Long Non-Coding RNA AC022306.2 Promotes Cell Proliferation, Migration, and Invasion by Mediating GALK2 in Epithelial Ovarian Cancer

Xin Liu

The Third Affiliated Hospital of Guangzhou Medical University

Zhenghao Huang

The Third Affiliated Hospital of Guangzhou Medical University

Honglei Qin

The Third Affiliated Hospital of Guangzhou Medical University

Jingwen Chen

The Third Affiliated Hospital of Guangzhou Medical University

Yang Zhao (✉ yida.zhaoyang@163.com)

Third Affiliated Hospital of Guangzhou Medical University <https://orcid.org/0000-0003-3226-8127>

Research

Keywords: long non-coding RNA, AC022306.2, ovarian cancer, miR-369-3p, GALK2

Posted Date: November 23rd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1083813/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background

Long non-coding RNA (LncRNA) has been exhibited to exert significant function among human cancers. AC022306.2, as a newly discovered lncRNA, has an unclear function in ovarian cancer (OC). This study aims to uncover the functional role of AC022306.2 in OC and discover its possible mechanism.

Methods

The expression of AC022306.2 and Galactokinase 2 (GALK2) in OC tissues and adjacent non-tumor tissues was detected via qRT-PCR. The CCK-8 assay, cell clonogenesis assay, scratch healing assay and trans-well assay were used to reveal the function of AC022306.2 and GALK2 in ovarian cancer cell lines. Mice xenografts experiment was performed. Bioinformatics predicted the microRNA (miRNA) that bond with AC022306.2 and GALK2, and dual luciferase reporter system confirmed it. Rescue experiments of miRNA mimics and siGALK2 transfection on the basis of AC022306.2 over-expression were carried out to uncover the mechanism by which AC022306.2 played cancer-promoting roles in ovarian cancer.

Results

It was found that AC022306.2 was up-regulated in EOC tissues compared with adjacent non-tumor tissues. The elevated expression of AC022306.2 was related to the FIGO stage of OC. Functional experiments showed that AC022306.2 overexpression accelerated proliferation and aggression of OC cells *in vitro* and accelerated tumor growth *in vivo*. We also found that GALK2 was up-regulated in OC tissues. The expression of GALK2 mRNA in OC tissue was positively associated with the expression of AC022306.2. After AC022306.2 was knocked down, the expression of GALK2 was down-regulated. In addition, GALK2 depletion restored the proliferation and aggression capabilities of OC cells after AC022306.2 overexpression. Mechanically, AC022306.2 acted as a competitive endogenous RNA (ceRNA) of miR-369-3p to modulate the expression of GALK2. The up-regulating of miR-369-3p or the down-regulating of GALK2 partially reversed the effect of AC022306.2 overexpressed on cell propagation and aggression in OC.

Conclusions

AC022306.2 is a new oncogene in the carcinogenesis and development of OC. AC022306.2 improves the development of OC by regulating the miR-369-3p / GALK2 axis, indicating that AC022306.2 may have the potential to become a new molecular target for the treatment of OC.

Background

Ovarian cancer (OC) is a gynecological malignant tumor with the second highest incidence after endometrial cancer and the highest mortality rate worldwide¹. Epithelial ovarian cancer (EOC) accounts for 85-90% among OCs and is the most common type of OC². OC lacks early typical symptoms and

mature and effective early diagnosis techniques, so when the clinical symptoms of OC appear, patients are often accompanied by a large area of abdominal metastasis, and the disease has often developed to an advanced stage with a poor prognosis³. Even if some patients achieve temporary remission after chemotherapy, there is still a large possibility of recurrence. For a long time, surgery and chemotherapy have been the mainstay of treatment for OC. In recent years, with the continuous improvement of molecular targeted drugs research, the treatment mode of OC has gradually changed, such as anti-angiogenic drugs, poly ADP-Ribose polymerase (PARP) inhibitors and other drugs have been used in the maintenance treatment and posterior therapy of OC, and have become an important part of the treatment of OC⁴. Nevertheless, prognosis is still poor due to recurrence and metastasis of OC. Therefore, the study of molecular targets that regulate the tumorigenesis and development of OC and the discovery of biomarkers for early detection of OC have important clinical value for early diagnosis and effective treatment.

