Journal of Ovarian Research

RESEARCH Open Access

Exploring lncRNA expression in follicular fluid exosomes of patients with obesity and polycystic ovary syndrome based on high-throughput sequencing technology

Xin Xin[1†], Li Dong[1†], Jiaxi Li[2], Wen Chen[1], Yue Qiu[3], Fang Lian[3*] and Haicui Wu[3*]

Abstract

Background Infertility is a reproductive health problem that attracts worldwide attention. Polycystic ovary syndrome (PCOS) is a major cause of female infertility and patients with obesity and PCOS are particularly common in clinical practice. Long non-coding RNA (lncRNAs) are a functional core in cells that regulate gene expression, transcription, and chromatin modification processes, and participate in epigenetics, cell cycle, and cell differentiation. LncRNAs are assumed to play a role in the occurrence and development of PCOS; however, their specific mechanism of action remains to be elucidated.

Methods High-throughput sequencing technology has been used to sequence and analyze lncRNAs in exosomes from the follicular fluid of patients with obesity and PCOS and those who underwent assisted reproductive therapy owing to male factors. Specific expression profiles of patients with obesity and PCOS were obtained and functional information analysis combined with a literature review were performed to screen for differentially expressed lncRNAs, which were validated using real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Results High-throughput sequencing analysis revealed that compared to normal patients with male infertility, patients with obesity and PCOS had a total of 20 lncRNAs with significant expression differences in follicular fluid exosomes. Among them, 17 lncRNAs were upregulated and three were downregulated. Functional analysis showed that differentially expressed genes were mainly enriched in "cell metabolism," "cell adhesion," and other aspects: related gene pathways mainly involved Huntington's disease, Parkinson's disease, spliceosomes, non-alcoholic fatty liver disease, and ribosomes. Verification of differentially expressed lncRNAs revealed that the expression of lncRNAs *TPT1-AS1*, *PTOV1-AS1*, *PTPRG-AS1*, and *SNHG14* in follicular fluid exosomes was consistent with the sequencing results.

Conclusion A preliminary differential expression profile of lncRNAs in exosomes of patients with obesity and PCOS was established by transcriptomic analysis of these individuals. Our bioinformatics analysis results may be applicable

†Xin Xin and Li Dong contributed equally to this work.

*Correspondence:
Fang Lian
lianfangbangong@163.com
Haicui Wu
haicui_w@163.com

Full list of author information is available at the end of the article

to further study of the impact mechanism involving obesity and PCOS. These differentially expressed lncRNAs maybe served as potential biomarkers for in-depth studies of the occurrence, development on Follicle quality and function for patients with PCOS in the future.

Keywords Polycystic ovary syndrome, LncRNA, Exosome, High throughput sequencing, Obesity

Introduction

The rapid development of society and the soaring level of human technology have optimized the human survival mode and changed human subjective ideology; however, these have also brought about environmental pollution and psychological pressure. Whether driven by human reproductive instincts or genetics, fertility treatment has always been a timeless topic among people of childbearing age. Infertility is related to family stability and social harmony and is a health issue that has attracted worldwide attention. The probability of infertility among Chinese couples of childbearing age is approximately 15% [1, 2]. Polycystic ovarian syndrome (PCOS) accounts for an important proportion of infertility in the female population [3], accounting for approximately 75% of female infertility caused by ovulation disorders [4]. The etiology and clinical manifestations of PCOS are complex and diverse, including amenorrhea, infertility, exuberant hair, acne, and obesity. It can also induce long-term complications in women, such as type 2 diabetes, endometrial cancer, and cardiovascular disease [5], which is an endocrine metabolic disease requiring long-term management. The onset of PCOS exhibits a clear familial genetic tendency, with complex genetic variations and epigenetic changes [6]. This suggests that genetic association research is of great value in understanding the mechanisms of PCOS occurrence and development, and genetic analysis has become an important means of understanding susceptibility to PCOS. Modern medicine has provided a better understanding of the etiology of PCOS. Genetic changes during intracellular development, such as exposure to excessive levels of anti-Müllerian hormone (AMH), androgens, or toxins (e.g., bisphenol A and endocrine disruptors) can lead to PCOS, and this association is more prominent in obese women [7]. Patients with obesity and PCOS are common in clinical practice, and a higher body mass index (BMI) exacerbates the clinical manifestations and long-term complications of PCOS. Obesity and PCOS often interact and pose a notable threat to health [8]. Research on the pathogenesis and treatment of PCOS in patients with obesity has always been challenging. Assisted reproductive technology is a commonly used fertility method in clinical practice for patients with infertility and PCOS. Obtaining as many high-quality oocytes as possible is key to improving pregnancy outcomes in such patients.

The proportion of DNA directly involved in protein coding functions in the human genome is <2%, containing a large number of non-coding sequences, including long non-coding RNA (lncRNAs) that were previously considered ineffective [9]; as a relatively long chain gene containing>200 nucleotides, only a few can be translated as short peptides and cannot directly encode proteins. LncRNAs regulate gene expression via RNA polymerase II (RNAPII) [10, 11]. A research team used whole exome detection technology in the blood of patients with PCOS, and the results showed that most PCOS susceptibility genes are located in intronic regions, indicating the potential value of ncRNA research in exploring the pathogenesis and diagnosis of PCOS [12]. LncRNA, serving as a set of functional RNA molecules, they are involved in the regulation of oocyte function, hormone metabolism, as well as the proliferation and apoptosis of granulosa cells [13]. Research has suggested that lncRNA regulation of epigenetic modifications is associated with the occurrence of PCOS [14, 15], while Huang et al. suggested that lncRNA *PWRN2* regulates the oocyte nuclear maturation process in patients with PCOS [16]. Guo et al. assumed that overexpression of *lncXIST* in the granulosa cells of patients with PCOS interferes with follicular recruitment and selection by inhibiting cell proliferation and promoting apoptosis [17], which might lead to the occurrence of PCOS.

Oocyte development and maturation are the result of the combined actions of endocrine and paracrine systems within the ovary, allowing for extensive bidirectional communication between oocytes and the various surrounding cell types. Follicular fluid, a complex bodily fluid that surrounds developing oocytes, contains secretions and metabolic components from different cells, including oocytes and granulosa cells, providing a critical microenvironment for oocyte development. Its biochemical composition helps to reveal the physiological and pathological states of follicles [18]. Analysis of the follicular fluid composition aids in better understanding the overall mechanisms of follicular development disorders across various diseases, serving as a critical tool in the diagnosis and treatment of reproductive disorders.

