LncRNA HOTAIRM1, miR-433-5p and PIK3CD function as a ceRNA network to exacerbate the development of PCOS

Hongmin Guo†, Ting Li‡ and Xinhui Sun*†

Abstract

Background: Currently, several non-coding RNAs (ncRNAs) were distinguished in polycystic ovarian syndrome (PCOS). This present study aims to explore the potential function of IncRNA HOTAIRM1/miR-433-5p/PIK3CD in ovarian granulosa cells.

Methods: We analyzed the expression profiles of HOTAIRM1, miR-433-5p and PIK3CD in PCOS samples by enquiring GEO database. GSEA was applied to enrich the pathways related to PCOS. The target association between HOTAIRM1 and miR-433-5p or the binding association between miR-433-5p and PIK3CD were assessed by online prediction tools and a dual luciferase reporter assay. qPCR and western blotting assays were used to detect PIK3CD expression after HOTAIRM1 and miR-433-5p treatment. The proliferation and apoptosis of ovarian granulosa cells were estimated by cell counting kit-8 and flow cytometry assays, respectively.

Results: The expression profiles of HOTAIRM1 and PIK3CD were increased, whereas miR-433-5p was decreased in PCOS tissues. PIK3CD expression was positively regulated by HOTAIRM1 and negatively modulated by miR-433-5p. Overexpression of HOTAIRM1 reduced the proliferative ability and increased the apoptotic ability of granulosa cells, whereas upregulation of miR-433-5p or downregulation of PIK3CD reversed the effects of HOTAIRM1 on granulosa cells. Moreover, overexpression of miR-433-5 displayed a results with increasing proliferative ability and decreasing apoptotic ability, but upregulation of PIK3CD eliminated the function of miR-433-5p on granulosa cells.

Conclusions: Our findings illustrated that HOTAIRM1 could sponge miR-433-5p to promote PIK3CD expression, thereby regulating the growth and apoptosis of granulose cells in PCOS.

Keywords: Apoptosis, ceRNA, Polycystic ovarian syndrome, Proliferation

Introduction

Polycystic ovarian syndrome (PCOS), as the most common endocrine disorder in women during their reproductive ages, is associated with chronic anovulation and androgen overproduction [2]. Besides that, PCOS is also involved in diverse metabolic perturbations, including insulin resistance, hyperinsulinemia, subclinical atherosclerosis, and so on [19]. The exact cause of PCOS remains unclear, but many studies have shown that PCOS is initially caused by ovarian abnormalities [3]. The latest treatments for PCOS include ovarian hippocampal signaling pathway blocking theory, leptin theory, inositol therapy, bilateral ovarian drilling to stimulate ovulation and assisted reproductive technology [12]. Since the current treatment methods cannot cure PCOS, life-long treatment is still the mainstream method for the treatment of PCOS [7]. Therefore, the molecular mechanism

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of the occurrence and development of PCOS remains to be further studied and explored in order to overcome the treatment scheme of PCOS.

Granulosa cells are somatic cells of the sex cord, which are related with the progression of oocytes in mammalian ovaries [1]. Additionally, granulosa cells are also involved in numerous ovary-related diseases, including PCOS [30]. The symptoms of PCOS can be alleviated by increased proliferation of granulosa cells and reduced apoptosis of granulosa cells [33]. Previous evidence has revealed that genetic factors were implicated in the progression of PCOS [9]. Interestingly, recent findings have suggested that several non-coding RNAs also regulate these female reproductive system dysfunctions [23].