Long non-coding RNA (LncRNA) is a class of RNA sequence that does not encode protein, and the length exceeds 200 nt⁵. Although LncRNA has no function of encoding protein, it plays a unique role in RNA processing, mRNA stability maintenance, regulation of translation and protein transport⁶. MicroRNA (miRNA) is a class of small RNA sequence that does not encode protein, and the length is generally no more than 25 bp⁷. Currently, it is recognized that miRNA is closely related to carcinogenesis and development, and regulates tumor biological behaviors such as propagation, aggression and apoptosis through a complex and intersecting molecular network⁸. Recent studies have revealed that LncRNAs are involved in post-transcriptional modulation by interaction with mRNA, miRNA and protein⁹. The intercommunication between LncRNA and miRNA is mainly through the RNA sponge effect of competitive endogenous RNA (ceRNA) binding, which has an irreplaceable important position in tumor pathophysiology¹⁰. The high expression of LncRNA SNHG12 in ovarian cancer is positively correlated with FIGO stage of ovarian cancer. The oncogenic mechanism of LncRNA SNHG12 is mainly realized through restricting the function of miR-129 by competing with miR-129¹¹. LncRNA XIST competes with miR-214-3p in vitro to hamper its expression, thereby restraining the occurrence of EOC and elevating the sensitivity of ovarian cancer cells to cisplatin¹². FEZF1-AS1 binds to miR-130a-5p in ovarian cancer cells by sponge competition, which upregulates the level of SOX4, the target gene of miR-130a-5p, resulting in enhanced OC cells capacity of proliferation and inhibition of cell apoptosis¹³.

Here, we aim to uncover the function of AC022306.2 in the EOC cells and tissues. This finding suggested the effect of AC022306.2 / miR-369 / GALK2 axis in the EOC carcinogenesis, providing novel insight into the molecular mechanism of EOC.

Methods

Cancer tissue collection

Forty-one cases of EOC tissues and 13 cases of noncancerous normal ovarian tissues were gathered from the Department of Gynecology of the First Affiliated Hospital of China Medical University during the surgical process. None of the patients had undergone chemotherapy or radiotherapy prior to surgery. Informed consent was obtained from all the patients or their families and the project was approved by the Ethics Committee of China Medical University (No:2018-132).

Cell culturing and transfection

The EOC cell lines A2780 and CAOV3 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100U/mL of penicillin/streptomycin and 10% fetal bovine serum (FBS). The OVCAR3 and HO8910 were cultured in RPMI 1640 supplemented with 100U/mL of penicillin/streptomycin and 10% FBS. All cells were cultured in 37°C atmosphere containing 5% CO₂.

RiboBio (Guangzhou, China) designed specific small interfering RNAs (siRNA) against AC022306.2 (si-AC022306.2#1, si-AC022306.2#2 and si-AC022306.2#3) and GALK2 (si-GALK2#1, si-GALK2#2) and si-GALK2#3) and their corresponding NC (siNC). The pCDNA3.1(+)-CMV-AC022306.2 plasmid and the control empty vector were designed and synthesized by Syngentech (Guangzhou, China). The miR-369-3p mimics and the negative control were purchased from Biomics. According to the instructions, Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was utilized for cell transfection, and qRT-PCR was used to analyze the transfection efficiency.

QRT-PCR

Total RNA was extracted from EOC cells and tissues using the RNAeasy™ Animal RNA Isolation Kit with Spin Column (Beyotime, China). After measuring OD_{260/280} with a spectrophotometer (Unico, Shanghai, China), the cDNA was reverse-transcribed using Hifair III 1st strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus, YEASEN, Shanghai, China). 2x SYBR Green qPCR Master Mix (Bimake, Shanghai, China) was used to carry out the qRT-PCR. The relative expression level was calculated by 2^{-ΔΔCt} method with 18S or U6 as internal reference.

CCK-8 assay

After the cells were maintained in a 37°C humidified/5% carbon dioxide atmosphere for 0h, 24h, 48h, and 72h, cell proliferation was examined with Cell Counting Kit-8 Reagent Kit. The absorbance at 450nm wavelength was detected by microplate reader.

Cell clonogenesis assay

Cells were planted in a six-well plate with a density of 500 cells per well and cultured in complete medium. On the 14th day, the medium was discarded and cells were washed with PBS. Subsequently, a 0.1% crystal violet-methanol solution was used for fixation and dyeing for 15min, and photographs were taken for observation.

Scratch healing assay

Cells were cultured in six-well dishes in complete medium. A wound was scratched along the diameter of the well with a 200 μ L pipette tip. The size of the wound (w0) was measured. The cells were then incubated in the serum-free medium for 48h. After 48h, the size of the wound (w48) was detected. The wound healing rate was measured by the way as follows: (size of 0h – size of 48h)/ size of 0h.

Trans-well assay

Twenty-four-well trans-well chambers (3422, Corning) were coated with Matrigel (Corning) in cell invasion assay, while the chambers were with no coating in cell migration assay. 25 000 cells in 100 μ L medium without serum were added to the upper chamber, and medium containing 10% FBS was implanted to the lower one. The cells were grown for 48h. Cotton balls were used to help gently removing the cells that did not pass through, and a 0.1% crystal violet-methanol solution was utilized to fix and stain the cells that passed through. The stained cells were counted with a microscope.

Western Blot

In short, the total protein was electrophoresed by 10% SDS-PAGE. Enhanced chemiluminescence (ECL) chromogenic reagent was utilized for probing GALK2 successively. An antibody against GALK2 was purchased from Proteintech (Wuhan, China). The anti-GAPDH antibody was served as a loading control.

