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Ghrelin O-acyltransferase could affect the cell proliferation, apoptosis and autophagy of gastric cancer by regulating AKT

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Abstract

Objective: Previous studies have shown that certain polypeptide molecules are crucial parts in the tumorigenesis of gastric cancer (GC). Herein, we explore the potential mechanism between Ghrelin-o-acyltransferase (GOAT) and the GC.

Methods: Firstly, GC cells SGC7901 and AGS were purchased and cultured. Then, the influence of over-expressed GOAT on the cell growth, cycle, apoptosis, and autophagy in SGC7901 and AGS cells were evaluated by RT-qPCR, CCK-8 detection, flow cytometry detection, and transmission electron microscopy, respectively. Meanwhile, we detected the over-GOAT effects on GC cells and protein expressions by RT-qPCR and western blotting (WB), compared with AKT and COX2 genes.

Results: Consequently, it was confirmed that over-expressed GOAT in SGC7901 and AGS cells accelerated cell proliferation, retarded cell apoptosis, delayed cycle arrest, and stimulated autophagy in GC cells. Besides, the expressions and protein levels of COAT, AKT and COX2 genes were all up-regulated in both cell lines with over-GOAT, but only AKT results were positive, which indicated that GOAT might only play a regulatory role in AKT.

Conclusions: Our findings suggest that GOAT, acting as an oncogenic factor in GC, promotes cell proliferation and autophagy, inhibits apoptosis by regulating the AKT. Therefore, our findings suggest that GOAT may become the potential diagnostic and therapeutic targets for GC patients.

Background

Gastric cancer (GC) is the second deadliest cancer in the world, and it frequently manifests itself insidiously. Every year, approximately 950,000 new cases are diagnosed, primarily men over the age of 50, with nearly 700,000 deaths [1,2]. At present, GC can be divided into early and advanced GC according to the degree of incidence. The former usually has symptoms, such as upper abdominal fullness and discomfort, acid regurgitation and so on, which is easily confused with chronic gastrointestinal diseases [3]. The latter often has irregular upper abdominal pain, nausea, vomiting, obstruction, and even blood vomiting, black stools, and significant weight loss [4]. As for the causation of GC, genetics [5], helicobacter pylori and unhealthy lifestyles like staying up late for extended periods, irregular meals and high levels of mental stress, will wreak havoc on the gastrointestinal tract's normal function and eventually lead to the incidence of GC [6,7]. Due to the low diagnostic rate of early-stage GC patients and the poor prognosis of advanced GC patients [8], it is still critical to explore new and effective diagnostic biomarkers and therapeutic targets for GC patients.

Ghrelin is a 28-amino-acid polypeptide generated mostly by P/D1 cells at the stomach's bottom and pancreatic ε cells [9,10]. Ghrelin receptors are found in the stomach, pancreas, gonad, and other organs, which indicates a wide range of biological roles of Ghrelin [11]. Ghrelin-o-acyltransferase (GOAT) belongs to the acyltransferase family, and it is the only one that has the capacity to acyl-modify Ghrelin [12]. Presently, GOAT has been proven to have a functional role in some diseases. For example, GOAT is regarded as a possible treatment target of obesity and diabetes mellitus for its activation in metabolic hormone [13,14]. Zhang SR et al. pointed out that knockdown of GOAT by siRNA could reduce steatosis in hepatocytes by regulating the AMPK/mTOR pathway and up-regulating autophagy activity [15]. In Kirchner H et al.'s study, GOAT acylated gastric-derived peptides and medium-chain fatty acids, thereby regulating gastric emptying and insulin secretion [16]. Nevertheless, the biological roles and activities of GOAT in the incidence and progression of GC are poorly understood.

This research plans to explore the functional roles of GOAT in GC by experiments, such as Real-time polymerase chain reaction (RT-qPCR), Cell Counting Kit 8 (CCK-8), Western blotting (WB), flow cytometry and others. These experiments above will provide new directions for the clinical strategies of GC.

Material and methods

Cell source

GC cells (SGC7901 and AGS) were acquired from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China), and grew in DEME containing 10% FBS with 5% CO_2 at 37°C.