Long non-coding RNAs (lncRNAs), as a subgroup of ncRNAs consisting of greater than 200 nucleotides, exert important roles in the pathogenesis of diverse human diseases [26]. It has been documented that the progression of PCOS is followed by changes in the expression levels of several lncRNAs, suggesting the importance of lncRNAs in the development of PCOS [10]. For example, lncRNA BANCR has been discovered to be involved in the progression of PCOS by promoting cell apoptosis [28]; upregulation of lncRNA H19 attenuated apoptosis [28]; upregulation of lncRNA SRLR in plasma could distinguish PCOS patients from healthy females [13]. LncRNA HOXA transcript antisense RNA myeloid-specific 1 (HOTAIRM1), located in healthy females [13]. LncRNA HOXA transcript antisense RNA myeloid-specific 1 (HOTAIRM1), located in healthy females [13]. LncRNA HOXA transcript antisense RNA myeloid-specific 1 (HOTAIRM1), located in healthy females [13]. LncRNA HOXA transcript antisense RNA myeloid-specific 1 (HOTAIRM1), located in healthy females [13]. LncRNA HOXA transcript antisense RNA myeloid-specific 1 (HOTAIRM1), located in healthy females [13]. LncRNA HOXA transcript antisense RNA myeloid-specific 1 (HOTAIRM1), located in healthy females [13]. LncRNA HOXA transcript antisense RNA myeloid-specific 1 (HOTAIRM1), located in healthy females [13]. LncRNA HOXA transcript antisense RNA myeloid-specific 1 (HOTAIRM1), located in healthy females [13]. LncRNA HOXA transcript antisense RNA myeloid-specific 1 (HOTAIRM1), located in healthy females [13]. LncRNA HOXA transcript antisense RNA myeloid-specific 1 (HOTAIRM1), located in healthy females [13]. LncRNA HOXA transcript antisense RNA myeloid-specific 1 (HOTAIRM1), located in healthy females [13]. LncRNA HOXA transcript antisense RNA myeloid-specific 1 (HOTAIRM1), located in healthy females [13]. LncRNA HOXA transcript antisense RNA myeloid-specific 1 (HOTAIRM1), located in healthy females [13]. LncRNA HOXA transcript antisense RNA myeloid-specific 1 (HOTAIRM1), located in healthy females [13].

Materials and methods
Bioinformatics analysis
Data from Gene Expression Omnibus (GEO) database (including 7 cases of PCOS patients and 3 cases of normal samples) with accession number GSE34526 was used to detect differentially expressed lncRNAs and mRNAs. Gene set enrichment analysis (GSEA) was applied to enrich the pathways related to PCOS. The differential genes co-expressed with HOTAIRM1 were analyzed by using the R language limma package. The miRNAs corresponding to PIK3CD or HOTAIRM1 were predicted by online tools of Targetscan or LncBase, respectively. GSE86241 dataset including 8 cases of PCOS patients and 9 cases of normal samples was used to determine the differentially expressed miRNAs.

Cell culture and treatment
KGN cells and IOSE80 cells were received from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and cultivated in DMEM medium including 10% fetal bovine serum (FBS) under a 37 °C humidified atmosphere with 5% CO2.

To upregulate HOTAIRM1 or PIK3CD, a cDNA coding HOTAIRM1 or PIK3CD was obtained and inserted into a pcDNA3.1-vector. si-HOTAIRM1–1 (5′-GCCGAAAGCC AGCCATAGT-3′) and si-PIK3CD (5′-CCACAAGTG TCAAAGACAA-3′) were applied to down-regulate HOTAIRM1 and PIK3CD expression, respectively. The sequence of si-negative control (NC, 5′-CGAACTCACA GTTCAGACC-3′) was regarded as a corresponding control. MiR-433-5p mimic, inhibitor and their NC were used to regulate miR-433-5p expression. All these molecules were obtained from Ribobio (Guangzhou, China) and transfected into KGN cells with the assistance of Lipofectamine 2000 (Invitrogen, USA).