Subcutaneous tumor formation experiment

BALB/c nude mice (Vital River Laboratories, Beijing, China) were raised in a specific sterile environment. In order to detect the tumorigenic ability of AC022306.2 *in vivo*, 1.0×10^7 AC022306.2 overexpression and control OVCAR3 cells were injected into the right axillary area of 4-week-old female nude mice. With the growth of the tumor, the long and short diameters of the subcutaneous tumor were measured with vernier calipers twice a week, and the tumor volume was calculated as follows: tumor volume = $1/2 \times \text{long diameter} \times \text{short diameter}^2$. Four weeks later, the tumor was peeled off and the long diameter and short diameter of the tumor were measured. Animal experiments are in accordance with the Guidelines for the Protection and Use of Laboratory Animals published by the National Institutes of Health and were supported by China Medical University Animal Care and Use Committee.

Dual luciferase reporter assay

The reporter gene vector and reporter gene point mutation vector were constructed by Shanghai genechem Co., Ltd. The vector is GV272. The luciferase reporter vectors were named mutant-type dual luciferase reporter plasmid (MUT) and wild-type dual luciferase reporter plasmid (WT), respectively. MiRNA mimics or miR-NC was co-transfected with above MUT and WT plasmids. After 48h of transfection, luciferase activity was measured by dual luciferase reporter gene detection system (Promega).

RNA immunoprecipitation (RIP) assay

The IP lysis buffer was used for cell lysis. The obtained lysates were incubated with magnetic beads conjugated with anti-CREB1 antibody (Proteintech) or IgG antibody (Proteintech) to adhere to the immunoprecipitation product. The RNA was extracted from immunoprecipitation after beads were washed. QRT-PCR was used for analysis.

Statistical analysis

With SPSS 20.0 (Chicago, IL, USA), all statistical analyses were performed. A two-tailed Student's t test was implemented to compare data between the two groups. The results were considered statistically significant only if $P \leq 0.05$.

Results

AC022306.2 is high-expressed in EOC

QRT-PCR was used to investigate the level of AC022306.2 in EOC tissues and non-cancerous ovarian tissues, and the results showed that AC022306.2 was more highly expressed in EOC tissues than in normal ovarian tissues (Figure 1A). According to the expression of AC022306.2, we divided 41 patients with ovarian cancer into AC022306.2 high-level group ($n=21$, with the median expression level of AC022306.2 as the demarcation of the cases) and AC022306.2 low-level group ($n=20$). Clinicopathological features were assessed according to AC022306.2 levels. The statistical results exhibited that AC022306.2 expression level was positively related with FIGO staging ($P=0.007$) (Table 1). The expression levels of AC022306.2 in 4 ovarian cancer cell lines (OVCAR3, HO8910, A2780, CAOVB3) were determined by qRT-PCR. The results exhibited that the expression level of AC022306.2 was relatively high in HO8910 cells, while that of OVCAR3 cells was relatively low (Figure 1B). Therefore, HO8910 cells were used for siRNA transfection, while OVCAR3 cells were used for AC022306.2 overexpressed plasmid transfection. To disclose the potential biological function of AC022306.2 in EOC cells, we first performed qRT-PCR analysis to detect the expression of AC022306.2 in HO8910 cells transfected with si-AC022306.2 and scrambled siRNA negative control and OVCAR3 cells transfected with pCDNA3.1(+) - CMV-AC022306.2 plasmid and empty vector. It can be seen from Figure 1C and D that the level of AC022306.2 in si-AC022306.2 #1 transfected cells was much lowest in the groups, while the level of AC022306.2 was increased in cells transfected with overexpressed plasmid. Subsequently, functional loss and functional gain tests were performed to explore the biological role of AC022306.2 in EOC cells.

Regulation of AC022306.2 on propagation and aggression of EOC cells *in vitro*

The effect of AC022306.2 on cell propagation was detected by CCK-8 assay and clonogenesis assay. As a result, it was found that cell growth was depressed when AC022306.2 was down-regulated in HO8910 cells compared with the control group (Figure 2A and B) while overexpression of AC022306.2 promoted cell proliferation (Figure 2C and D). In order to further determine whether AC022306.2 is related to EOC

progression, we investigated the effect of AC022306.2 on EOC cell migration and aggression. Compared with the si-NC group, the scratch healing ability of HO8910 cells was impaired when AC022306.2 was depleted, while the ability of the overexpressed AC022306.2 group was significantly improved compared with the control group (Figure 2E and F). It was found that aggression of HO8910 cells were significantly weakened after AC022306.2 was knocked down (Figure 2G). In contrast, overexpression of AC022306.2 in OVCAR3 cells accelerated cell migration and invasion (Figure 2H). In summary, these results verified that AC022306.2 played a vital role in progression of EOC.

