Ghrline Promot the Ovarian Cancer Cell Autophagy by LncRNA linc00598

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Abstract

Objective

To explore the autophagy effect of ghrelin on the ovarian cancer cell line SK-OV-3. And the lncRNA which regulate the ghrelin effect SK-OV-3 autophagy was showed.

Methods

the expression of ghrelin in the ovarian cancer tissues was analyzed according GEPIA database and HPA database. The CCK-8 was used to detect the the optimal concentration of ghrelin effect on the SK-OV-3. The influence on the SK-OV-3 cell autophagy by ghrelin was showed by detecting the expression of Beclin-1, LC3 and LC3 using western blot. Linc00598 selected as the effecting the SK-OV-3 cells autophagy by ghrelin using RNA-Seq. And the Linc00598 which was silenced or overexpressed promote the SK-OV-3 cells autophagy treated by ghrelin though western blot.

Results

Ghrelin was expressed low in the ovarian cancer tissues. Ghrelin concentratio of 600 ng/ml was the optimal concentration o and 24 h was the optimal time. Ghrelin can promote the SK-OV-3 cell autophagy. Ghrelin mainly through linc00598 to promote the SK-OV-3 cells autophagy. When the linc00598 silenced, ghrelin promote SK-OV-3 cells autophagy was inhibited. And When the linc00598 overexpressed, ghrelin promote SK-OV-3 cells autophagy was inhanced.

Conclusions

Ghrelin promote SK-OV-3 cells autophagy. Additionally, we proved that ghrelin regulated the progression of SK-OV-3 cells autophagy by linc00598/ Beclin1 axis.

Introduction

Ovarian cancer is the common gynecologic cancer death in worldwide. It threat to women's health(Wu, Gao et al. 2021; Xie, Wang et al. 2021). And a large number of women was died from ovarian cancer each year. The overall 5-year survival rate of ovarian cancer is about 47%. However, in the early stage, the typical symptoms was lacked in the patients of ovarian cancer(Wang, Chen et al. 2021; Wei, Lv et al. 2021). More than 2/3 cancer cases was diagnosed at the advanced stage, and the 5-year survival rate was less than 25%. Hence, the diagnosis and treatment on ovarian cancer gave a big challenge(Wang and Zheng 2020; Venkatappa and Sarkar 2021; Wang, Yan et al. 2021; Wei, Feng et al. 2021). To find the biomarkers and therapeutic targets were the useful way to improve the diagnosis and prognosis in the early stage ovarian cancer(Wang, Peng et al. 2018; Sun, Hu et al. 2020; Takagi, Yagi et al. 2021; van der Wel, Uijterwaal et al. 2021).

Ghrelin is one of the endogenous ligands of growth hormone secretagogue receptor, which can promote secretion of growth hormone and is proven to be with the orexigenic and adipogenic effects(Leng, Zhao et al. 2021). Moreover, the positive effect of Ghrelin on metabolism of bone has been observed through regulation of by proliferation ovarian cancer cells(Lin and Hsiao 2017; Khatib, Shankar et al. 2018; Asadi, Farahani et al. 2021).

Long non coding RNA (lncRNA) is about 200nt, and cannot translated into proteins(Meng, Zhou et al. 2020; Wang, Ding et al. 2021; Yang and Dong 2021). However, lncRNA can regulate many cell physiological process, such as cell proliferation, autophagy, apoptosis. lncRNA is also the potential prognostic marker for many tumors(Chen, Xie et al. 2021; Guo, Du et al. 2021).

Cell autophagy can clean out the damaged organelles, proteins in normal(Sun, Hu et al. 2020). However in the apoptotic cells, autophagy can prevent the necrosis, and local inflammation(Meng, Zhou et al. 2020; Cai, An et al. 2021). Hence, in this study, the autophagy effect of ghrelin on the ovarian cancer cell line SK-OV-3 was explored. And the lncRNA which regulate the ghrelin effect SK-OV-3 autophagy was showed.