RNA extraction and qPCR
TRizol™ was used to extract the total RNA from the cells according to the direction of the supplier. Reverse transcription was conducted using the PrimeScript™ RT Master Mix. The PCR was performed based on the direction of SYBR Green PCR kits on a 7500 sequence detection system. GAPDH was acted as the internal control for detection of HOTAIRM1 and PIK3CD expression, and U6 was taken as the standard control for analysis of miR-433-5p. The relative expression of HOTAIRM1, miR-433-5p and PIK3CD were analyzed by 2^{-ΔΔCT} method. The sequences of primers were listed as below:

HOTAIRM1: F: 5′-CCCACCGTTCATGAAG-3′, R: 5′-GTTCACAAACACCACTTTC-3′;
PIK3CD: F: 5′-TGCCAACACCTCCCCATCTC-3′, R: 5′-CATCTCGTTGGCGTAGAAC-3′;
mir-433: F: 5′-TGATGGCTCCTCGGT-3′, R: 5′-GAACATGTCTCGATTC-3′;
U6: F: 5′-CTCGCTTCGGCCACAGCATATCT-3′, R: 5′-ACGGTTCAAGATTTTGGTGC-3′;
GAPDH: F: 5′-TGTTGTCCTGCTTGAGATCT-3′, R: 5′-CCTGCTTCACCCACCTTCTG-3′.

Western blotting
Total protein was extracted from the cells by RIPA buffer, isolated by SDS-PAGE, and blotted onto the PVDF
membranes. Then, the membranes were blocked in 5% skimmed milk for 1 h and probed with the antibodies against PIK3CD (CST, 34050, 1:1000) and GAPDH (CST, 5174, 1:2000) at 4 °C for 24 h. Next, the membranes were probed with secondary antibody for 1 h at ambient temperature. The protein bands were visualized with enhanced chemiluminescence and analyzed by Image J software.

**Cell growth detection**
A cell counting kit-8 (CCK-8, Beyotime, China) was applied to examine the function of HOTAIRM1, miR-433-5p and PIK3CD on cell viability. In brief, cells were implanted in a 96-well plate with the concentration of 2 × 10⁵ cells/well and cultivated for 24, 48, and 72 h. At each specified time point, 15 μL of CCK-8 solution was loaded and the cells were cultured for another 1.5 h. Finally, a microplate reader was applied to analyze the absorbance at 450 nm, and the proliferation curve was drew by Graphpad Prism 6.0.

**Cell apoptosis detection**
A fluorescein-conjugated annexin V (annexin V-FITC)/propidium iodide (PI) staining kit (BD Biosciences, USA) was applied to analyze the apoptotic of KGN cells based on the supplier’s direction. Briefly, the treated cells were rinsed with PBS and resuspended in 100 μL of binding buffer, and added 10 μL of FITC-annexin V and 10 μL of PI in order. After incubated at ambient temperature for 15 min away from the light, 400 μL of binding buffer was added and apoptosis was analyzed. The apoptosis of KGN cells was analyzed by flow cytometry.

**Dual luciferase assay**
Luciferase plasmids were generated, including wild-type (pGL3-HOTAIRM1-WT) or mutated (pGL3-HOTAIRM1-MUT) that may be used to bind miR-433-5p targeting sites. Luciferase plasmids including wild-type (pGL3-PIK3CD-WT) or mutated (pGL3-PIK3CD-MUT) potential miR-433-5p targeting sites from the 3’-UTR of PIK3CD were also generated. The plasmids were acquired from Sangon.

To analyze the targeted relationship between HOTAIRM1 and miR-433-5p, pGL3-HOTAIRM1-WT or pGL3-HOTAIRM1-MUT was co-transfected with miR-433-5p mimic/inhibitor/NC into cells with the support of Lipofoxetamine 2000. After 48 h of cultivation, cells were gathered and luciferase activity was analyzed by a dual-luciferase reporter assay system. Detection of targeted association between miR-433-5p and PIK3CD was conducted in the same way.

**Statistic analysis**
All data were processed by SPSS22.0 and GraphPad Prism 6.0 software and presented as the mean ± standard deviation (SD). Student’s test was used to detect the difference between HOTAIRM1 expression in KGN and IOSE80 cells. While the comparisons among multiple groups were analyzed by ANOVA followed by LSD post hoc test. P values less than 0.05 were deemed as statistically significant.