Regulation of AC022306.2 on proliferation of EOC cells *in vivo*

In order to determine whether AC022306.2 affects EOC cell growth *in vivo*, we established a xenograft model. The mice were sacrificed after 4 weeks, and the subcutaneous tumors were removed and measured. It was found that overexpressed AC022306.2 promoted tumorigenicity *in vivo* (Figure 3A) and the tumor volume elevated in AC022306.2 overexpressed group compared with the control group (Figure 3B). These results indicated that AC022306.2 significantly promoted the growth of EOC cells *in vitro* and *in vivo*, and could be regarded as a cancer-promoting molecule.

GALK2 is at high level in EOC and positively associated with AC022306.2 expression

AC022306.2 is located on the forward strand of chromosome 15 (15q21.2) and it overlaps with part of the intron region of gene that encodes GALK2 (Figure 4A). Therefore, we hypothesized that GALK2 may be regulated by AC022306.2. To verify this hypothesis, we first disclosed the level of GALK2 mRNA in the same EOC tissues and non-cancerous normal ovarian tissues by qRT-PCR. The results exhibited that the level of GALK2 mRNA in EOC tissues was also significantly higher than that in normal ovarian tissues (Figure 4B). Moreover, the level of GALK2 mRNA was significantly positively related with AC022306.2 expression in EOC tissues ($r=0.329$, $P<0.05$; Figure 4C). In addition, GALK2 mRNA and protein levels were measured by qRT-PCR and Western Blot. The results displayed that both GALK2 mRNA and protein levels were down-regulated after AC022306.2 was knocked down (Figure 4D and E). These results disclosed that GALK2 expression in EOC was up-regulated and positively associated with AC022306.2 expression level, and the expression of GALK2 may be regulated by AC022306.2.

GALK2 plays an oncogene role in EOC

Aiming to reveal the potential role of GALK2 in EOC cells, we performed a loss-of-function test. We first performed qRT-PCR analysis to determine the expression of GALK2 in HO8910 cells transfected with si-GALK2 and scrambled siRNA negative control. As the transfection efficiency showed in Figure 5A, siGALK2#1 was suitable for the experiments followed by. The CCK-8 experiment was used to detect the propagation potential of EOC cells, and it was found that the proliferation ability of the GALK2 knockdown group was decreased (Figure 5B). The clone formation experiment was utilized to detect the cell propagation ability again, and the same experimental result as CCK-8 assay was obtained (Figure 5C). Scratch healing experiment exhibited that the migration ability of EOC cells was inhibited when GALK2 knockdown (Figure 5D). In addition, trans-well assay was utilized to discover cell migration and

invasion ability, and the same results as the scratch healing experiment were obtained (Figure 5E). The above experimental results showed that GALK2 depletion hampered the propagation and aggression of EOC cells, demonstrating that GALK2 may play an oncogene role in ovarian cancer.

Downregulated GALK2 reverts the ability of cell propagation and aggression after AC022306.2 overexpression

To further explore whether AC022306.2 plays a carcinogenic role in EOC through positive regulation of GALK2, we constructed a co-transfection model in OVCAR3 cell line for rescue experiments: si-NC + Vector, si-NC + pCDNA3.1-AC022306.2, siGALK2 + pCDNA3.1-AC022306.2. QRT-PCR analysis showed that after co-transfection of siGALK2 with AC022306.2 overexpression plasmid, the expression of GALK2 mRNA was restored (Figure 6A). The CCK-8 assay showed that GALK2 depletion restored the enhanced cell propagation capacity induced by up-regulation of AC022306.2 (Figure 6B). Wound healing assay and trans-well assay showed that decreased GALK2 expression reverted the enhanced cell aggression ability caused by up-regulation of AC022306.2 (Figure 6C and D). In conclusion, these results suggested that GALK2 knockdown restored the role of AC022306.2 in promoting EOC cell propagation and aggression. These results also indicated that the carcinogenic effect of AC022306.2 in EOC was at least partially leant on the positive modulation of GALK2.

AC022306.2 is competitively sponge-combined with miR-369-3p

In order to determine the in-depth mechanism of AC022306.2 in regulating EOC cell function, a bold conjecture comes to mind that AC022306.2 may serve as a ceRNA that protects mRNA by competing to target miRNAs. Subsequently, we used the bioinformatics database (starBase V2.0, Targetscan and DIANA) to predict that miR-369-3p might target both AC022306.2 and GALK2 mRNA (Figure 7A). Therefore, we first transfected miR-369-3p mimics in OC cell line and verified the transfection efficiency (Figure 7B). Subsequently, dual luciferase reporter gene assay was performed to confirm miR-369-3p worked as the functional target of AC022306.2 and GALK2 mRNA. As shown in Figure 7C and D, miR-369-3p mimics significantly decreased the luciferase activity of WT-AC022306.2 and WT-GALK2, and the luciferase activity of reporter vectors MUT-AC022306.2 and MUT-GALK2 had little changed. In addition, AC022306.2 deletion significantly increased miR-369-3p expression (Figure 7E), while cells transfected with miR-369-3p mimics significantly reduced AC022306.2 and GALK2 expression (Figure 7F and G). Importantly, cells transfected with miR-369-3p mimics reversed the increase in GALK2 mRNA and protein levels provoked by AC022306.2 overexpression (Figure 7H and I), suggesting that AC022306.2 may regulate GALK2 expression by sponging miR-369-3p in EOC.