Exosomes are small vesicles with diameters of approximately 40–150 nm that are secreted by multiple cell types in physiological and pathological states [19]. They regulate target cells through plasma membrane release [20] and are important structures for material and information exchange between cells. By containing source cell-specific proteins, microRNAs, lncRNAs, and circular RNAs, exosomes regulate the function of target

cells, and their unique bilayer structure ensures stability and prevents pathway interruptions [21]. The presence of exosomes increases the likelihood of lncRNAs acting as biomarkers [22]. Recently, exosomes have attracted widespread attention because of their high stability, low immunogenicity, and unique advantages in targeting cells. By detecting the content of specific lncRNAs in exosomes to explain the process of disease occurrence and development, identify diagnostic and therapeutic biomarkers, and develop targeted drug therapies, exosomes would have more efficient and stable value.

This study explored the mechanisms underlying the occurrence and development of obesity-related PCOS by studying the expression profiles of lncRNAs in follicular fluid exosomes. The lncRNAs identified by sequencing have the potential to serve as biomarkers of obesity-related PCOS, furthur study will help to understand its pathogenesis, and to provide directions for future research concerning the pathogenesis and diagnosis of PCOS.

Materials and methods

Object

Patients who need in vitro fertilization embryo transfer technology (IVF-ET) to assist in reproduction at the Reproductive and Genetic Department of Shandong University of Traditional Chinese Medicine Affiliated Hospital between September 2021 and September 2022 were enrolled. The inclusion criteria for the PCOS group were those that met the diagnosis of PCOS, namely those with hyperandrogenism; imaging examination suggested polycystic changes in bilateral or unilateral ovaries, and the diagnosis could be made by excluding diseases that cause elevated testosterone levels and ovulation disorders, with a body mass index≥25 kg/m^2. The control group consisted of women who were infertile due to factors related to their husbands, with normal ovarian function, menstrual cycle, basal hormone levels, basal antral follicle count, and BMI. Both groups of patients were women aged 25–35 years. Patients with a history of recurrent miscarriage, endocrine disorders such as insulin resistance, hyperprolactinemia, thyroid diseases, ovarian dysfunction such as premature ovarian failure, uterine fibroids, endometriosis, or abnormal results from peripheral blood chromosome tests were excluded.

This study had been registered with the Chinese Clinical Trial Registration Center (ChiCTR210052331||http://www.chictr.org.cn/) on October 24, 2021. The research had also been approved by The Reproductive Medicine Ethics Committee of Shandong University of Traditional Chinese Medicine Affiliated Hospital (batch number: SDSZYYSZ20211009) and to exempt the informed consents. All patients signed informed consent forms before enrollment.

Treatment protocol

All patients were treated with antagonist stimulation method for assisted reproduction. Daily injection of, recombinant FSH Purecon (Merck Corporation, Canada) was adopted to initiate the regimen from the 2nd to 3rd day of their menstrual cycles to stimulate follicle growth. On the 6th to 7th day of stimulation, the gonadotropin-releasing hormone (GnRH) antagonist 0.25 mg (Ganirelix, Mercadon, USA) was start to used. When at least three follicles with a diameter of 17 millimeters or two follicles with a diameter of 18 millimeters were observed under ultrasound, a 10,000 IU of human chorionic gonadotropin (Merck, Canada) was injected intramuscularly to ultimately promote oocyte maturation. 34–36 h after trigger, mature oocytes were retrieved under vaginal ultrasound guidance and corresponding follicular fluid was collected.

Follicular fluid collection

Collect the follicular fluid from the largest and first punctured dominant follicle during the follicle retrieval process. All follicles were recorded and their diameters tracked using transvaginal ultrasound prior to puncture, ensuring that follicular fluid was collected from a mature follicle exceeding 18 cm in diameter for each patient. All selected mature oocytes were surrounded by the cumulus-oocyte complex (COC). Each follicle was aspirated independently then with oocyte isolated. The collected follicular fluid was examined for red blood cells. Follicular fluid with red blood cells was excluded from the study.

Enrichment and identification of exosomes in follicular fluid

A total of 12 patients with obesity and PCOS and 12 patients with infertility due to male factors were treated for controlled ovulation induction and follicular fluid collection. Considering the limited content of exosomes in follicular fluid and the need for a higher initial RNA level for lncRNA detection for library construction, the specimens of 6 different patients in the same group were randomly paired and mixed together, therefore actually divided into 3 experiment groups and 3 control groups for subsequent library construction and sequencing, etc. While another 6 patients with obesity and PCOS and 6 patients with infertility due to male factors were used for subsequent validation.

Exosomes in follicular fluid were obtained by ultracentrifugation at 2,000 × *g* in a centrifuge for 10 min to collect supernatants. The upper clear liquid was transferred to a centrifuge tube at 4℃ at 10,000 × *g* and was then centrifuged for 30 min. The obtained upper clear liquid was centrifuged at 100,000 × *g* in an ultracentrifuge for 70 min, and the precipitate was collected. Phosphate Buffer Saline (PBS) was added to the sediment until the

tube was fully filled, then centrifuged at 100,000 × g for 70 min. The precipitate was retained.

Transmission electron microscopy (TEM) was used to determine the integrity of the exosomes, which were transparent cystic structures. The concentration range and diameter distribution of exosomes were determined to verify whether their content met the requirements for library sequencing, and the surface marker protein CD9/63/81/TSG101 was detected to assist in the identification of exosomes.

Library preparation and sequencing

RNA library for lncRNA-seq was prepared with Ovation SoLo RNA-Seq System(nugen). Briefly, the genome from the total RNA was depleted. The construction of exosomal RNA library (nugen) using the method of removing ribosomal RNA and micro amplification. Divalent cations were used to fragment exosomal RNA into short lengths at 95℃. Then, using the fragmented RNA as a template to synthesize the first strand of cDNA by reverse transcription, followed by the incorporation of deoxyribonucleotide triphosphate, RNase H, and Escherichia coli DNA polymerase I to synthesize the second strand. After end-repairing, A-tailing and adapter ligation and purification, the first round of PCR amplification was carried out and purified. Then, the second round of amplification was subjected to remove ribosomal RNA by gene-specific InDA-C primers. In order to select cDNA fragments of preferentially 250 ~ 300 bp in length, the library fragments were purified with AMPure XP system.