**Results**

**High expression of HOTAIRM1 suppressed the viability of KGN cells**
By consulting GSE34526, we observed that HOTAIRM1 was highly expressed in PCOS patients (n = 7) compared with the normal samples (n = 3, Fig. 1a). Then, results from qPCR indicated that HOTAIRM1 was also upregulated in KGN cells (Fig. 1b). To further detect the function of HOTAIRM1 in PCOS, we used pcDNA3.1-HOTAIRM1 to increase HOTAIRM1 expression in KGN cells (Fig. 1c). Afterwards, we found that the viability of KGN cells was decreased significantly when HOTAIRM1 was upregulated (Fig. 1d), indicating that HOTAIRM1 affected the function of KGN cells.

**Bioinformatics analysis was applied to detect the downstream miRNA and mRNA related to HOTAIRM1**
To further explore molecular mechanism related to PCOS development, GSEA was used to enrich the pathways related to PCOS. The apoptotic pathway was obtained, due to its highest correlation (Fig. 2a). The genes enriched in the apoptotic pathway were intersected with the differentially expressed genes in PCOS, and 9 genes were obtained, namely BID, PIK3CD, TNFRSF10C, TNFRSF1A, MYD88, PIK3R5, PRKACA, MAP 3 K14, PPP3R2 (Fig. 2b). Through co-expression analysis with HOTAIRM1, PIK3CD with the strongest co-expression relationship was selected for further study. Data from GSE34526 (Fig. 2c) displayed that PIK3CD was highly expressed in PCOS patients (n = 7) compared with the normal samples (n = 3). The positive relationship between HOTAIRM1 expression and PIK3CD expression was displayed in Fig. 2d, further confirming the association between HOTAIRM1 and PIK3CD. Next, we used Targetscan or LncBase website severally to detect the corresponding miRNA related to PIK3CD or HOTAIRM1, and a total of 31 common miRNAs were obtained (Fig. 2e). The 31 miRNAs were intersected with the 72 downregulated miRNAs in PCOS to acquire miR-433-5p (Fig. 2e). The expression level of miR-433-5p in PCOS was presented in Fig. 2f, indicating that miR-433-5p was under expressed in PCOS samples (n = 8) compared with the normal (n = 9). Based on the bioinformatics, we constructed a ceRNA network among HOTAIRM1, miR-433-5p and PIK3CD that may be involved in the development of PCOS.
Associations among HOTAIRM1, miR-433-5p and PIK3CD were identified by biological experiments

The predicted binding sites between HOTAIRM1 and miR-433-5p, as well as miR-433-5p and PIK3CD were displayed in Fig. 3a-b. The results of a dual luciferase reporter experiment indicated that in WT-HOTAIRM1 group, miR-433-5p mimic treatment reduced the relative luciferase activity whereas miR-433-5p inhibitor treatment increased the relative luciferase activity. However, in MUT-HOTAIRM1 group, the relative luciferase activity has no obvious change after either miR-433-5p mimic or inhibitor treatment (Fig. 3c). Similarly, the luciferase activity in WT-PIK3CD group was also significantly decreased or increased in KGN cells after miR-433-5p mimic or inhibitor treatment, while the luciferase activity in MUT-PIK3CD group has no obvious change no matter with miR-433-5p mimic or inhibitor treatment (Fig. 3d). We next detected the mRNA and protein expression of PIK3CD under the abnormal expression of HOTAIRM1 and miR-433-5p. As displayed in Fig. 3e-g, we observed that PIK3CD expression was increased when HOTAIRM1 was upregulated, whereas overexpression of miR-433-5p reduced PIK3CD expression. However, depletion of HOTAIRM1 reduced PIK3CD expression, whereas PIK3CD expression was increased when miR-433-5p expression was suppressed (Fig. 3h-j). All these findings not only emphasized the ceRNA mechanism among HOTAIRM1, miR-433-5p and PIK3CD, but also found that the expression of PIK3CD was positively related to HOTAIRM1 and negatively correlated with miR-433-5p.