AC022306.2 / miR-369-3p / GALK2 axis regulates EOC propagation and aggression

Our existing research results showed that AC022306.2 up-regulated the expression of GALK2 in EOC cells through sponge adsorption of miR-369-3p. Therefore, we confirmed the relationship among AC022306.2, miR-369-3p and GALK2 in EOC by salvage experiments. The CCK-8 assay exhibited that transfection with miR-369-3p mimics or siGALK2 significantly reduced the enhanced proliferation capacity of EOC cells

induced by AC022306.2 overexpression (Figure 8A). The results of scratch healing and trans-well chamber assay demonstrated that transfection with miR-369-3p mimics or siGALK2 significantly reverted the elevated migration and invasion ability of EOC cells provoked by AC022306.2 up-regulation (Figure 8B and C). Moreover, with RIP assay, we found that AC022306.2 targeted to the oncogene CAMP response Element Binding 1 (CREB1) in downstream (Figure 8D). These results suggest that the AC022306.2 / miR-369-3p / GALK2 / CREB1 axis regulates EOC progression.

Discussion

LncRNAs play a vital role in modulating tumor signaling pathways, and affect various aspects of carcinogenesis and development, including propagation, aggression and maintenance of genomic stability^{14–16}. Although improvement has been achieved in the pathogenesis of tumor development, the role of lncRNAs in the pathogenesis and development of ovarian cancer is far from being completely clarified.

LncRNA AC022306.2 has 647 nucleotides in length and is located on chromosome 15q21.2. The Ensembl Gene ID is ENSG00000275580. At present, there are no reports of AC022306.2 in tumors. In this study, we demonstrated the role and molecular mechanism of AC022306.2 in ovarian cancer. AC022306.2 was up-regulated in EOC tissues. The association between the expression of AC022306.2 and clinicopathological factors was analyzed, and it was verified that AC022306.2 was positively linked with FIGO stage. It suggests that AC022306.2 may be involved in the carcinogenesis and development of EOC. *In vitro* and *in vivo* experiments showed that AC022306.2 played a vital role in propagation, migration and invasion of EOC cells. LncRNAs exert the function in a variety of ways, one of which is to modulate the expression of adjacent protein-coding genes¹⁷. We found that the gene encoding AC022306.2 and GALK2 were located at the same locus. GALK2 is an N-acetylgalactosaminase (GalNAc) kinase, which also has galactokinase activity at high galactose concentration^{18, 19}. It is one of the important molecules that regulate carbohydrate metabolism during cell growth²⁰.

In the process of tumor cell metabolism, the Warburg effect, that is, the cancer cell undergoes metabolic reprogramming from oxidative phosphorylation to aerobic glycolysis, is an emerging hallmark of cancer cell²¹. Abnormal expression of GALK2 in tumors may play a tumor-promoting role by influencing tumor cell metabolism. In prostate cancer cells, GALK2 knockdown hampers the proliferation of prostate cancer cells, and the mRNA level of GALK2 increases with the progression of prostate cancer²². However, GALK2 has not been reported in the study of EOC. In this research, we demonstrated that GALK2 was up-regulated in EOC tissues. *In vitro* experiments showed that GALK2 accelerated the propagation and aggression of EOC cells, and played a carcinogenic role in EOC. Further, we investigated whether AC022306.2 modulated GALK2, and whether GALK2 was a mediator molecule of AC022306.2 carcinogenesis in EOC. We found that GALK2 mRNA expression in EOC tissue was significantly positively associated with AC022306.2 expression. In EOC cells, down-regulation of AC022306.2 caused down-regulation of GALK2 mRNA and protein expression. In addition, GALK2 depletion inhibited the

carcinogenic effect of AC022306.2 in EOC cells. These results reveal that AC022306.2 may promote the progression of EOC by regulating GALK2.

Recent studies have verified that lncRNAs participate in post-transcriptional regulation via the interaction with mRNAs, miRNAs and protein²³. The interaction between lncRNA and miRNA is mainly through the RNA sponge effect of ceRNA binding, which has an irreplaceable important position in tumor pathophysiology^{24, 25}. For example, FGD5-AS1 can be used as the ceRNA of miR-330-3p²⁶. lncRNA PSMA3-AS1 promotes the propagation and aggression of ovarian cancer cells through miR-378a-3p²⁷. lncRNA H19 can be used as the ceRNA of miR-29b-3p to regulate carboplatin chemotherapy resistance of ovarian cancer²⁸. miR-369-3p has been identified as tumor suppressor in several human tumors^{29–31}. In this work, miR-369-3p was predicted to directly bind to AC022306.2 and GALK2 mRNA. Our experimental results revealed that overexpression of miR-369-3p restored the increase in the expression of GALK2 mRNA and protein caused by AC022306.2 up-regulation. These results disclose that AC022306.2 may regulate the expression of GALK2 in EOC cells through sponge adsorption of miR-369-3p.