After library preparation was completed, Qubit 2.0 (Invitrogen, Singapore) was used for preliminary quantification, diluting the library to 1 ng/μl. Further, a highly sensitive Agilent2100 (Agilent Technologies, America) instrument was used to detect the insert size of the library. Once the insert size met expectations, qPCR was used to accurately quantify the effective concentration of the library (>2nM) to ensure its quality. Subsequent to passing warehouse inspection, we strictly followed Illumina Novaseq (Thermo, America) instructions after pooling according to the requirements of effective concentration and target offline data volume ™ 6000 instruction sequencing library. Sequencing read segments were stored in FASTQ format and the raw data were filtered. The sequencing error rate and Q20, Q30, and GC content distributions were evaluated. HISAT2 software (v2.0.4) was used to map each sample read to reference genome, and the results were transmitted to the StringTie program for transcription assembly. We used Cuffmerge software to merge the transcripts obtained from splicing each sample, and selected transcripts with an exon count≥2 and a transcript length>than 200 nt; using Cuffcompare software to compare with known databases, we filtered-out known transcripts in the database, and performed coding potential prediction (CPC2/Pfam/CNCI) on the newly screened transcripts. Referring to the nomenclature guidelines of the HGNC (HUGO Gene Nomenclature Committee) for lncRNAs, candidate novel lncRNAs were screened and named based on their positional relationships to the coding gene.

Function and pathway analysis

Gene ontology (GO, https://www.geneontology.org) analysis was performed on the genes corresponding to each group of differentially expressed transcripts using Goseq software (R-3.3.2). The method is based on Wallenius non central hypergeometric distribution, which is characterized by a different probability of extracting an individual from a certain category compared to extracting an individual from outside of a certain category, allowing for more accurate calculation of the probability of GO term enrichment by the source gene. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysisis was conducted on the genes corresponding to each group of differentially expressed transcripts using Kobas software (V2.0). The main statistical analysis method is hypergeometric test. The P-value and FDR (false discovery rate) value were used to define the threshold for significance. The FDR correction method is Benjamini and Hochberg, with the adjusted p-value<0.05 indicating significant enrichment.

Quantitative real-time PCR (RT-qPCR)

Further, 1 ml of RNA isolation solution was added to 200 μl of exosomes to prepare a lysis solution. Subsequently, a 1/5 volume of chloroform solution was added. After sufficient shaking and settling, we added 400 μl of the topmost aqueous phase to 400 μl of isopropanol, which was mixed well, and centrifuged again. Then, 1 ml of 75% ethanol (RNase-free H_2O) was added to the precipitate. The supernatant was discarded after centrifugation. The experiments were repeated once. 2 ml of 75% ethanol solution was added to the centrifuge tube to dissolve the precipitate and extract the RNA. The TURE-Script first-strand cDNA synthesis kit (Aidlab, Beijing, China) was used to reverse transcribe total RNA into cDNA. The specific primers used for RT- qPCR are listed in (Table 1). Using *GAPDH* as an internal reference gene, the relative expression level was determined by the $2^{-\triangle\triangle Ct}$ method.

Statistical analysis

We established an original database using Excel and SPSS 23.0 to statistically analyze clinical and experimental data, and GraphPadPrism 8.0.2 to analyze experimental results and create graphs. The Shapiro-Wilk test was used to evaluate the normal distribution of data. Measurement data are expressed as mean±standard deviation.

Table 1 Primer synthesis information

Name	Sequence (5′→3′)
Homo-TPT1-AS1-F	CTTCCAGTACGACCACGAG
Homo-TPT1-AS1-R	GCCATCCCCATCATTGT
Homo-PTOV1-AS1-F	CCCACGCTTGTGAACAG
Homo-PTOV1-AS1-R	TCCGATGACTGCACAGC
Homo-SH3BP5-AS1- F	CCTGTCTCTCCACTTTCCC
Homo-SH3BP5-AS1- R	TGTAGCTTTGGGCTTCGT
Homo-PTPRG-AS1-F	ATCCACGGCCAAGGTAG
Homo-PTPRG-AS1-R	ACAGCCCAACTAATGGTCA
Homo-SNHG14-F	TCAAATGTCCTGCCTCTTCA
Homo-SNHG14-R	GCTCTCCCTCACTCCAACA
Homo-ACTIN-F	GGAAATCGTGCGTGACAT
Homo-ACTIN-R	GTGATGACCTGGCCGTT

Student's *t*-test or Welcht's test was used to compare continuous variables. Differences were considered statistically significant at $P<0.05$.

Results

Comparison of general baseline data between two groups of patients

Compared with the control group (non-PCOS), the experimental group (PCOS) had higher BMI levels and higher levels of AMH, basal luteinizing hormone (bLH), and basal testosterone (bT) in peripheral blood. The experimental group had more oocytes than the control group ($P<0.05$). There were no significant differences in the other basic data (Table 2).

Exosome identification and sequencing data volume

The morphology under TEM, nanoparticle tracking analysis, and surface protein molecular marker detection (Fig. 1) indicated that the extracellular vesicles in follicular fluid have been successfully isolated and can be used for subsequent sequencing analysis. The number of raw data reads generated by the IlluminaNovaseq ™ 6000 sequencing platform was 557,404,142, and after filtering out a small number of reads of joints or poor-quality raw data, 543,103,780 sequencing reads were obtained. The result showed that Q20 and Q30 reads accounted for 92.6%, while GC accounted for 50%, indicating that the quality of data obtained from sequencing was good (Table 3).

Screening and identification of lncRNA

We compared transcript lengths, numbers of exons, and open reading frame (ORF) lengths between lncRNAs and mRNAs to determine differences between them and verified whether the screened lncRNAs conformed to general characteristics. As shown in Fig. 2, the median length of lncRNA transcripts was approximately 1250 nt, which was less than the median length of mRNAs, which was 2000 nt. The number of exons in lncRNA transcripts was significantly lower than that in mRNA transcripts. The lengths of predicted ORFs in lncRNAs were similar to those of known lncRNAs, and the predicted novel lncRNAs conformed to general characteristics.