Cell transfection

We constructed targeting GOAT plasmids from Ribobio (Guangzhou, China) as instructed. Firstly, SGC7901 and AGS cells were transfected with 10 μM GOAT and NC control plasmids, and then RT-qPCR was performed to detect gene changes at the RNA level for transfection verification.

Cell proliferation assay

To study the influence of GOAT overexpression on cell growth, we first seeded 1×10^3 SGC7901 and AGS cells in 96-well plates, and transfected them with over-GOAT and over-NC, respectively. Then, they were incubated with 10 μl/well CCK-8 solution in the dark for some time. At last, the optical density (OD) values at 450 nm were observed with a microplate reader at 0, 1,2,3,4, and 5 days, respectively.

Real-time PCR

To detect gene changes at the RNA level, we applied TRIzol reagent (Invitrogen, Carlsbad, CA, USA) to extract all RNAs from GC cells, and then they were reversely transcribed into complementary deoxyribonucleic acid (cDNA) according to the instructions of the RT-qPCR system.

Western blotting assay

In GC cells AGS and SGC7901, the changes of COX2, AKT and GAPDH (internal reference) protein levels after GOAT overexpression were detected by WB. The specific operations were as follows: the concentrations of protein were measured by bicinchoninic acid technique. 10-12% SDS-PAGE isolated protein samples of the same amount (40 g/lane), and then they were transferred to PVDF membranes. Membranes were blocked at room temperature for 1 hour with 5% BSA. Next, we probed primary antibody (Akt 1:1000, COX2 1:1000, GAPDH 1:1000) into the membranes overnight in the icebox at 0-4°C, and then used the secondary antibody (goat anti-rabbit IgG 1:5000) for another 1 hour in the icebox at 4°C. Enhanced chemiluminescence (ECL) kit was for visualizing protein bands, and Image Quant LAS 4000 Mini system (GE Healthcare Life Sciences, Chalfont, UK) capturing the images.

Flow cytometry detection

The cell apoptosis was detected using the Annexin V-FITC/PI Apoptosis Kit (BioVision, Inc. Milpitas, CA, USA). After culture overnight in 6-well plates, cells were harvested, centrifuged, washed and placed in that kit. Finally, Guava Easy Cytometer (Germany) was applied to observe cell apoptosis.

As for cell cycle detection, 1×10^6 cells were collected and centrifuged to discard the supernatant, and then washed once with PBS and centrifuged to discard the supernatant again. Next, we supplemented 10 μL Permeabilization and 1 ml DNA Staining solution and blended for 5-10 seconds, and then cells were incubated out of the sun at indoor temperature for 30 minutes. Finally, observation was realized by flow cytometry detection.

Transmission electron microscopy (TEM)

Cells with 5 or more autophagic vacuoles are called autophagic vacuoles. Cell slides were counterstained with 0.3% lead citrate, and TEM [17] was applied to detect the microstructural changes and autophagic vacuoles of autophagosomes.

Statistical analysis

Statistics were processed by GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA), two-tailed t-test and Chi-square test. When $P<0.05$, it meant statistical significance.

Results

GOAT promotes GC cell progression through regulating AKT gene

Previously, AKT and COX2 are reported to have regulatory functions in GC development. To determine the relationship among GOAT, AKT and COX2 genes, we first used RT-qPCR to detect their expression changes at the RNA level. In comparison with NC, GOAT had a higher in over-GOAT group (Figure 1A). Also, the corresponding levels of AKT and COX2 were increased in GC cells with overexpressed GOAT (Figure 1B and 1C). Besides, we performed WB experiments in SGC7901 and AGS cells (over-GOAT group vs NC group) and observed changes in the expression levels of AKT and COX2 proteins. WB results showed that in the two cells, the expressions of AKT and COX2 proteins were both elevated, compared with control group, but only the result of AKT was positive (Figure 1D). This indicated that GOAT might play a regulatory function on AKT in GC progression. To sum up, GOAT may promote the occurrence and development of GC cells through AKT.