CREB1, as a cancer-promoting factor, promotes the occurrence and development of tumors such as breast cancer³², renal cell carcinoma³³, acute myeloid leukemia³⁴ and glioma³⁵ through various ways. Chhabra et al.³² revealed that CREB1 was up-regulated in breast cancer tissues and improved cell metastasis. A study by Friedrich et al.³⁶ verified that high expression of CREB1 was closely correlated to the clinical pathological characteristics of renal cell carcinoma such as tumor stage, grade and lymphatic invasion. Importantly, several studies have unveiled that CREB1 may promote the carcinogenesis and development of ovarian cancer as a cancer-promoting factor^{37–39}. In this study, using RIP assay, we found that AC022306.2 directly targeted the downstream oncogenic factor, CREB1, to further exert its cancer-promoting effect and promote the development of EOC.

Conclusions

In brief, it is found for the first time that lncRNA AC022306.2 is up-regulated in EOC tissues and related to FIGO staging. Mechanically, AC022306.2 performs as a ceRNA to modulate GALK2 expression in EOC through sponging miR-369-3p, and exerts a cancer-promoting effect; besides, it further promotes progression of EOC by directly targeting and binding to the oncogene CREB1. The results above suggest that it will be helpful in the search for new and promising therapeutic targets for epithelial ovarian cancer.

Abbreviations

lncRNA: Long non-coding RNA

OC: Ovarian cancer

GALK2: Galactokinase 2

miRNA: MicroRNA

ceRNA: Competitive endogenous RNA

EOC: Epithelial ovarian cancer

PARP: Poly ADP-Ribose polymerase

DMEM: Dulbecco's modified Eagle's medium

FBS: Fetal bovine serum

SiRNA: Small interfering RNAs

RIP: RNA immunoprecipitation

CREB1: CAMP response Element Binding 1

Declarations

Ethics approval and consent to participate

This project was approved by the ethic committee of China Medical University (No:2018-132) and obtained informed consents from patients or their families. All animal experiments conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were supported by the China Medical University Animal Care and Use Committee.

Consent for publication

Not applicable

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by National Natural Science Foundation of China (No. 81772776; 81872115), The Young Science-technology Talents Support Project of Guangzhou (No. X20200301068) and The Project for Key Medicine Discipline Construction of Guangzhou Municipality (No. 2021-2023-17).

Authors' contributions

Yang Zhao and Xin Liu conceived the study. Xin Liu wrote the manuscript. Xin Liu, Zhenghao Huang, Honglei Qin and Jingwen Chen performed experiments and statistics. All authors read and approved the final manuscript.

Acknowledgements

Not applicable

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin*. 2020;70(1):7–30.
2. Kurman RJ, Shih Ie M. The Dualistic Model of Ovarian Carcinogenesis: Revisited, Revised, and Expanded. *Am J Pathol*. 2016;186(4):733–47.
3. Kuroki L, Guntupalli SR. Treatment of epithelial ovarian cancer. *BMJ*. 2020;371:m3773.
4. Lheureux S, Gourley C, Vergote I, et al. Epithelial ovarian cancer. *Lancet*. 2019;393(10177):1240–53.
5. Huang JZ, Chen M, Chen, et al. A Peptide Encoded by a Putative lncRNA HOXB-AS3 Suppresses Colon Cancer Growth. *Mol Cell*. 2017;68(1):171–84 e6.
6. Schmitz SU, Grote P, Herrmann BG. Mechanisms of long noncoding RNA function in development and disease. *Cell Mol Life Sci*. 2016;73(13):2491–509.
7. Lu TX, Rothenberg ME. MicroRNA. *J Allergy Clin Immunol*. 2018;141(4):1202–7.
8. Iorio MV, Croce CM. MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. *EMBO Mol Med*. 2012;4(3):143–59.
9. Quinn JJ, Chang HY. Unique features of long non-coding RNA biogenesis and function. *Nat Rev Genet*. 2016;17(1):47–62.
10. Chan JJ, Tay Y. Noncoding RNA:RNA Regulatory Networks in Cancer. *Int J Mol Sci*. 2018;19(5).
11. Sun D, Fan XH. LncRNA SNHG12 accelerates the progression of ovarian cancer via absorbing miRNA-129 to upregulate SOX4. *Eur Rev Med Pharmacol Sci*. 2019;23(6):2345–52.
12. Wang C, Qi S, Xie C, et al. Upregulation of long non-coding RNA XIST has anticancer effects on epithelial ovarian cancer cells through inverse downregulation of hsa-miR-214-3p. *J Gynecol Oncol*. 2018;29(6):e99.
13. Sun Z, Gao S, Xuan L, et al. Long non-coding RNA FEZF1-AS1 induced progression of ovarian cancer via regulating miR-130a-5p/SOX4 axis. *J Cell Mol Med*. 2020;24(7):4275–85.
14. Liu SJ, Dang HX, Lim DA, et al. Long noncoding RNAs in cancer metastasis. *Nat Rev Cancer*. 2021;21(7):446–60.
15. Peng WX, Koirala P, Mo YY. LncRNA-mediated regulation of cell signaling in cancer. *Oncogene*. 2017;36(41):5661–7.

