ANGPTL4 expression in ovarian granulosa cells is associated with polycystic ovary syndrome

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Abstract

ABSTRACT Objectives:To characterize the expression of ANGPTL4 in ovarian granulosa cells (GCs) and its association with polycystic ovary syndrome. Design: A retrospective study. Setting: University-based center for reproductive medicine. Participants:This study included 104 PCOS patients and 112 control women undergoing in vitro fertilization-embryo transfer (IVF-ET) from the reproductive hospital affiliated with Shandong University between 2019 and 2021. Methods: The mRNA expression of ANGPTL4 in GCs were assessed by reverse transcription and real-time quantitative (RT-q)PCR, then clinical information for these patients were reviewed and analyzed. Main outcome measures: ANGPTL4 expression in GCs in participants, correlation between ANGPTL4 expression level and metabolic characteristics of patients and predictive value of ANGPTL4 expression for PCOS. Results:The RT-qPCR results showed that ANGPTL4 expression in the control group is significantly lower than that in the PCOS group(P=0.000). It indicated positive association with AMH(r=0.211), HOMA-IR(r=0.174), LDL/HDL(r=0.176), ApoB/ApoAI(r=0.155) and TC/HDL(r=0.189). Additionally, the ANGPTL4 expression in the ovarian granulosa cells might be a independent factor in PCOS(OR:3.345, 95%CI:1.951–5.734) and served as a good predictor for PCOS (AUC0.704, 95%CI 0.633-0.774,P<0.001). Conclusions:For the first time our study revealed on the higher ANGPTL4 expression in ovarian GCs with PCOS, and its association with glucose and lipid metabolism showed that ANGPTL4 might be a predictor for PCOS and play an important role in metabolism and pathogenesis of PCOS. Funding: National Key R&D Program of China (2018YFC1003202, 2016YFC1000604) and Taishan scholar project special funds (No. ts201712103). Key words: polycystic ovary syndrome, angiopoietin-like protein 4, mRNA, ovarian granulosa cell, glycolipid metabolism

INTRODUCTION

Polycystic Ovary Syndrome (PCOS) is one of most common and complicated endocrine and metabolic disorder, affecting about 6%-20% women of reproductive age[1].In addition to reproductive dysfunction, PCOS can also manifest abnormal glycolipid metabolism while the insulin resistance (IR) is an independent risk factor for several metabolic abnormalities, including dyslipidemia, impaired glucose tolerance, cardiovascular disease and metabolic syndrome(MetS)[2-4]. The process of follicular development is crucial for reproduction. It needs bi-directional signaling between oocytes and granulosa cells. For patients with PCOS, the associated endocrine and glycolipid metabolic disorder can disrupt normal signal transmission, and then leads to the block of follicular growing, a decrease of high-quality embryos and even reduce the rate of transplantation [5,6].

ANGPTL4 is a member of angiopoietin-like proteins (Angptls) family and is known as a regulator of lipid

and glucose metabolism. It is proposed to inhibit activity of the enzyme lipoprotein lipase (LPL), which hydrolyzes triglyceride(TG) core of TG-rich lipoproteins, chylomicrons and very low-density lipoproteins (VLDL), and regulates their distribution to peripheral tissues[7-9]. A research reported that, there was a significant increase of ANGPTL4 level in serum of patients with PCOS compared with healthy subjects[10]. Evidence has shown that ANGPTL4 level in serum may positively correlate with PCOS.

Research on ANGPTL4 expression in ovarian granulosa cells (GCs) is still deleted. Therefore, our study aims to investigate the relative expression levels of ANGPTL4 in ovarian granulosa cells in PCOS and examined their possible associations with the glucose and lipid metabolism.