HOTAIRM1 suppressed the proliferation and promoted the apoptosis of KGN cells by regulation of miR-433-5p/PIK3CD

We used CCK-8 and flow cytometry assays to detect the function of HOTAIRM1, miR-433-5p and PIK3CD on KGN cells growth and apoptosis. Analysis from Fig. 4a indicated that the viability of KGN cells was suppressed by HOTAIRM1 upregulation, whereas overexpression of miR-433-5p promoted the viability of KGN cells. Moreover, miR-433-5p mimic treatment could reduce the inhibitory effect of HOTAIRM1 on KGN cells viability. However, overexpression of PIK3CD inhibited the promoting effect of miR-433-5p on KGN cells viability, while knockdown of PIK3CD suppressed the inhibitory
We also observed that overexpression of HOTAIRM1 increased the apoptosis of KGN cells, whereas the apoptosis of KGN cells was suppressed by miR-433-5p mimic treatment. As well, overexpression of miR-433-5p suppressed the promoting effect of HOTAIRM1 on KGN cells apoptosis. But the inhibitory effect of miR-433-5p mimic on KGN cells apoptosis was suppressed by PIK3CD upregulation, while depletion of PIK3CD blocked the promoting effect of HOTAIRM1 on KGN cells apoptosis (Fig. 4c). Additionally, we also discovered that depletion of HOTAIRM1 increased the viability of KGN cells, and knockdown of miR-433-5p reduced the viability of KGN cells. Moreover, miR-433-5p depletion suppressed the promoting effect of si-HOTAIRM1 on the viability of KGN cells. Whilst, depletion of PIK3CD alleviated the inhibitory effect of miR-433-5p inhibitor on KGN cells viability, but the promoting effect of si-HOTAIRM1 on the viability of KGN cells was suppressed by PIK3CD upregulation (Fig. 4b). Furthermore, the apoptosis of KGN cells was reduced when HOTAIRM1 was depletion, whereas miR-433-5p inhibitor treatment increased the apoptosis of KGN cells. And miR-433-5p inhibitor suppressed the inhibitory effect of si-HOTAIRM1 on KGN cells apoptosis. The inhibitory effect of si-HOTAIRM1 on KGN cells apoptosis was
blocked when PIK3CD expression increased, whereas knockdown of PIK3CD suppressed the promoting effect of miR-433-5p inhibitor on KGN cells apoptosis. Thus, these data revealed that HOTAIRM1 suppressed the growth of KGN cells and promoted the apoptosis of KGN cells by regulation of miR-433-5p/PIK3CD.

Discussion
PCOS is a kind of common endocrine disease, which troubles many women of childbearing age [22]. A study has revealed that HOTAIRM1 was associated with the recurrence of ovarian cancer [27]. Moreover, HOTAIRM1 was also reported to be a tumor suppressor by affecting a series of malignant behaviors related to ovarian cancer [5]. This report was conducted to detect the function of HOTAIRM1/miR-433-5p/PIK3CD in PCOS, and we discovered that HOTAIRM1 can modulate the progression of PCOS by regulating miR-433-5p and PCOS.

Accumulated reports have suggested that lncRNAs exerted important roles in numerous biological processes and disease progression, including PCOS [11].
this study, we discovered that HOTAIRM1 was highly expressed in PCOS tissues and cells, and upregulation of HOTAIRM1 suppressed the proliferation and promoted the apoptosis of ovarian granulose cells (KGN), which suggested that HOTAIRM1 may perform a crucial role in the progression of PCOS.