16. Bhan A, Soleimani M, Mandal SS. Long Noncoding RNA and Cancer: A New Paradigm. *Cancer Res.* 2017;77(15):3965–81.
17. Huang X, Zhou X, Hu Q, et al. Advances in esophageal cancer: A new perspective on pathogenesis associated with long non-coding RNAs. *Cancer Lett.* 2018;413:94–101.
18. Agnew A, Timson D. Mechanistic studies on human N-acetylgalactosamine kinase. *J Enzyme Inhib Med Chem.* 2010;25(3):370–6.
19. Termini JM, Silver ZA, Connor B, et al. HEK293T cell lines defective for O-linked glycosylation. *PLoS One.* 2017;12(6):e0179949.
20. Thoden JB, Holden HM. The molecular architecture of human N-acetylgalactosamine kinase. *J Biol Chem.* 2005;280(38):32784–91.
21. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* 2011;144(5):646–74.
22. Whitworth H, Bhadel S, Ivey M, et al. Identification of kinases regulating prostate cancer cell growth using an RNAi phenotypic screen. *PLoS One.* 2012;7(6):e38950.
23. Ye S, Yang L, Zhao X, et al. Bioinformatics method to predict two regulation mechanism: TF-miRNA-mRNA and lncRNA-miRNA-mRNA in pancreatic cancer. *Cell Biochem Biophys.* 2014;70(3):1849–58.
24. Cesana M, Cacchiarelli D, Legnini I, et al. A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. *Cell.* 2011;147(2):358–69.
25. Kong X, Qi J, Yan Y, et al. Comprehensive analysis of differentially expressed profiles of lncRNAs, mRNAs, and miRNAs in laryngeal squamous cell carcinoma in order to construct a ceRNA network and identify potential biomarkers. *J Cell Biochem.* 2019;120(10):17963–74.
26. Gao SJ, Ren SN, Liu YT, et al. Targeting EGFR sensitizes 5-Fu-resistant colon cancer cells through modification of the lncRNA-FGD5-AS1-miR-330-3p-Hexokinase 2 axis. *Mol Ther Oncolytics.* 2021;23:14–25.
27. Xu Z, Jin H, Duan X, et al. LncRNA PSMA3-AS1 promotes cell proliferation, migration, and invasion in ovarian cancer by activating the PI3K/Akt pathway via the miR-378a-3p/GALNT3 axis. *Environ Toxicol.* 2021;36(12):2562–77.
28. Tian X, Zuo X, Hou M, et al. LncRNA-H19 regulates chemoresistance to carboplatin in epithelial ovarian cancer through microRNA-29b-3p and STAT3. *J Cancer.* 2021;12(19):5712–22.
29. Liu P, Ma C, Wu Q, et al. MiR-369-3p participates in endometrioid adenocarcinoma via the regulation of autophagy. *Cancer Cell Int.* 2019;19:178.
30. Xu Q, Liu K. MiR-369-3p inhibits tumorigenesis of hepatocellular carcinoma by binding to PAX6. *J Biol Regul Homeost Agents.* 2020;34(3):917–26.
31. Li P, Dong M, Wang Z. Downregulation of TSPAN13 by miR-369-3p inhibits cell proliferation in papillary thyroid cancer (PTC). *Bosn J Basic Med Sci.* 2019;19(2):146–54.
32. Chhabra A, Fernando H, Watkins G, et al. Expression of transcription factor CREB1 in human breast cancer and its correlation with prognosis. *Oncol Rep.* 2007;18(4):953–8.

33. Zhang Z, Guan B, Li Y, et al. Increased phosphorylated CREB1 protein correlates with poor prognosis in clear cell renal cell carcinoma. *Transl Androl Urol.* 2021;10(8):3348–57.
34. Shankar DB, Cheng JC, Kinjo K, et al. The role of CREB as a proto-oncogene in hematopoiesis and in acute myeloid leukemia. *Cancer Cell.* 2005;7(4):351–62.
35. Chen J, Zhang C, Mi Y, et al. CREB1 regulates glucose transport of glioma cell line U87 by targeting GLUT1. *Mol Cell Biochem.* 2017;436(1-2):79–86.
36. Friedrich M, Heimer N, Stoehr C, et al. CREB1 is affected by the microRNAs miR-22-3p, miR-26a-5p, miR-27a-3p, and miR-221-3p and correlates with adverse clinicopathological features in renal cell carcinoma. *Sci Rep.* 2020;10(1):6499.
37. Tong L, Wang Y, Ao Y, et al. CREB1 induced lncRNA HAS2-AS1 promotes epithelial ovarian cancer proliferation and invasion via the miR-466/RUNX2 axis. *Biomed Pharmacother.* 2019;115:108891.
38. Li CJ, Lin LT, Chu PY, et al. Identification of Novel Biomarkers and Candidate Drug in Ovarian Cancer. *J Pers Med.* 2021;11(4).
39. Yan H, Silva MA, Li H, et al. Long noncoding RNA DQ786243 interacts with miR-506 and promotes progression of ovarian cancer through targeting cAMP responsive element binding protein 1. *J Cell Biochem.* 2018;119(12):9764–80.