MATERIALS and METHODS

Patients

In the present study, a total of 104 PCOS patients (PCOS group) and 112 control women (control group) younger than 40 years old who were undergoing their in-vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) cycles were retrospectively recruited in reproductive hospital affiliated with Shandong University from 2019 to 2021.To diagnose the polycystic ovary syndrome, we referred to the modified Rotterdam criteria[11,12], including oligo- or anovulation combined with either hyperandrogenism or polycystic ovaries. Other causes of hyperandrogenism and ovulation dysfunction were excluded. Inclusion criteria for patients in control group were as follows: normal menstrual cycle, no endocrine abnormalities and normal ovarian and uterine morphology confirmed by either ultrasound or histological examination. The study was approved by the Ethics Committee of Reproductive Medicine of Shandong University with approval number 58, and signed informed consent was obtained from all participants.

Medication Protocol

All the subjects received a standardized long-term protocol in mid-luteal phase or gonadotropin-releasing–hormone (GnRH) antagonist regimen for ovarian stimulation. For long-term protocol, GnRH agonist (GnRHa) was used to downregulate the function of the pituitary gland on day 21 of the previous menstrual cycle. A dose of recombinant FSH ranging from 75 to 225 IU was initiated after down-regulation. As for GnRH antagonist regimen, recombinant FSH at a dose of 75 to 225 IU was administered on day 3 of the menstrual cycle. GnRH antagonist at a dose of 0.25mg daily was initiated when at least one follicle was 12 mm or more in the mean diameter. For all two protocols, urinary human chorionic gonadotropin (hCG) at a dose of 4000 to 8000IU was intramuscularly injected when at least two dominant follicles reached 18–20 mm in diameter and oocyte retrieval was performed 36–48 h later. 50ml follicular fluid was collected after oocyte retrieval.

Isolation of Ovarian Granulosa Cells

Ovarian GCs were collected from follicular fluid which is aspired on the oocyte retrieval day. Follicular fluid was centrifuged at 2000rcf for 10 minutes at 4°C and the supernatant was discarded. Added 1mg of Hyaluronidase (Solarbio, China) per 1ml of Hanks Balanced Salt Mixture (Solarbio, China) and 1ml of the mixture was added to the sample tube. Then incubated the mixture at 37 for 20 minutes. Added the mixture to the upper liquid of 4 ml of Human Peripheral Blood Lymphocyte Separation Solution (tbdscience, China) and avoided to mix with the underlying liquid. The mixture was centrifuged at 1700rcf for 10 minutes. Collected the middle liquid containing GCs and went through a Pre-Separation Filters (70 μm) (MACS, Germany) to eliminate cell clumps that may be inserted into the column. The mixture was centrifuged at 6000rcf for 5 minutes and discarded the supernatant. The cells were transferred to a new microfuge tube by PBS resuspending. The new microfuge tube with GCs suspension was centrifuged at 6000rcf for 3 minutes. Fresh GCs were stored at -80°C after discarding the supernatant.

RNA Extraction, Reverse Transcription and Quantitative Real-time PCR

Isolated ovarian GCs were lysed by Trizol reagent (Thermo Fisher Scientific, Inc.) for 30min to extract total RNA according to the guidance. The concentration and purity of the total RNA was determined

by UV spectrophotometry. The ratio of the optical density at 260/280 nm was between 1.8 to 2.1. Total RNA was reverse transcribed into cDNA using Prime Script RT Reagent Kit (TaKaRa, Japan) for real-time quantitative PCR(RT-qPCR) carried out by TB Green Premix Ex Taq II (TaKaRa, Japan) and performed on Roche Light Cycle 480 (Hoffman-La Roche, Switzerland). ANGPTL4 mRNA expression levels were normalized against the corresponding levels of GAPDH mRNA, which were served as an internal control and the $2^{-[?][?]Ct}$ method[13] was applied to determine ANGPTL4 expression level. Primer sequences were as follows: ANGPTL4 (forward primer, 5'-TCCTGGACCACAAGCACCTAGAC-3'; reverse primer, 3'-CGGTTGAAGTCCACTGAGCCATC-5'), GAPDH (forward primer, 5'-GCACCGTCAAGGCTGAGAAC-3'; reverse primer, 3'-TGGTGAAGACGCCAGTGGA-5').