Differential expression profile of LncRNA in exosomes

Significant differences were analyzed using edgeR. The screening criteria for lncRNAs with significant differential expression were determined based on difference multiples and significance tests, with a value of ($|\log2(\text{FoldChange})| \geq 1$, $P<0.05$). In both patient groups, we identified 20 lncRNAs with significant differential expression. Among them, 17 lncRNAs were upregulated (four known lncRNAs) and three were downregulated (two known lncRNAs) (Fig. 3A). A volcano plot was used to visually display the overall distribution of genes with significant differences in expression (Fig. 3B). Upregulated genes are represented by red dots, downregulated genes by green dots, and blue dots represent genes that did not undergo any significant changes. We calculated the average expression of lncRNAs in the experimental group and control group separately, screened for genes with a Fragments Per Kilobase of exon model per Million mapped fragments (FPKM) value < 0.1 per thousand exon

Table 2 Comparison of baseline levels between the two groups

	Experiment group ($n=6$)	Control group ($n=6$)	t	*P*
Female age at oocyte retrieval (year)	28.83 ± 3.25	30.17 ± 1.17	−0.945	0.367
Duration of infertility (year)	3.33 ± 1.86	3.17 ± 1.33	0.178	0.862
BMI (kg/m^2)	31.50 ± 4.09	20.90 ± 2.17	5.601	***< 0.001***
AMH (ng/mL)	9.63 ± 3.60	2.52 ± 0.91	4.689	***0.004***
bFSH (mIU/mL)	7.41 ± 1.03	6.48 ± 1.51	1.247	0.241
bLH (mIU/mL)	14.40 ± 3.36	4.03 ± 1.43	6.948	***< 0.001***
bE_2 (pg/mL)	41.29 ± 18.84	47.07 ± 27.27	−0.427	0.679
bP (ng/mL)	0.62 ± 0.37	0.60 ± 0.19	0.128	0.901
bT (ng/mL)	0.90 ± 1.17	0.44 ± 0.58	4.274	***< 0.001***
No. of oocytes retrieved	19.50 ± 8.60	9.83 ± 3.97	1.895	***0.031***

Data are presented as mean ± SD and proportion (%). Abbreviations: AMH:, anti-Müllerian hormone; BMI, body mass index; bFSH, basic follicle-stimulating hormone; bLH, basic Luteinizing Hormone; bT, basal testosterone; bE_2, basic estrogen; bP, basic progesterone

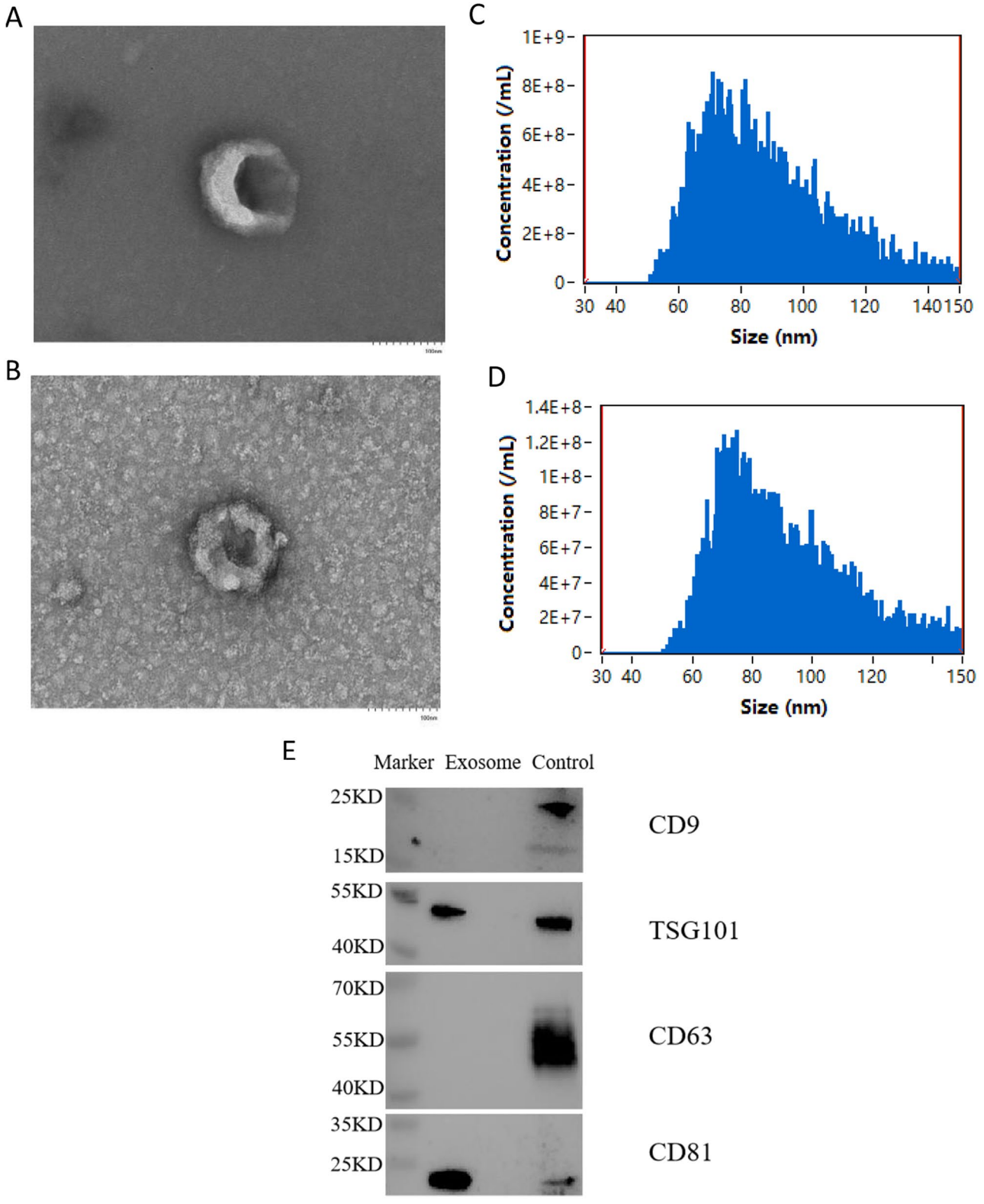

Fig. 1 Identification of exosomes. **A**: Representative TEM image of the exosome in PCOS, scale bar = 100 nm; **B**: Representative TEM image of the exosome in non PCOS, scale bar = 100 nm; **C**: Nanoparticle Tracking Analysis of exosome in PCOS; **D**: Nanoparticle Tracking Analysis of exosome in non PCOS; **E**: CD9, CD81, CD63, TSG101 expressions of exosome detected by western blot