MiRNAs are crucial members of the ncRNA family with nearly 22 nucleotides in length [18]. MiRNAs can suppress gene expression at post transcriptional level by targeting to 3'-UTR of specific mRNA [4]. In addition, a growing number of evidences revealed that IncRNAs could target to miRNAs to affect the functions of miRNAs. Based on this, the hypothesis of ceRNA was put forward. Mechanically, IncRNAs served as endogenous sponge to competitively target miRNAs, thus affecting functions of miRNAs and their target genes [20]. In this study, we discovered that HOTAIRM1 was negatively correlated to miR-433-5p expression and overexpression of miR-433-5p reduced the impacts of HOTAIRM1 on KGN cells growth and apoptosis. MiR-433 has been reported to play a role in numerous kinds of physiological processes, especially in tumors [21]. For instance, miR-433 was found to be lower expressed in hepatocellular carcinoma and gastric carcinoma [17, 25]. Moreover, miR-433 negatively modulated the expression of thymidylate synthase responsible for 5-fluorouracil sensitivity in HeLa cells [8]. Besides, miR-433 inhibited cell proliferation and strengthened chemosensitivity by targeting...
CREB in glioma [21]; miR-433 suppressed liver cancer cells migration by repressing CREB [29]. These findings revealed that a miRNA exerting various functions could be connected with the downstream target gene expression and function. Additionally, the regulatory capacities of miRNAs could be modulated by ceRNAs. Herein, we discovered that overexpression of miR-433-5p obviously inhibited PIK3CD expression in ovarian granulose cells. Moreover, upregulation of PIK3CD reduced the effects of miR-433-5p on KGN cells proliferation and apoptosis. These observations revealed that the function of HOTAIRM1 in KGN cells might be achieved through targeting miR-433-5p to regulate the expression of PIK3CD.

To identify that HOTAIRM1 affects the proliferation and apoptosis of ovarian granulose cells by regulating PIK3CD, we co-transfected si-HOTAIRM1 and si-PIK3CD or HOTAIRM1-OE and PIK3CD-OE into KGN cells. The results exhibited that overexpression of PIK3CD significantly increased the adverse effect of HOTAIRM1 on KGN cells including suppressing cell growth and promoting cell apoptosis. Moreover, depletion of PIK3CD also strengthened the positive effect of si-HOTAIRM1 on KGN cells containing increasing cell growth and inhibiting cell apoptosis. Accumulated evidence has shown that PIK3CD was primarily expressed in leukocytes and involved in the development of several hematological malignancies and solid tumors, such as glioma, neuroblastoma, glioblastoma and breast cancer [6]. However, this is the first time we have reported the function of PIK3CD on KGN cells growth and apoptosis.

Even though, several limitations in this study should be noted. First, the sample size of GEO dataset used for detection of HOTAIRM1, miR-433-5p and PIK3CD expression was small, and a large sample size is needed to verify the expression of HOTAIRM1, miR-433-5p and PIK3CD in future. Second, we only analyzed the effects of HOTAIRM1, miR-433-5p and PIK3CD on KGN cells viability and apoptosis, more biological behaviors and indicators of the KGN cells should be detected in future. Third, we just detected the function of HOTAIRM1, miR-433-5p and PIK3CD on KGN cells in vitro, we will explore the function of HOTAIRM1, miR-433-5p and PIK3CD in vivo.

Conclusion

In summary, this study revealed that HOTAIRM1 and PIK3CD expression levels were elevated and miR-433-5p was reduced in PCOS samples, and that increased HOTAIRM1 could inhibit miR-433-5p, thereby elevating the expression of PIK3CD. Moreover, overexpression of HOTAIRM1 suppressed cell proliferation and promoted apoptosis of ovarian granulose cells, whereas upregulation of miR-433-5p or downregulation of PIK3CD reversed the effects of HOTAIRM1 on ovarian granulose cells. Nevertheless, the role and mechanism of HOTAIRM1/miR-433-5p/PIK3CD axis in PCOS need to be further studied.

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Authors’ contributions

HG and TL designed the experiments, performed the experiments, analyzed the data and wrote the manuscript. XS conceived the research program and reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors agreed to be published.

Competing interests

The authors declare that they have no competing interests.

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