Statistical Analysis

Data analysis was performed using SPSS (version 20; SPSS Inc). Continuous variables of clinical characteristics were displayed as mean+-standard deviation and compared by t-test. Parameters not normally distributed were presented as median(inter-quartile range) and compared by nonparametric test. Correlation between different variables was analyzed by Pearson correlation analysis and linear regression analysis. Receiver operating characteristic (ROC) curve were used for the independent predictive analysis. Binary logistic regression was used to assess the relationships between variables and incidence of PCOS. All values of $P < 0.05$ for two-side tests were considered statistically significant.

RESULTS

Baseline and Metabolic Characteristics

A total of 216 patients participated in the study, including 104 PCOS patients and 112 control women. The clinical baseline characteristics of all subjects were summarized in **Table 1.** Statistically significant differences in age, E2, P, PRL,THS were not found between the two groups(P¿0.05). However, compared with control group, there was a significant increase in the levels of BMI, LH, T, dehydroepiandrosterone(DHEA-s), AMH, but a decline in FHS in PCOS group(P¡0.05) as expected, which was according with the clinical features of PCOS. **Table 2** showed the glucose and lipid metabolic characteristics in participants. LDL, triglyceride(TG), LDL/HDL, ApoB, ApoB/ApoAI, total cholesterol(TC) and TC/HDL levels were elevated in the PCOS group (P¡0.05). In other hand, fasting blood glucose(FBG), fasting insulin and HOMA-IR had no statistically significant between two groups (P¿0.05).

The expression of ANGPTL4 in ovarian GCs

The expression of ANGPTL4 in ovarian GCs was measured in the two groups by qRT-PCR. Compared with the control women, the expression of ANGPTL4 was significantly increased in the PCOS patients [(1.05+-0.60) vs (1.75+-1.12), p =0.000] as shown in **Fig.1** .

The correlation between the expression of ANGPTL4 and the clinical characteristics of patients

It was found that the expression level of ANGPTL4 was positively correlated with AMH (r= 0.211, P=0.002), HOMA-IR (r= 0.174, P=0.028), LDL/HDL (r=0.176, P=0.013), ApoB/ApoAI (r=0.155, P=0.028) and TC/HDL (r=0.187, P=0.007) in all patients after correlation analysis (**Fig.2**). At the same time, the multiple liner regression analysis was used to investigate the association between ANGPTL4 expression and clinical characteristics supplementally. Just as showed in **Table 3** , ANGPTL4 expression in ovarian GCs was related to PCOS, FBG, FINS, HOMA-IR, TG and ApoAI (P¡0.05)。

Predictive value of ANGPTL4 expression in ovarian GCs for PCOS

In the present study, ROC cure was used to analyze the specificity and sensitivity of ANGPTL4 expression in ovarian GCs for PCOS to explore its predictive value (**Fig.3**). For predicting PCOS, the area under curve (AUC) of ANGPTL4 expression was 0.704 (95%CI 0.633-0.774,P¡0.001) with sensitivity (67.3%) and specificity (70.5%), compared with T (AUC 0.742; 95% CI 0.675– 0.808) , AMH (AUC 0.817; 95% CI 0.761–0.873) and LH/FSH (AUC 0.801; 95% CI 0.742–0.860) .Binary logistic regression was used to assess the relationships between variables above-mentioned and incidence of PCOS. The results (**Table 4 and Fig.4**

) showed that after adjusting AMH, LH/FSH and T, the incidence of PCOS was correlated to ANGPTL4 expression in ovarian GCs (OR:3.345, 95%CI:1.951–5.734).

DISCUSSION

Main Findings

To our knowledge, this is the first research finding that ANGPTL4 expression level in ovarian GCs had a significant increase in PCOS patients compared with women with regular ovulatory cycle. In addition, we also found that the expression level of ANGPTL4 was positively correlated with AMH, HOMA-IR, LDL/HDL, ApoB/ApoAI and TC/HDL after correlation analysis with the corresponding clinical data in all participants. According to the results of multiple liner regression analysis, ANGPTL4 expression was also related to PCOS, FBG, FINS, HOMA-IR, TG and ApoAI. Those showed that the ANGPTL4 expression in ovarian GCs was closely associated with glucose and lipid metabolism in PCOS.