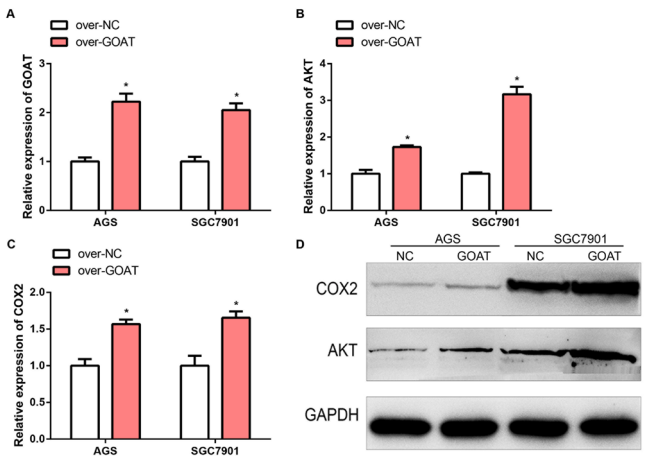

Figure 1: GOAT promotes GC cell progression through regulating AKT gene.
(A) The expression of GOAT was increased in the over-GOAT group.
(B) The expression of AKT was increased in the over-GOAT group.
(C) The COX2 expression level was increased in the over-GOAT group.
(D) Expression changes of COX2 and AKT in GC cells with over-GOAT by WB. **P*<0.05.

Over-GOAT promotes the proliferation of GC cells

Next, we deeply explore the role of GOAT in GC cell proliferation. CCK-8 assay demonstrated a higher proliferation rate after over-GOAT transfection in SGC7901 cells (Figure 2A). In addition, the results were the same in AGS cells (Figure 2B). These findings showed over-GOAT could facilitate the cell growth of GC.

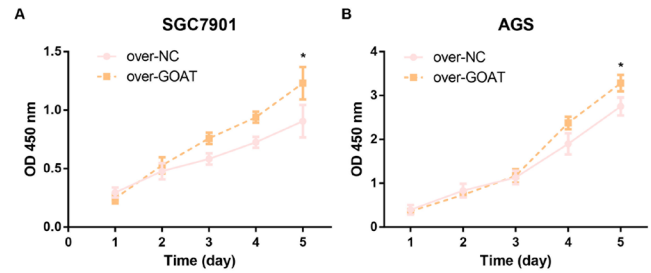

Figure 2: Up-regulation of GOAT promotes the proliferation of GC cells.
(A) SGC7901 cells with over-GOAT had higher proliferation rate.
(B) AGS cells with over-GOAT had higher proliferation rate.
**P*<0.05.

Upregulation of GOAT induces cell cycle acceleration in GC

Cell cycle acceleration is one of the mechanisms for the rapid progression of GC. Therefore, flow cytometry was performed to study cell cycle development upon GOAT overexpression. As shown in Figure 3A and 3B, compared with SGC7901-NC, SGC7901 cells were significantly blocked in the G1 phase, when GOAT was overexpressed. However, cells in the S phase and G2 phase were obviously elevated. Cells in the G1 phase were decreased in the GOAT overexpression group, while cells in the and S phase and G2 phase were increased (Figure 3C, 3D). Our findings suggest that overexpression of GOAT enhances the cell proliferation ability was enhanced, and is associated with G1 phase arrest of cell cycle in GC.

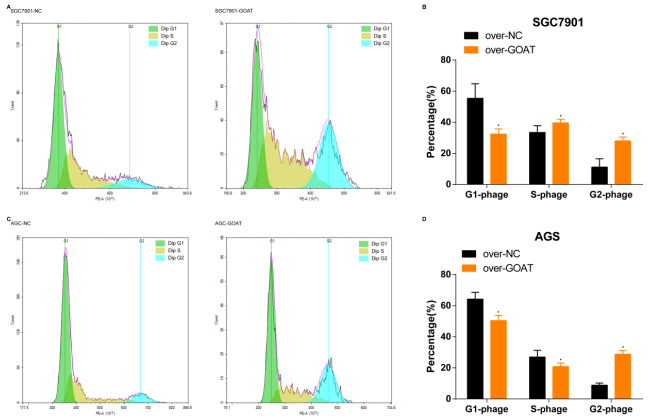

Figure 3: Up-regulation of GOAT induces cell cycle acceleration in GC.
(A,B) GC cell cycle changes in SGC7901 with over-GOAT.
(C,D) GC cell cycle changes in AGS with over-GOAT. **P*<0.05.

