 related proteins in gastric cancer cells. The results showed that with the increase of 18β-GRA concentration, the expression levels of MRPL35 and BCL2L1 proteins were gradually down-regulated, while the expression of TP53 proteins was up-regulated. The expression of COPS5, the protein linking MRPL35 and TP53, was down-regulated, and the expression levels of pro-apoptotic proteins BAX and BAD were up-regulated. These results confirm the results of our proteomics and bioinformatics analysis above (Figure 11).

DISCUSSION

In this study, the functional changes of GC cells after 18β-GRA intervention were tested by *in vitro* experiments, and the results showed that 18β-GRA had little effect on the survival rate of normal gastric epithelial GES-1 cells, and significantly inhibited the

proliferation of BGC-823 and MGC80-3 cells. Next, we detected the cell cycle found that 18β-GRA could arrest the GC cell cycle in G0/G1 phase. After drug intervention in GC cells, the cells can either exit the cell cycle reversibly, by initiating quiescence, or irreversibly, by senescence or apoptosis. The decision to exit the cell cycle solely depends on the DNA damage checkpoint [20]. In response to irreparable DNA damage, DNA damage checkpoints can initiate quiescence, senescence or programmed cell death, primarily through TP53-dependent pathways[21, 22]. Moreover, TP53 mutations are the most common mutations in cancer. However, even if cancer-related mutations prevent cell cycle exit, continuous proliferation of cells can still be prevented by blocking the entry of G1 cell cycle prior to replication, which relies on the activation of E2F-dependent transcription [23, 24]. We investigated the effect of 18β-GRA on the apoptosis of two types of GC cells, and confirmed the results of cell cycle arrest. It was found that the cells chose to undergo apoptosis and exit the cell cycle, and TP53 played a crucial role in this process.

The cell invasion and migration experiment showed that cell invasion and migration ability were significantly suppressed after 18β-GRA intervention in GC cells. EMT plays an important role in the occurrence of metastasis and invasion of malignant tumors [25]. The loss of epithelial characteristics and the acquisition of mesenchymal features reduce cell adhesion and increase cell mobility. In addition, our previous experiments also showed that LINC00514 was up-regulated in GC specimens compared with non-tumor specimens, and over-expression of LINC00514 induced GC cell growth and EMT progression[26].We used immunodeficient nude mice for tumor-formation experiment *in vitro*. It was found that subcutaneous tumor volume was significantly inhibited in nude mice after two wk of intragastric administration of 18β-GRA compared with the control group, and the expression level of *MRPL35* in the transplanted tumor tissues of 18β-GRA group was significantly decreased by qRT-PCR assay, which also confirmed the results of cell function *in vivo*.

Based on the above results, we further investigated the mechanism of 18β-GRA in inhibiting GC cell proliferation, and promoting cell apoptosis. TMT analysis showed

that there were 609 DEPs in GC cells treated with 18β-GRA, among which the expressions of MRPL35, TP53 and BCL2L1 were significantly altered. GO analysis found that all these DEPs were mainly involved in cellular processes, biological regulation and molecular metabolism, and were enriched in membrane attack complexes and mitochondrial large ribosomal subunits. In addition, subcellular localization analysis of differentially expressed proteins showed that DEPs were mainly distributed in the nucleus, cytoplasm, extracellular space and mitochondria. Our previous study showed that the high expression of MRPL35, a member of the large subunit family of MRPs, was correlated with the poor survival rate in GC, and knocking down MRPL35 could inhibit GC cell proliferation. Interestingly, KEGG results showed that these DEPs were enriched in the TP53 signaling pathway, which was ranked tenth in the analysis. TP53 is an important protein involved in the process of cell apoptosis. As a transcription factor, TP53 is mutated in most human cancers, oncogenes and DNA damage [27]. To inhibit cancer, TP53 protein regulates the transcription of many different genes, including *BAX*, *NOXA1*, and *GADD45A*, in response to multiple stress signals, such as DNA repair, cell cycle arrest, senescence, and apoptosis, while tumor mutations can make them resistant to apoptosis induced by the TP53 pathway. Combined with the expression changes of BCL2L1, we speculated that 18β-GRA might play a role in inhibiting the proliferation of GC by regulating TP53 apoptosis-related signaling pathways through MRPL35. Enrichment analysis of DEP domains showed that they were significantly enriched in immunoglobulin-like folding domains, TSP1 repeats and superfamily, fibronectin type III and superfamily protein domains. Hence, we hypothesized that 18β-GRA might regulate the expression of MRPL35, induce the changes of apoptosis-related proteins TP53 and BCL2L1, and play a role in inhibiting the proliferation of GC cells.

Finally, we analyzed the input STRING database of these three signaling proteins, and found that COPS5 is the protein connecting MRPL35 and TP53. Therefore, we believe that MRPL35 is likely to play a pro-apoptotic role by regulating COPS5. In addition, we also found that TP53 and BCL2L1 were closely related to BAX and BAD.

As a major regulator of mitochondrial involvement in the intrinsic apoptosis pathway, BAX controls a cell's commitment to the pathway by changing the physiology of resident mitochondria. BAX undergoes a series of ordered events that lead to the formation of pores in the outer membrane of mitochondria, facilitating the release of signaling molecules such as cytochrome C [28]. This event is commonly considered as the "point of no return" in cell death[29]. BAD, a ligand of the pro-survival protein 14-3-3 is an on-off protein in the mitochondria-induced apoptotic pathway [30]. When separated from 14-3-3, BAD may interact with BCL-2/BCL2L1 and release BAX, revealing its apoptotic activity. Binding of BAD to BCL2/BCL2L1 has also been reported to release the activator BH3-only molecules, such as Bim, Bid, and Puma, to directly activate BAX[31, 32].