Materials And Methods

Cell culture

The ovarian cancer cell line SK-OV-3 () containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 0.1 mg/mL streptomycin, with 5% CO₂.

Cell counting kit-8 (CCK-8) assay

Cell proliferation after ghrelin treatment was assessed using the CCK-8 (K1018, Apexbio, Houston, TX, USA) kit. Cells (1×10^4 cells per well) were plated in the 96-well plates (100 μ L/well) and 10 μ L of CCK-8 solution was added at each time point (1d, 2d, 3d, 4d, 5d, 6d, and 7d) for 2 h at 37°C. The absorbance values at 450 nm were then measured using an enzyme marker.

RNA extraction and sequencing

Sequencing libraries were generated using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA). Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in an Illumina proprietary fragmentation buffer. First strand cDNA was synthesized using random oligonucleotides and SuperScript II. Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities and the enzymes were removed. After adenylation of the 3' ends of the DNA fragments, Illumina PE adapter oligonucleotides were ligated to prepare for hybridization. The library fragments were purified using the AMPure XP system (Beckman Coulter, Beverly, CA, USA). DNA fragments with ligated adaptor molecules on both ends were selectively enriched using Illumina PCR Primer Cocktail in a 15 cycle PCR

reaction. Products were purified (AMPure XP system) and quantified using the Agilent high sensitivity DNA assay on a Bioanalyzer 2100 system (Agilent). The sequencing library was then sequenced on a HiSeq platform (Illumina) by Seqhealth Technology Co., Ltd., Wuhan, China. Differentially expressed genes were then identified by applying a FDR cutoff of 0.05.

Gene function annotation and pathway analysis

Identification of enriched KEGG pathways in the upregulated and downregulated gene lists was performed with DAVID (Database for Annotation, Visualization, and Integrated Discovery) v6.8. Pathway analysis was performed using Ingenuity Pathway Analysis (IPA), version to infer the functional roles and relationships of the differentially expressed genes based on the log₂ fold-change value of each gene.

Cell transfection

The mimic of miR-378b as well as negative control (NC mimic) were purchased from Invitrogen (Carlsbad, CA, USA). The full length of SNHG10 was ligated in pcDNA3.1 vector (pcDNA3.1SNHG10) was purchased from GenePharma (Shanghai, China) with empty plasmid as negative control (pcDNA3.1). Cell transfection was conducted using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). After 48 h, cells were harvested for following experiments.

Real-Time qPCR analysis

The total RNA was extracted from the SK-OV-3 cells by using the commercial TRIzol reagent (Invitrogen, USA) following the manufacturer's instruction. The quality of the total RNA was determined by agarose electrophoresis, and the RNA was reversely transcribed into complementary DNA (cDNA) by using the TIANScript RT kit (Tiangen Biotech, China). Then, the SYBR Premix Ex Taq™ II Kit (Takara, Japan) was used to determine relative gene expression levels and enrichment. The up-primer sequences for linc00598 is CCTCCCCTACTATCAACATCCC and down primer is TGCCAAGAACGAGCCCTA. The expression levels were normalized by GAPDH (up primer CAATGCCTCCTGCACCACCAACTGC and down primer GCAGTTGGTGGTGCAGGAGGCATTG)

Western blot

Total tissues or cellular proteins were extracted using high performance radio immunoprecipitation analysis lysis buffer (C0481, Sigma-Aldrich) containing 1% protease inhibitor and 1% phosphatase inhibitor (Beyotime). The protein concentration of each sample was determined by a bicinchoninic acid kit (23227, Thermo Fisher Scientific). The proteins were separated by polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) which were blocked with 5% bovine serum albumin (BSA) at room temperature for 1 h. Primary antibodies including rabbit anti-Beclin-1 (1:1000, 3495, CST) rabbit anti-LC3 α and β (1:1000, 4108, CST), and rabbit anti-GAPDH (1:3000, a5174, CST) were incubated with membranes overnight at 4°C. On the second day, the membranes were washed with TBST, and incubated with horseradish peroxidase (HRP)-labeled goat anti-

rabbit immunoglobulin G (IgG; 1:20000, ab205718, Abcam) dilutions at room temperature for 1.5 h. After incubation, the membranes were developed by developing liquid (NCI4106, Pierce, Rockford, IL, USA).