Interpretation

As we all know, Polycystic ovary syndrome (PCOS) is a highly heterogeneous endocrine disorder characterized by hyperandrogen, rare ovulation and polycystic ovary, and its pathogenesis is debated[14-16].Previous studies have demonstrated that the core etiology and primary endocrine characteristics of PCOS are insulin resistance (IR) and hyperandrogenemia (HA), and they can interplay each other leading to metabolic syndrome, especially dyslipidemia[17-21]. AMH are often used as clinical observation indicators reflecting the ovarian reserve[22,23], and the LH/FSH and T levels were significantly higher in PCOS patients compared with control patients[24,25].Compared with those characteristics related to PCOS, the expression level of ANGPTL4 in ovarian GCs might be a independent factor which affected incidence of PCOS and could serve as a new predictor for PCOS according to results of ROC cure and binary logistic regression analysis.

As a member of angiopoietin-like proteins (Angptls) family, ANGPTL4 has been extensively investigated, reporting its involvement in physiological and pathological conditions including energy metabolism, tumorigenesis, vascular homeostasis, and inflammation[26-30]. One of the extensively investigated roles of ANGPTL4 is its role in lipid metabolism, specifically regulating LPL activity to clear triglycerides (TG) from the circulation[8].In addition, a recent study demonstrated that ANGPTL4 knock out mice markedly improved glucose tolerance with increased insulin levels[31].Güneş M et al[10] revealed that there was a significant increase in level of serum ANGPTL4 compared to control women and IR was significantly associated with ANGPTL4 concentrations in patients. Those were corresponding to the result in our study that the ANGPTL4 expression in ovarian GCs was positively correlated with HOMA-IR, as well as related to lipid metabolic characteristics such as LDL/HDL, ApoB/ApoAI and TC/HDL. In view of the finding of our study, ANGPTL4 might participant in the glucose and lipid metabolism in the ovarian surroundings, which might affect the occurrence and development of PCOS. In addition, ANGPTL4 also acts as an apoptosis survival factor for vascular endothelial cells. It plays a key role in the late stages of folliculogenesis and participant in providing oxygen and nutrients to growing follicles [32,33]. Our study showed the positively correlation between ANGPTL4 expression and AMH, and indicated its possible impact on follicular development. All of our findings were preliminary, more mechanisms research are still expected.

High expression of ANGPTL4 in ovarian GCs with PCOS and its strongly association with multiple glucose and lipid metabolism characteristics suggests that ANGPTL4 expression level might play an important role in pathogenesis and development of PCOS, and could offer a granulosa-cell–derived marker for PCOS prediction. Our findings also raise serious of important new questions. What is mechanism of ANGPTL4 expression in PCOS? What is the role of ANGPTL4 signaling for metabolism in the ovarian GCs? All these questions have not yet been reported.

Strengths and limitations

The main strength of the study is that we focused on the relationship between ANGPTL4 and PCOS and linked them together for the first time, and the ANGPTL4 expression in ovarian GCs might be a risk factor for the occurrence and development of PCOS by taking part in glucose and lipid metabolism. This has not

been confirmed before. But it also had limitations, small sample size and retrospective study design made it essential to take further large sample and basic experimental research to investigate the pathogenesis of ANGPTL4 in PCOS.

Conclusion

Our study showed that differential expression of ANGPTL4 in ovarian GCs was identified between PCOS patients and control women, and its association with glucose and lipid metabolism showed that the expression of ANGPTL4 might be a predictor for PCOS and play an important role in metabolism and pathogenesis of PCOS.

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Disclosure of interests

The authors report no conflict of interest.

Contribution to authorship

Yuhua Shi conceived and designed this study. Qi Jiang contributed to experiments, statistical analysis, interpretation of data and draft of the manuscript. Yanjun Zheng and Ping Li performed statistical analysis and participated in the discussion. Wenqi Wang, Tian Song and Guihua Wu acquired the data. Yuehong Bian analyzed and interpreted the data. Yuhua Shi participated in the discussion and critically revised the manuscript. All authors read and approved the final manuscript.