Overexpression of GOAT blocks the apoptosis of GC cells

We also studied the apoptotic results after GOAT overexpression in GC cells. As a result, lower percentages of Q3-2 and Q3-4 quadrants in SGC7901 and AGS cells with over-GOAT were observed, indicating the less cell apoptosis (Figure 4A-4D). Our experiments indicated the percentage of apoptosis was reduced after the upregulation of GOAT, and confirmed the active role of GOAT in apoptosis tolerance in SGC7901 and AGS cells.

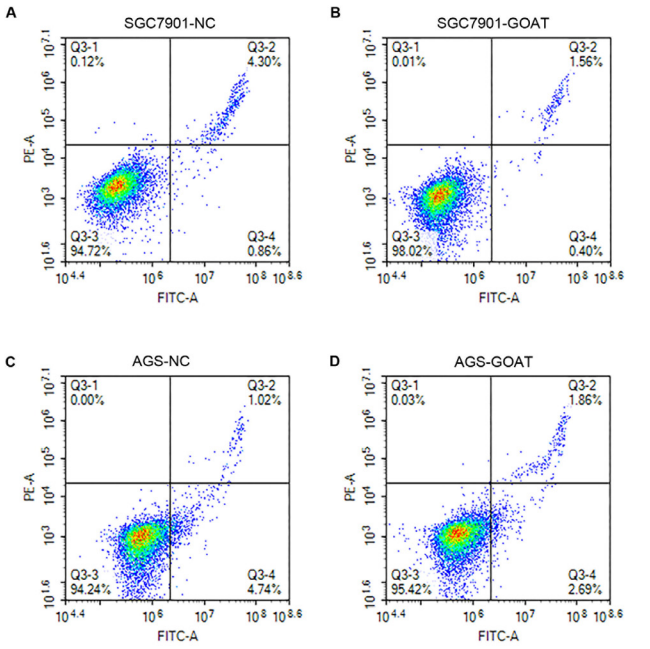

Figure 4: Overexpression of GOAT inhibits the apoptosis of GC cells.
(A,B) Cell apoptosis scatter plot of SGC7901-NC and SGC7901-GOAT.
(C,D) Cell apoptosis scatter plot of AGS-NC and AGS-GOAT. *P<0.05.

GOAT overexpression induces cell autophagy in GC

Autophagy acts a vital part in many pathophysiological processes, including cell development, proliferation and apoptosis. It also plays a crucial role in the tumorigenesis of malignant tumors. To determine the effect of over-GOAT on autophagy in GC cells, we observed the morphological changes of SGC7901 and AGS cells with GOAT overexpression by TEM. As shown in Figure 5, more autophagic vesicles and autophagosomes with double-membrane vacuolar structures appeared in over-GOAT-

treated SGC7901 and AGS cells than control group. These results suggest that GOAT is closely related to the cell autophagy in SGC7901 and AGS cells, and involved in the tumorigenesis of GC.

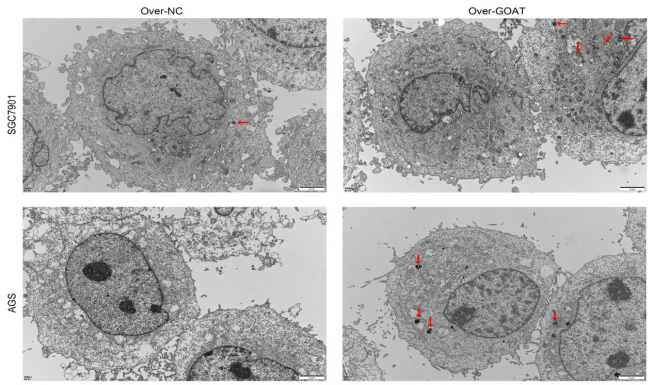

Figure 5: Overexpression of GOAT inhibits the apoptosis of GC cells. **(A,B)** Cell apoptosis scatter plot of SGC7901-NC and SGC7901-GOAT.
(C,D) Cell apoptosis scatter plot of AGS-NC and AGS-GOAT. *P<0.05.