Statistical analysis

Statistical analyses of data were performed using Graphpad Prism 8.0 (GraphPad Software, San Diego, CA, USA). The measurement data were expressed as mean $\pm$ standard deviation. Independent sample t test were used in comparisons between groups, and one-way analysis of variance (ANOVA) was used for comparison among multiple groups, followed by Tukey's post hoc tests. Comparison of data between groups at different time points was performed using two-way ANOVA. A $p < 0.05$ indicated statistically significant.

Results

Ghrelin down expression in the ovarian cancer tissues

To obtain the ghrelin expression in the ovarian cancer tissues, the GEPIA database was used to analyse. The ghrelin expression was lower in the ovarian cancer tissues than the normal tissues (Fig. 1A). And in HPA database ghrelin was lowly expressed in ovarian cancer tissues compared with normal tissues ($P < 0.05$, Fig. 1B). Hence, the ghrelin was expressed low in the ovarian cancer tissues.

Ghrelin promoted the cell autophagy and inhibited the cell viability in SK-OV-3 cells

To obtain the optimal concentration of ghrelin effect on the SK-OV-3, the SK-OV-3 cells were treated with ghrelin with the concentrations of 400, 500, 600, 700 and 800 ng/ml. Compared with the blank control group (control), the cell survival rate of 400, 500, 600, 700 and 800 ng/ml group decreased at 24 h, 48 h and 72 h ($P < 0.01$). When SK-OV-3 cells were treated with 600 ng/ml ghrelin for 24 h, 48 h and 72 h, the cell survival rates were around 50% (Fig. 2A). Hence, the ghrelin concentration of 600 ng/ml was the optimal concentration and 24 h was the optimal time.

The influence on the SK-OV-3 cell autophagy by ghrelin was showed by detecting the expression of Beclin-1, LC3 and LC3 (Fig. 2B). The expression of Beclin-1 and LC3 in SK-OV-3 cell treated with the 600 ng/ml ghrelin 24h was higher than without ghrelin treated (Fig. 2C&D). To explore the function of ghrelin on the SK-OV-3 cell autophagy further, the D-Lys3-GHRP6, a ghrelin receptor antagonist, was used. The expression of Beclin-1 and LC3 in SK-OV-3 cell treated with ghrelin was higher than ghrelin+ D-Lys3-GHRP6 treatment (Fig. 2E-G).

Linc00598 selected as the effecting the SK-OV-3 cells autophagy by ghrelin using RNA-Seq

To found the LncRNAs which effected the SK-OV-3 cells autophagy by ghrelin, the SK-OV-3 cells treated with ghrelin was analyzed by RNA-Seq. The differential expression of LncRNA was analyzed by DESeq. The conditions for screening differentially expressed genes were as follows: the multiple of expression difference $|\log_2\text{foldchange}| > 1$, $P < 0.05$. Compared with the control group, 236 LncRNAs were

differentially expressed (up-regulated 130 and down regulated 106), in the ghrelin treatment group. The volcanic map shows the distribution of LncRNAs, the difference of LncRNAs expression fold and significance results (Fig. 3A). In Fig. 3A, the red dot represents the differentially up-regulated LncRNAs, the blue dot represents the differentially down-regulated LncRNAs, and the gray dot represents the non significant differentially expressed LncRNAs.

The go enrichment analysis results of differentially expressed LncRNAs are classified, according to molecular function MF, biological process BP and cell component CC. The top 10 go term items with the smallest p-value, i.e. the most significant enrichment, are selected for display in each go classification (Fig. 2B). In the figure 2B, the orange histogram represents the top 10 cell components with the most significant enrichment, the green histogram represents the top 10 molecular functions with the most significant enrichment, and the blue histogram represents the top 10 biological processes with the most significant enrichment. According to the go enrichment results, the enrichment degree is measured by rich factor, FDR value and the number of LncRNA enriched on the GO term. The larger the rich factor, the greater the degree of enrichment. The general value range of FDR is 0-1. The closer is closed to zero, the more significant the enrichment. The first 20 GO term entries with the lowest FDR value, are selected for display (Fig. 2C). Go enrichment analysis showed that these differentially expressed LncRNAs were mainly related to arginine and lysine transmembrane transport, oxidative stress response and developmental process.