Details of ethics approval

This study was approved by the Ethics Committee of Reproductive Medicine of Shandong University with approval number 58 on December 28, 2018, and signed informed consent was obtained from all participants.

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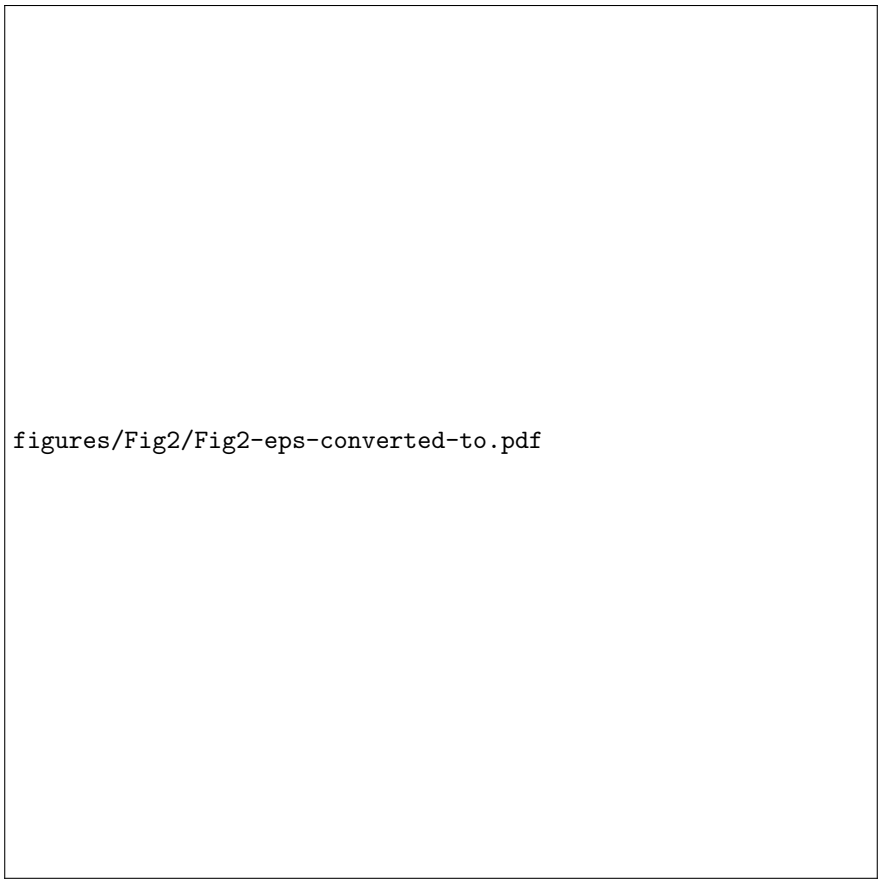