Representative transmission electron microscopy images of SGC7901 and AGS cells treated with over-NC or over-GOAT. Red arrows point autophagic vacuoles.

Discussion

GC is a malignant tumor that causes huge harm to human health. Studies have pointed out that polycyclic aromatic hydrocarbons, helicobacter pylori infection, gastric ulcer, remnant stomach and others are all dangerous risks of GC [18]. Although gastroscopy and surgical resection are currently available for targeted treatment, most patients with advanced disease still have poor prognosis [19]. Based on this, it is necessary to explore the pathogenesis of GC and find new and effective therapies for GC patients.

Herein, we conducted multiple experiments on GOAT and GC progression to find new directions of GC diagnosis and treatment. The experimental analysis of SGC7901 and AGS cells in this study found over-GOAT significantly enhanced the proliferation of SGC7901 and AGS cells, shortened the cycle of cell division, and slowed down the process of cell apoptosis. Likewise, when GOAT was up-regulated, SGC7901 and AGS cells were found to promote cell autophagy compared with controls, and significantly increased autophagic vesicles were observed in TEM images. Besides, we also found that the expressions of AKT and COX2 genes and proteins were up-regulated by overexpressed GOAT through PCR and WB assays, and only AKT had positive results in both cell lines during GOAT upregulation. This indicates that GOAT may play a regulatory role in AKT. Therefore, we believe that GOAT can regulate the expression of AKT, thereby affecting the progression of GC.

The cell cycle encompasses the entire period from the conclusion of one cell division to the beginning of the next [20]. Many studies have previously demonstrated that the length of the cell cycle is related to the cell type of the species, and the disorder of this process can easily lead to the occurrence of tumors [21]. For example, Zhang L et al. confirmed by cellular experiments that lncRNA CASC11 could promote GC cell proliferation, migration and invasion by regulating the cell cycle [22]. In the study of Wu L et al., it was pointed out that in GC tissues, germacrene could block the cell cycle of BGC823 and promote cell apoptosis, thereby exerting an anti-tumor effect [23]. Through cell cycle experiments, our results showed that overexpression of GOAT in GC cells led to a cell reduction of the G1 phase and increase in the S and G2 phases.

Autophagy is a cytoplasmic protein and organelle degradation process by lysosomal pathway, which are highly conserved in evolution and widely present in animal cells [24]. We understand that in cancer cells, cells are already controlled by various mechanisms of tumorigenesis [25]. In many cases, cells are in a state of hypoxia, which induces mitophagy to maintain cell survival [26]. In addition, genomic instability can lead to protein folding errors, and at the same time produce a large number of ubiquitin-proteases. The large accumulation of these junk proteins is bound to deter the survival of cancer cells. At this time, cells selectively activate intracellular autophagy receptors for survival, making autophagy very active in cancer cells [27,28]. In recent years, it has been gradually discovered autophagy is a certain regulatory role in the occurrence and development of tumors, which provides new clues for new therapies of diseases. Wang X mentioned that inhibition of autophagy resulted in the buildup of p62/SQSTM1 and the activation of nuclear factor (NF)-B, thereby reducing PD-L1 expression in GCs [29]. Li et al. showed that DAPK3 as a regulator of autophagy could inhibit GC progression by over activating ULK1-dependent autophagy [30]. In this experiment, we found that over-GOAT reduced cell apoptosis, and autophagic vesicles were significantly increased in SGC7901 and AGS cells treated with over-GOAT. The above findings indicated over-GOAT could promote cell autophagy in GC progression, which sheds new light on new GC treatment methods.

Through a series of functional experiments, we conclude that the overexpression of GOAT promotes cell proliferation, shortens the cell division cycle, inhibits apoptosis and promotes autophagy by regulating AKT *in-vitro*. Therefore, GOAT may serve as a potentially effective diagnostic biomarker and clinical target for GC patients.

Declarations

Conflict of interest: None.

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

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