Based on the above mentioned proteomics and STRING analyses, we concluded that 18β-GRA is likely to inhibit the proliferation of GC cells by regulating the expression of MRPL35, thereby affecting the expression of TP53, BCL2L1, COPS5, BAX and BAD proteins. To verify the above hypothesis, the expression of MRPL35, COPS5, apoptosis-related protein TP53, pro-apoptotic protein BAX, BAD and anti-apoptotic protein BCL2L1 were down-regulated after 18β-GRA intervention. The anti-tumor activity of GRA and its derivatives are associated with the mitochondrial apoptotic pathway of mitochondrial membrane potential depolarization, which leads to the activation of caspase-9 and caspase-3, and the production of ROS. Cancer cells can avoid apoptosis by destroying the balance between pro-apoptosis and pro-survival, so that the apoptotic signal of mitochondria is insensitive to cell death. Studies have shown that 18β-GRA may potentiate the heat shock protein 90 inhibition-induced apoptosis in ovarian carcinoma cell lines *via* the activation of the apoptosis-related proteins and the mitochondria-mediated cell death pathway, leading to activation of caspases [33].

MRPs play a critical role in protein synthesis and mitochondrial function, and also participate in the regulation of cell apoptosis, protein biosynthesis and signal transduction. MRPL35 and its partner MRP7 play a key role in coordinating the synthesis of the COX1 subunit with its assembly into the COX enzyme [15]. The

expression of MRPL35 was found to be higher in CRC tissues than in matched cancer-adjacent tissues. *In vitro*, down-regulation of MRPL35 Led to increased production of ROS along with DNA damage, decrease in mitochondrial membrane potential and autophagy induction, thereby inhibiting the progression of CRC [16]. Oncomine showed that the expression of MRPL35 in GC tissues was significantly higher than that in adjacent tissues. Our previous results also showed that the expression of MRPL35 was significantly up-regulated in GC, which was associated with poor survival rate of GC patients. Moreover, the knockdown of MRPL35 could inhibit the proliferation and colony formation of GC cells and induce apoptosis [18].

This study demonstrated that 18-GRA inhibited the proliferation and promoted apoptosis of GC cells by down-regulating the expression of MRPL35, which regulated TP53 and BCL2L1 proteins, both *in vivo* and *in vitro*. Our proposed research in the future will be divided into three stages: In the first stage, the signaling pathways of MRPL35, TP53 and BCL2L1 proteins will be explored, possibly through methylation, ubiquitination, acetylation and phosphorylation. The second stage will be to evaluate the interaction mechanism between MRPL35 and COPS5 proteins. Two modes of action will be considered: direct action and through tool protein action. Co-immunoprecipitation (Co-IP) or yeast two-hybrid system may be used. In the third stage, MRPL35 and COPS5 protein interaction sites will be explored. After silencing the *MRPL35* and *COPS5* gene domains, GST pull-down and Co-IP will be used to verify whether the two proteins interact. The present study aimed to provide a scientific basis for the treatment of GC and identify an effective drug target for the early prevention of GC.

CONCLUSION

18β-GRA can down-regulate the expression of MRPL35 protein, suppress the expression of anti-apoptotic protein BCL2L1, and promote the pro-apoptotic protein TP53, BAX and BAD through COPS5, thus inhibiting the proliferation of gastric cancer cells.

ARTICLE HIGHLIGHTS

Research background

Gastric cancer (GC) is a common malignant tumor of digestive tract in the world, with more than 1 million new cases to be expected each year since 2020. MRPL35 is a member of the large subunit family of mitochondrial ribosomal proteins, which plays an important role in the development of cancer.

Research motivation

At present, the treatment of gastric cancer is mainly surgery, chemotherapy and radiotherapy, and the first-line treatment drugs are mainly harmful due to side effects.

Research objectives

The purpose of this study was to investigate the correlation between MRPL35 apoptosis related signaling pathway and GC.

Research methods

Cell functional indexes were determined by CCK-8, flow cytometry, Transwell and cell scratch. The effect of 18β-GRA on proliferation of gastric cancer cells was observed by BABL/ C tumor-forming experiment in nude mice. TMT labeling combined with LC-MS/MS to screen for differentially expressed proteins extracted from GC cells and control cells after 18β-GRA intervention. Moreover, STRING database was used to predict PPI relationships and western blotting was used to detect the expression of proteins of interest in GC cells.

Research results

18β-GRA can inhibit the proliferation of gastric cancer cells, induce apoptosis, and arrest the cell cycle at G0/G1 phase, and inhibit the migration and invasion of gastric cancer cells. 18β-GRA can inhibit tumor formation in BALB/c nude mice. Compared

with the control group, MRPL35 and BCL2L1 expressions were significantly down-regulated and TP53 expression was up-regulated after 18β-GRA intervention. STRING analysis showed that COPS5, BAX and BAD proteins could form PPI network with MRPL35, TP53 and BCL2L1 proteins. After 18β-GRA intervention, MRPL35, TP53 and BCL2L1 were up-regulated/down-regulated in a dose-dependent manner, as were COPS5, BAX and BAD.

Research conclusions

18β-GRA can inhibit the proliferation of GC cells by regulating MRPL35, COPS5, TP53, BCL2L1, BAX and BAD.

Research perspectives

MRPL35 can be used for targeted therapy of GC, and can also be used as a new biomarker for GC.

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