Table 3 Summary table of sequencing quality

Sample	Raw data	Effect data	Raw data volume (G)	Effect data volume (G)	Error rate (%)	Q20 (%)	Q30 (%)	GC (%)
NP1	97,879,650	96,996,184	13.88	13.75	0.03	97.54	93.23	47.94
NP2	92,293,656	91,383,202	12.99	12.86	0.03	97.42	93.06	51.42
NP3	84,644,962	83,756,690	11.81	11.68	0.03	97.33	92.94	51.05
P1	88,842,488	88,093,244	12.87	12.76	0.03	97.47	92.98	50.58
P2	85,546,166	84,611,804	12.41	12.27	0.03	97.21	92.6	50.82
P3	99,160,058	98,262,656	14.38	14.25	0.03	97.55	93.12	50.51

Q20%: The proportion of bases with a quality value ≥ 20; Q30%: The proportion of bases with a quality value ≥ 30 (error rate < 0.1%); P: PCOS; NP: non PCOS

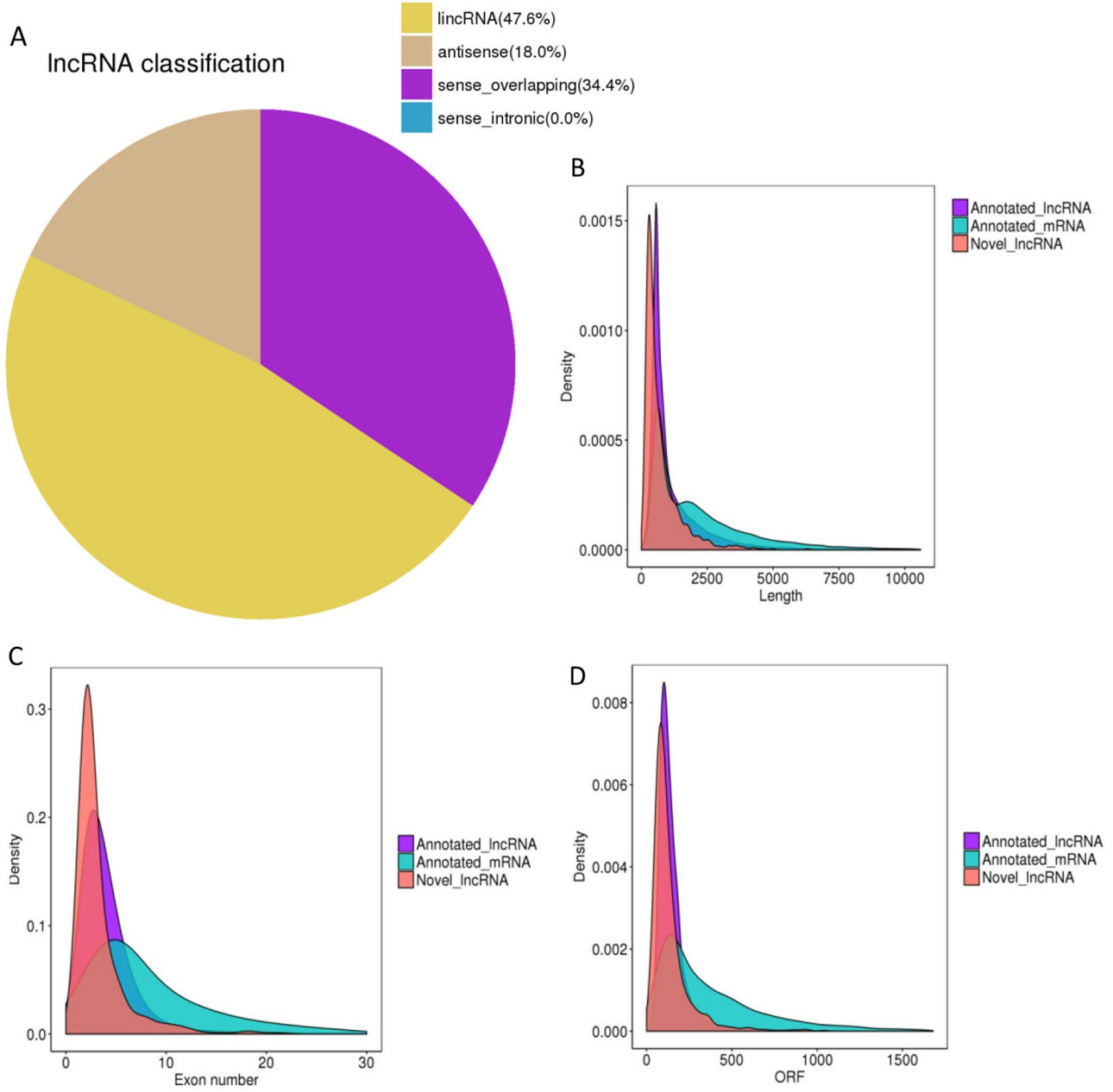

Fig. 2 Features of lncRNAs. **A**: Distribution map of lncRNA types; **B**: Density distribution map of length comparison between lncRNAs and mRNAs; **C**: Exon number density plot of lncRNAs and mRNAs; **D**: ORF length density distribution map of lncRNAs and mRNAs