Tables

Table 1 Relationship between AC022306 expression and the clinicopathologic features of EOC patients.

Factors	Cases	AC022306 Expression		<i>P</i>
		Low (20)	High (21)	
Age (years)				.853
< 55	24	12	12	
≥55	17	8	9	
The pathology type				.819
Serous carcinoma	28	14	14	
Other pathology types	13	6	7	
Pathology classification				.638
Well	8	5	3	
Mod+Poor	33	15	18	
FIGO stage				.007
I–II	22	15	7	
III–IV	19	5	14	

Figures

Figure 1

AC022306.2 is high-expressed in ovarian cancer AC022306.2 was up-regulated in ovarian cancer (A). The expression levels of AC022306.2 in 4 ovarian cancer cell lines (OVCAR3, HO8910, A2780, CAOV3) (B). The efficiency of AC022306.2 interference in HO8910 cell line evaluated by qRT-PCR (C). The efficiency of AC022306.2 overexpression in OVCAR3 cell line measured by qRT-PCR (D). Data are shown as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 2

AC022306.2 promotes the progression of ovarian cancer in vitro CCK-8 assay for AC022306.2-depletion HO8910 cells (A). Clonogenesis assay for the treated HO8910 cell line (B). CCK-8 assay for AC022306.2-overexpressing OVCAR3 cells (C). Colony formation assay in AC022306.2-overexpressing OVCAR3 cells (D). Wound healing assay for AC022306.2-depletion HO8910 cells (E) and the overexpressed AC022306.2

OVCAR3 cells (F). Trans-well assay of AC022306.2-depletion HO8910 cells (G) and AC022306.2-overexpressing OVCAR3 cells (H). Data are shown as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 3

AC022306.2 promotes the growth of ovarian cancer in vivo The tumorigenesis was significantly strengthened and the tumor volumes were greater during identical observation period when the expression of AC022306.2 in OVCAR3 cells was elevated (A and B). Data are shown as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 4

GALK2 was highly expressed in ovarian cancer and positively correlated with AC022306.2 expression. Schematic representation of location of AC022306.2 and GALK2 in chromosome (A). GALK2 was up-regulated in ovarian cancer (B). QRT-PCR showed that there was a positive correlation relationship between AC022306.2 and GALK2 (C). GALK2 mRNA and protein levels were down-regulated after AC022306.2 was knocked down (D and E). Data are shown as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 5

GALK2 promotes ovarian cancer progression The siRNA of GALK2 was examined by qRT-PCR (A). GALK2 depletion decreased the proliferation, migration, invasion ability of ovarian cancer cells (B, C, D, E). Data are shown as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 6

Downregulated GALK2 reverted the enhanced ability of cell migration, invasion and proliferation provoked by AC022306.2 overexpression. QRT-PCR analysis showed that after co-transfection of siGALK2 with AC022306.2 overexpression plasmid, the expression of GALK2 mRNA was restored (A). The CCK-8 assay results (B). The Wound healing assay results (C). The trans-well assay results (D). Data are shown as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 7

AC022306.2 regulates GALK2 expression by competing for miR-369-3p Potential binding sites of miR-369-3p with AC022306.2 and GALK2 respectively (A). The transfection efficiency of miR-369-3p mimics evaluated by qRT-PCR (B). The interaction between AC022306.2 and miR-369-3p was testified by dual luciferase reporter assay (C). The interaction between GALK2 and miR-369-3p was verified by dual luciferase reporter assay (D). AC022306.2 deletion increased miR-369-3p expression (E). The ovarian

cancer cells transfected with miR-369-3p mimics reduced the expression of AC022306.2 (F) and GALK2 (G). The expression of GALK2 mRNA (H) and protein (I) was down-regulated after miR-369-3p was overexpressed in AC022306.2 up-regulating ovarian cancer cells. Data are shown as the mean $\pm$ SD. *P < 0.05, **P<0.01 and ***P<0.001.

Figure 8

AC022306.2/miR-369-3p/GALK2 axis regulates ovarian cancer cell proliferation, migration and invasion The cell proliferation ability of ovarian cancer cells transfected with NC, AC022306.2 overexpression plasmid, AC022306.2 + miR-369-3p mimics and AC022306.2 + si-GALK2 (A). The migration and invasion ability of transfected cells (B and C). The RIP assay demonstrated a direct binding between AC022306.2 and CREB1. Data are shown as the mean $\pm$ SD. *P < 0.05, **P<0.01 and ***P<0.001.