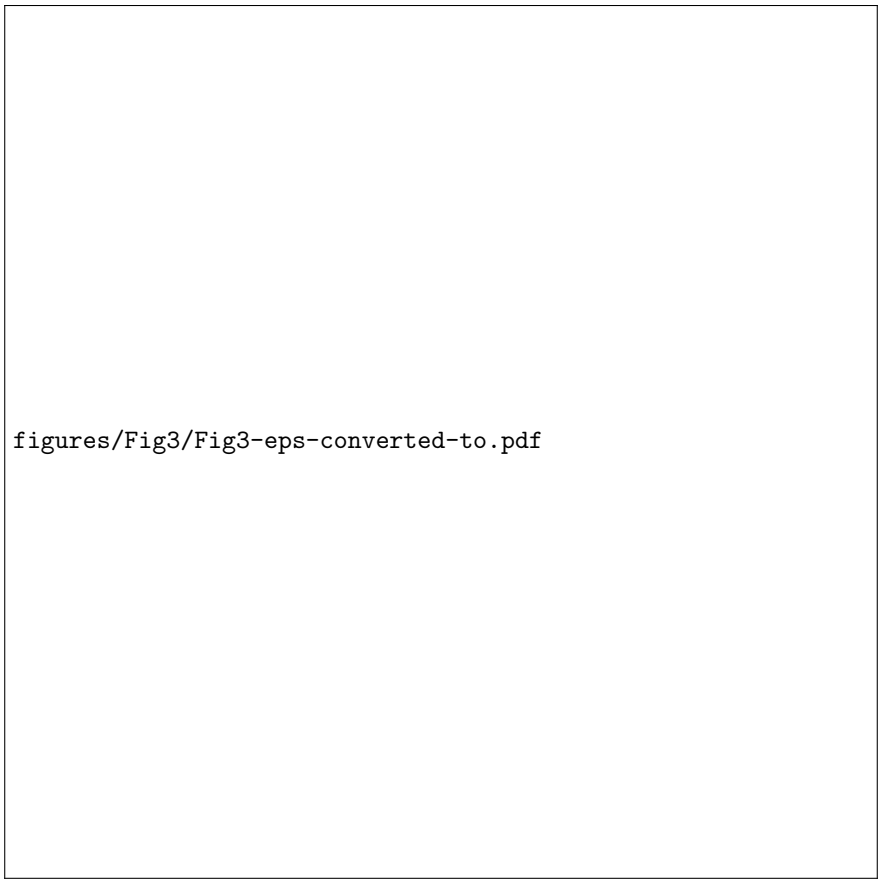

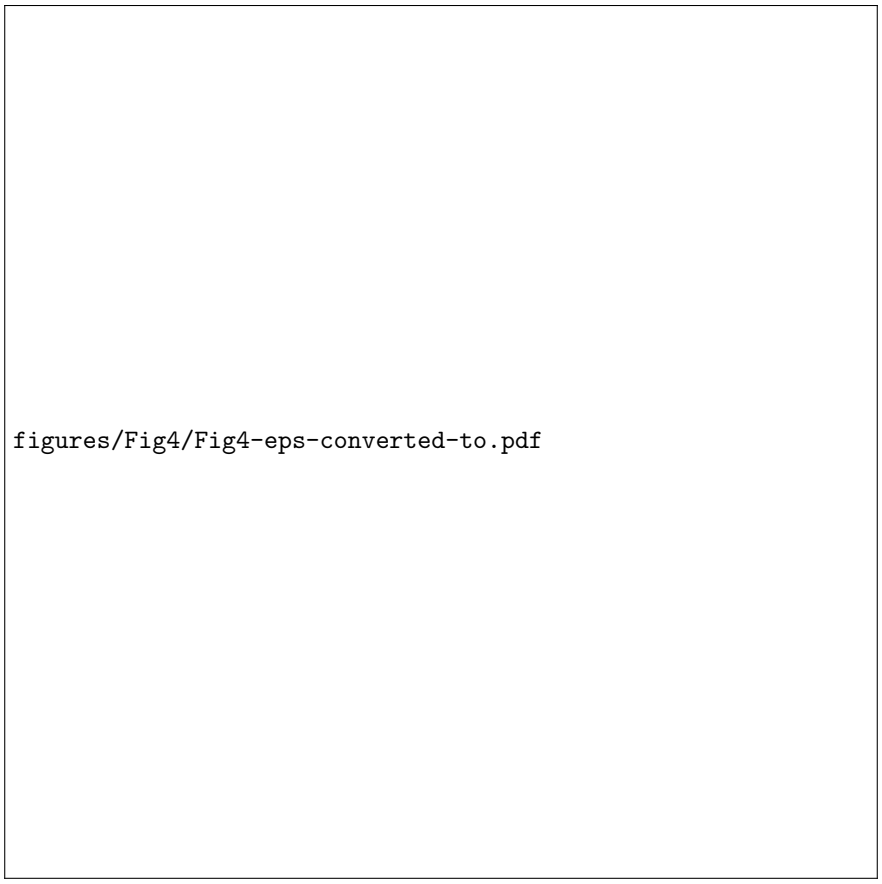

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