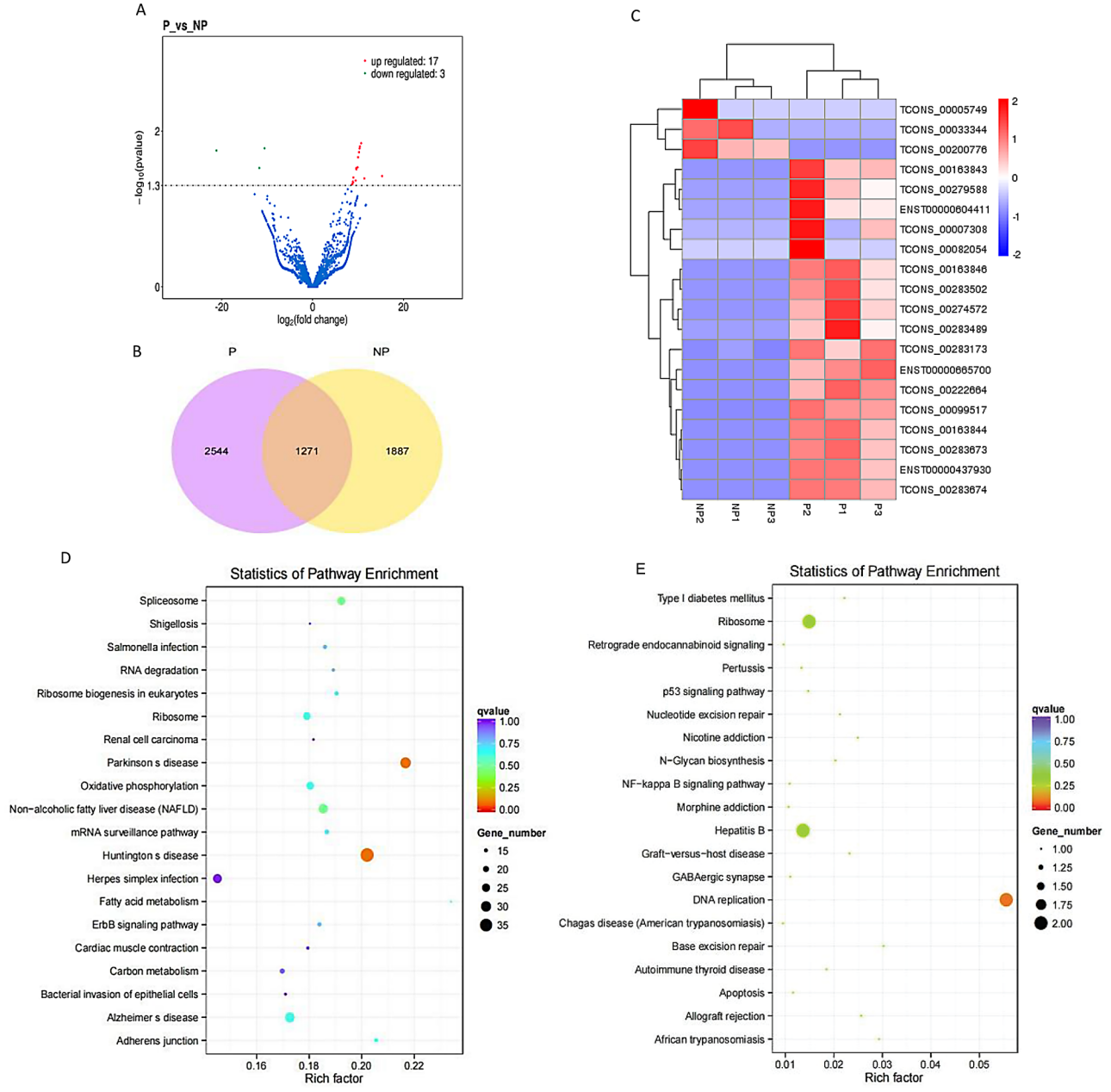

Fig. 3 Differential expression profile of lncRNAs and functional analysis. **A**: Differential lncRNA volcano map; **B**: Venn diagram showing the number of unique and common lncRNAs between two groups; **C**: Cluster analysis of differential lncRNA expression levels; **D**: Scatter plot of KEGG enrichment for co-expression candidate target genes; **E**: Scatter plot of KEGG enrichment for co-location candidate target genes

model fragment/million, and crossed plot the two groups into Venny diagrams. Genes were clustered according to the degree of similarity in gene expression, expression of genes with consistent functions, or participation in consistent metabolic and signaling pathways in different samples. The relative expression of these genes gradually increased (shown as blue to red). lncRNAs in follicular fluid exosomes of the experimental group exhibited differential expression compared to those of the control group, and the gene expression trend between the two groups was generally consistent (Fig. 3C).

lncRNAs regulate target genes by various mechanisms, the most common of which is through co-localization and co-expression. Co-localization refers to the potential regulatory effect of lncRNAs on neighboring protein-coding genes, analyzed by searching for genes within 100 kb upstream and downstream of the lncRNA, and refers to the mode of action of lncRNA transcription on distant target genes, analyzed through expression

correlation involving multiple samples. The prediction of target genes for lncRNA co-localization regulation was performed using Python scripts, covering the 100 kbp upstream and downstream of lncRNA sites. GO analysis was used for target gene functional enrichment analysis, and 677 GO terms were significantly enriched. In biological processes (BP), they were mainly enriched in cellular metabolism (GO: 0071840, GO: 00703030), cellular components (CC) were mainly enriched in organelles and DNA polymerase complex terms (GO: 0042575), and molecular function (MF) terms were mainly enriched in protein binding and adhesion terms (GO: 005488, GO: 005515) (Supplementary File 1 and Supplementary File 2); Draw the terms of the top 20 respectively. KEGG analysis was used to identify signaling pathways that were significantly enriched in lncRNA target genes. The size of the circle in the scatter plot was positively correlated with the number of genes enriched in this pathway. The significance of enrichment is represented by color, with red representing more significant enrichment. Selecting the pathway enrichment items in the top 20 through scatter plots: expression related target gene pathways mainly included Huntington's disease and Parkinson's disease, spliceosome, and non-alcoholic fatty liver disease pathways. The position-related target gene pathway mainly involved "DNA replication" and "ribosome" pathways (Fig. 3D and E).

RT-qPCR detection of related lncRNA expression in exosomes

To verify the sequencing results, combined with sequencing analysis and a literature review, RT-qPCR experiments based on different 6 patients with obesity and PCOS and 6 patients with infertility due to male factors were performed on the following lncRNAs to detect their expression levels: non-active specific transcript antisense RNA (*TSIX*) and actin α 2 (*ACTAA2*). Smooth muscle antisense RNA1 (*ACTA2-AS1*), ubiquitin specific peptidase 3 antisense RNA1 (*USP3-AS1*), protein tyrosine phosphatase receptor G-type antisense RNA1 (*PTPRG-AS1*), small nucleolar RNA host gene 14 (*SNHG14*), PTOV1 extended AT hook containing adapter protein antisense RNA1 (*PTOV1-AS1*), tumor protein translation control 1 antisense RNA1 (*TPT1-AS1*), SH3 domain binding protein 5 antisense RNA1 (*SH3BP5-AS1*). Six PCOS patients with obesity and six healthy female who were infertile due to factors related to their male partner were randomly selected. Follicular fluid exosomes were extracted and analyzed by RT-qPCR. The baseline data of the two groups of patients were compared as follows (Table 4), and except for significant differences in AMH, BMI, bLH, bT, and the number of retrieved oocytes, the remaining baseline data were also different.