The top 20 pathways with the lowest p-value value are displayed, according to the KEGG enrichment analysis the differentially expressed LncRNAs (Fig. 3D). The path in the figure mainly involves four aspects: environmental information processing (orange part), human diseases (green part), metabolism (blue part) and organic systems (purple part). According to the KEGG enrichment results, the enrichment degree, the top 20 KEGG pathways with the lowest FDR value are selected. (Fig. 3E). KEGG enrichment analysis showed that these differentially expressed LncRNAs were mainly enriched in cytokine receptor signaling pathway, glucagon and insulin signaling pathway and cancer-related signaling pathway.

According to the prediction of target genes and the enrichment analysis results of go and KEGG pathway, the linc00598 was selected as the potential relationship with autophagy, and the linc00598 was differential up-regulation of fold change > 2 times. The expression of linc00598 in SK-OV-3 cells was detected by qPCR. The expression of linc00598 in the ghrelin treated group was higher than blank group.

Linc00598 promote the SK-OV-3 cells autophagy treated by ghrelin

To show the function of linc00598 on the SK-OV-3 cells autophagy, the linc00598 was silenced and overexpressed (Fig. 4A&B). And the expression of Beclin1 and LC3 in the linc00598 silence group (si-linc00598) was lower than the control group and si-NC group (the blank plasmid, Fig. 4C,D,F). However, in the linc00598 overexpressed group (h-linc00598), the expression of Beclin1 and LC3 was high (Fig. 4C,E,G). The effect of linc00598 silenced or overexpressed on the SK-OV-3 cells autophagy treated by ghrelin was also explored. The expression of Beclin1 and LC3 in the Ghrelin + Si-linc00598 group was

lower than the ghrelin group(Fig. 4A,B,D). And the expression of Beclin1 and LC3 in the Ghrelin + h-linc00598 was higher than ghrelin group(Fig. 4A,C,E).

Discussion

Ovarian cancer has a high mortality rate(Rogalska, Gajek et al. 2019; Shao, Liu et al. 2019; Quan, Xiong et al. 2021). Patients have chemotherapy resistance highly and therapeutic effect poorly.(Badi, Khaleel et al. 2020; Chen, Sun et al. 2020) Hence, to show the molecular mechanism is very urgently. Ghrelin as a growth hormone secretagogue receptor has many function such as promote the oocyte(Bai, Zhao et al. 2012; Sirini, Anchordoquy et al. 2019), anti-cancer and the osteocyte differentiation(Ye and Jiang 2015; Ye, Wang et al. 2018; Liu, Pan et al. 2020; Ye, Yang et al. 2020). In study, we found ghrelin can promote the SK-OV-3 cells autophagy. In the GEPIA database and HPA database, the ghrelin expression was lower in the ovarian cancer tissues than the normal tissues. Hence, the ghrelin was used to treat the ovarian cancer cell line SK-OV-3. And the ghrelin can promote the SK-OV-3 cell autophagy.

Many study has show the LncRNA can influence the development of ovarian cancer(Meng, Zhou et al. 2020). Particular, inhibiting growth, promoting autophagy were the focus of anti cancer research(Gu, Li et al. 2020; Guo, Du et al. 2021; Wang, Ding et al. 2021). In this study, the RNA was sequenced in the SK-OV-3 cells treated by ghrelin. And the linc00598 was selected as the closely related to cell autophagy effected by ghrelin. Linc00598 can regulate the transcription of specific target genes, including those for cell cycle regulators and autophagy(Jeong, Chae et al. 2016). To identify linc00598 can regulate the SK-OV-3 cells autophagy, the linc00598 was silenced and overexpressed. And the linc00598 can promote the SK-OV-3 cells autophagy.