Table 4 Comparison of basic data between two groups using RT-qPCR

	Obese PCOS Group ($n=6$)	Male Factors Group ($n=6$)	t	*P*
Female age at oocyte retrieval (years)	28.33 ± 1.86	26.67 ± 1.21	0.964	0.096
Duration of infertility (years)	3.09 ± 1.26	3.00 ± 1.10	0.739	0.894
BMI (kg/m^2)	30.62 ± 0.89	20.50 ± 2.19	6.750	***< 0.001***
AMH (ng/mL)	8.22 ± 1.10	3.35 ± 0.88	0.189	***< 0.001***
bFSH (mIU/mL)	6.25 ± 1.03	7.35 ± 1.83	0.936	0.228
bLH (mIU/mL)	9.26 ± 1.62	5.28 ± 1.50	0.243	***0.001***
bE_2 (pg/mL)	45.36 ± 4.37	47.87 ± 12.68	1.963	0.657
bP (ng/mL)	0.81 ± 0.10	0.62 ± 0.21	2.256	0.073
bT (ng/mL)	0.83 ± 0.25	0.43 ± 0.12	1.303	***0.006***
No. of oocytes retrieved	20.00 ± 3.85	6.67 ± 3.33	0.313	***< 0.001***

The RT-qPCR test results showed that the expression trends of lncRNAs *ACTA2-AS1*, *USP3-AS1*, *PTPRG-AS1*, *SNHG14*, *TPT1-AS1*, and *PTOV1-AS1* were consistent with the sequencing results. No significant differences were observed in the expression of *TSIX* or *SH3BP5-AS1* between the two groups (Fig. 4).

Discussion

PCOS is assumed to be a chronic inflammation caused by abdominal obesity, with most obese individuals consuming an abnormal diet. Excessive accumulation of glucose or fat induces oxidative stress and stimulates inflammation. Obesity leads to a higher incidence of insulin resistance and other diseases [23], exacerbating abnormalities in reproductive-related metabolic indicators. Researchers have proposed that fat-ovarian crosstalk is a key mediator in the pathogenesis of PCOS [24], and the impact of obesity on PCOS remains an attention-seeking problem. Xiang et al. found differential gene expression in granulosa cells of obese patients with PCOS and assumed that differentially expressed genes are related to cellular processes, such as immune responses, cell proliferation, gene transcription regulation, and inflammation [25]. Patients with obesity and PCOS have significantly higher serum levels of miR-194-3p, miR-296-3p, and AMH than those in non-obese groups [26]. A research team detected serum lncRNA expression in patients with PCOS of different body weights, and the lncRNA expression pattern was related to body weight [27].

In this study, we selected follicular fluid exosomes from patients with obesity and PCOS and patients with male infertility for high-throughput sequencing to obtain lncRNA expression profiles. Owing to their relatively long chain states, lncRNAs only exist in a fragmented form in exosomes. In addition, exosome content in follicular fluid was lower than that in blood samples. By sequencing analysis, only 20 lncRNAs with significant

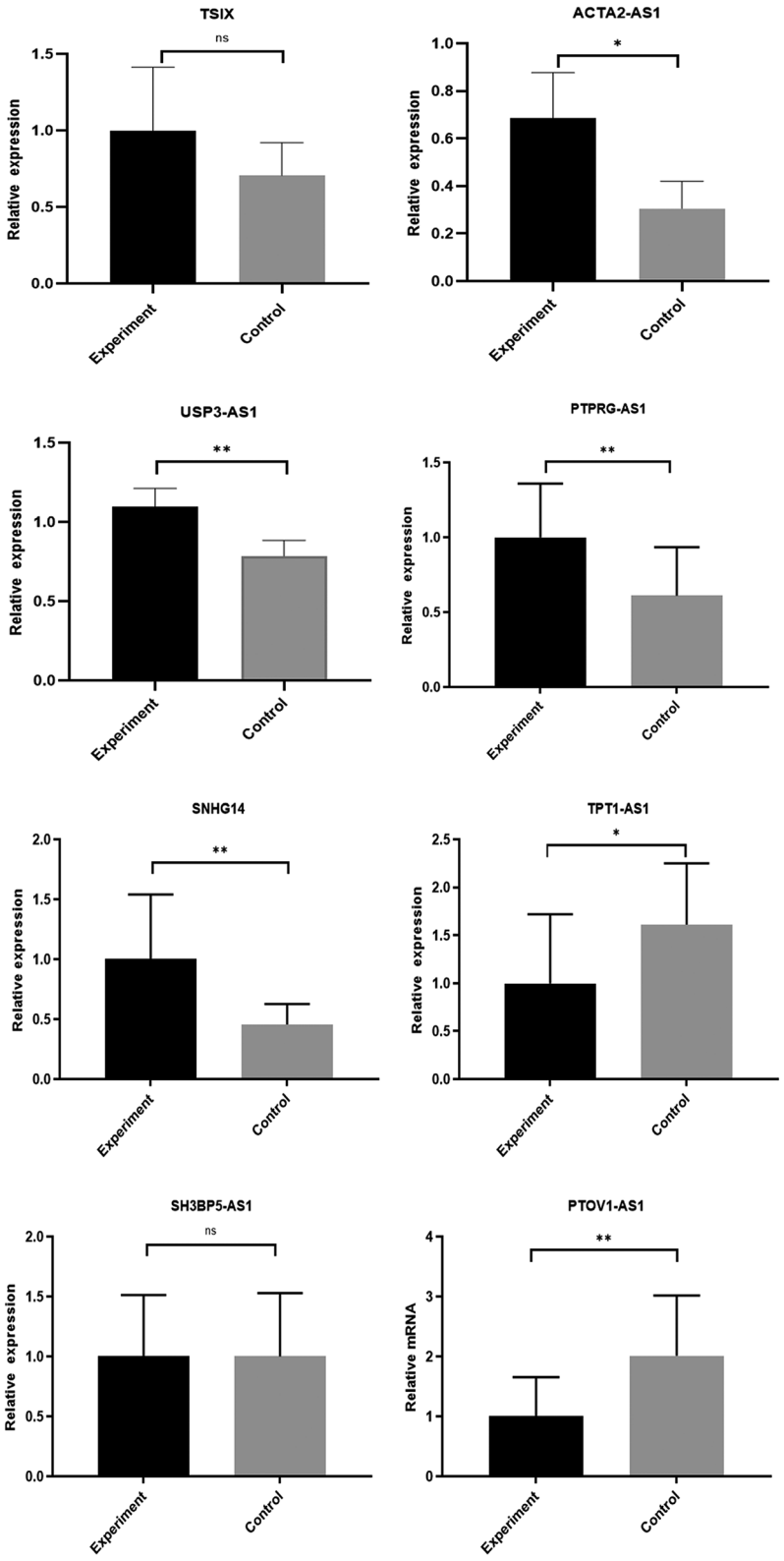

Fig. 4 Comparison of lncRNA expression levels between two groups. RT-qPCR was used to measure lncRNA expression levels. ns: $P > 0.05$; *: $P < 0.05$; **: $P < 0.01$