Ghrelin and linc00598 can promote the SK-OV-3 cells autophagy. The linc00598 was silenced and overexpressed in the SK-OV-3 cells. Then the ghrelin was used to effect the SK-OV-3 cells. We found ghrelin mainly through linc00598 to promote the SK-OV-3 cells autophagy. When the linc00598 silenced, ghrelin promote SK-OV-3 cells autophagy was inhibited. And When the linc00598 overexpressed, ghrelin promote SK-OV-3 cells autophagy was enhanced.

Conclusions

In conclusion, ghrelin promote SK-OV-3 cells autophagy. Additionally, we proved that ghrelin regulated the progression of SK-OV-3 cells autophagy by linc00598/ Beclin1 axis.

Declarations

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Authors' contributions

Ruixia Bai^{1a}, Wanying Song and Pengwei Zhao wrote this article. Xiumei Wang, Pengwei Zhao designed, organized and reviewed this article. All authors have read and agreed to the published version of the manuscript.

Availability of data and materials

All data generated or analyzes during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Declaration of Interest Statement

The authors declare that they have no competing interests.

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Figures

A

B

Figure 1

Expression of ghrelin in different ovarian cancer tissues. A. The expression levels of ghrelin in different cancer tissues were provided by GEPIA database. B. The expression levels of ghrelin in ovarian cancer tissues were provided by HPA database. * $P < 0.05$ was regarded as statistical significance.

Figure 2

Influence of ghrelin on SK-OV-3 Cell viability and the autophagy pathway. A. Effect of ghrelin with 400, 500, 600, 700 and 800 ng / mL on SK-OV-3 Cell viability at 24h, 48h and 72h. B-D Western Blot analysis was conducted to examine the protein levels of Beclin-1, LC3 and LC3 in SK-OV-3 cells after ghrelin added. E-G Western Blot analysis was conducted to examine the protein levels of Beclin-1, LC3 and LC3 in SK-OV-3 cells after ghrelin and D-Lys3-GHRP6 added. Single experiment had 3 repetitions, and * $P < 0.05$ was regarded as statistical significance.

Figure 3

LINC00598 selected on the SK-OV-3 cells treated by ghrelin using RNA-Seq. A. Volcano Plot of differentially expressed lncRNA. the red dot represents the differentially up-regulated LncRNAs, the blue dot represents the differentially down-regulated LncRNAs, and the gray dot represents the non significant differentially expressed LncRNAs. B&C. Histogram of GO enrichment analysis. the orange histogram represents the top 10 cell components with the most significant enrichment, the green histogram represents the top 10 molecular functions with the most significant enrichment, and the blue histogram represents the top 10 biological processes with the most significant enrichment in fig 3B. D&E. Bubble chart of KEGG enrichment analysis. environmental information processing (orange part), human diseases (green part), metabolism (blue part) and organic systems (purple part) in fig 3D. F. The effect of Ghrelin on the expression of LINC00598 was detect by qRT-PCR. Single experiment had 3 repetitions, and * $P < 0.05$ was regarded as statistical significance.

Figure 4

Effect of LINC00598 on the SK-OV-3 cells autophagy pathway. A&B. the expression of LINC00598 in the SK-OV-3 cells after the LINC00598 silenced or overexpressed. C-F. Western Blot analysis was conducted to examine the protein levels of Beclin-1, LC3 I and LC3 II in SK-OV-3 cells after the LINC00598 silenced or overexpressed. Single experiment had 3 repetitions, and * $P < 0.05$ was regarded as statistical significance.

Figure 5

Influence of ghrelin on autophagy pathway of SK-OV-3 cells after the LINC00598 silenced or overexpressed. A. Western Blot analysis was conducted to examine the protein levels of Beclin-1, LC3 β and LC3 α . B-E. Relative expression of Beclin-1, LC3 β and LC3 α . Single experiment had 3 repetitions, and * $P < 0.05$ was regarded as statistical significance.