differential expression trends were found, of which 17 were significantly upregulated and three significantly downregulated, including *TSIX*, *ACTA2-AS1*, and *USP3-AS1*. *TSIX* is considered important in the mechanism of imprinting inactivation. A recent study reported that lncRNAs *TSIX* and *XIST* are related to the expression of programmed cell death 1 (PD-1) protein, which can be used as a noninvasive immune biomarker to reflect the extent to which breast cancer cells escape anti-tumor immunity [28]. A research team sequenced small RNAs in stem cells during aging, suggesting that the upregulation of *TSIX* may be related to aging cellular replication [29]. Overexpression of *TSIX* in the follicular fluid of patients with obesity and PCOS is speculated to affect cellular immunity or alter cell division by affecting cellular replication and aging, leading to the occurrence of PCOS. Therefore, *ACTA2-AS1* can be used in studies concerning cancer drug resistance. *ACTA2-AS1* inhibits the activity of colorectal cancer cells, regulates cancer progression by affecting cell proliferation, migration, and invasion processes [30], and targets the miR-4428/KLF9 axis to inhibit the suppresses metastasis in papillary thyroid cancer cells [31], which is a significantly differentially expressed lncRNA in obese PCOS patients. Further research surrounding *ACTA2-AS1* in cellular processes will help deepen the relationship between obesity, cell proliferation, and apoptosis in patients with PCOS. *USP30-AS1* is a negative regulator of mitochondrial autophagy [32], and animal experiments have shown that fat can cause infertility in PCOS rats by damaging mitochondrial function. Mitochondrial function may be related to infertility in patients with obesity and PCOS. Cellular functional analysis of differentially expressed lncRNAs in patients with obesity and PCOS further confirmed that fat may affect the occurrence and development of PCOS by regulating cell division, inflammation, proliferation, apoptosis, and mitochondrial function. The establishment of lncRNA differential expression profiles will further our understanding of PCOS pathogenesis and provide effective treatments.

This study used two public databases, GO and KEGG, to conduct informatics analysis on lncRNAs obtained from sequencing to identify their enrichment in terms of biological functions and pathways. The GO term for lncRNA target genes was enriched in organelles and protein adhesion, and mainly participates in splicing bodies, Huntington's disease, Parkinson's disease, non-alcoholic fatty liver disease, and ribosomal pathways. According to previous reports, the spliceosome pathway regulates cancer occurrence [33], affects gene transcription and expression, and participates in the removal of introns and the connection of remaining exons. A better understanding of the role of spliceosomes in cancer development can help formulate strategies for treating patients with cancer [34]. Ingvar et al. found that the fat breakdown rate of visceral adipocytes in patients with PCOS has a specific defect, which may be caused by a unique change in the non-enzymatic splicing form of hormone sensitive lipase (HSL) in adipocytes [35]; XU et al. performed DNA methylation analysis of genes in the granulosa cells of obese individuals with PCOS to explore the biological functions of related genes in regulatory networks. The results showed that they were significantly enriched in splicing variation and selective splicing terms, and that the spliceosome pathway contributes to a deeper understanding of the occurrence of obese PCOS [36]. International research teams conducted transcriptomic analysis on adipose tissue of PCOS and healthy women and observed that differentially expressed genes are not only enriched in cell proliferation and apoptotic regulatory and metabolic pathways, but are also involved in cancer pathways and brain diseases such as Huntington's disease and Parkinson's disease [37], consistent with the results of this study. Lipid peroxidation, iron accumulation, and neurodegenerative diseases, such as Parkinson's and Huntington's diseases, are related [38]. Mitochondrial dysfunction, autophagy destruction, and iron-mediated cell death are considered the key pathological mechanisms of lipid peroxidation and iron accumulation in these diseases. This suggests that the interaction between obesity and PCOS may be related to abnormal egg cell proliferation and apoptosis caused by lipid peroxidation and iron deficiency. Non-alcoholic fatty liver disease (NAFLD) is associated with abnormal cellular autophagy. The accumulation of fat throughout the body, especially in the abdomen, can easily inhibit autophagy in liver cells, leading to a large accumulation of lipids. Autophagy disorders are of great significance in the development of obese PCOS [39]. The incidence of NAFLD is significantly increased in women with overweight/obesity and PCOS, and NAFLD exacerbates liver and systemic insulin resistance through necrotizing inflammation as well as various pro-inflammatory, procoagulant, and fibrotic mediators, playing an important role in the pathophysiology of PCOS [40]. In addition, a research team has observed through animal experiments that differentially expressed genes in PCOS mouse oocytes are enriched in ribosome related pathways, which may explain the developmental disorders of PCOS follicles [41], while PCOS patients with visceral obesity often have β diversity changes in intestinal flora, the diversity changes may affect lipid homeostasis and visceral fat accumulation through pathways such as ribosome and fatty acid biosynthesis pathways [42]. Patients with obesity and PCOS who undergo assisted reproductive assistance because of male factors exhibit differential expression of lncRNAs in follicular fluid exosomes. Adipose tissue may regulate biological processes such as cell

proliferation, apoptosis, oxidative stress, and autophagy, by regulating the expression of related genes, which can affect the occurrence and progression of PCOS.

However, this study still had certain limitations. Firstly, the study only investigated 6 patients with obesity complicated by PCOS and 6 male factor infertility patients, which may not fully reflect the actual situation due to the small sample size, .and also poses a challenge to clarify the sensitivity and specificity of certain lncRNA as clinical diagnostic biomarkers. Secondly, the study only focused on patients with obesity and PCOS, and the single research object results may not be applicable to other types of infertility patients. Besides, the study used high-throughput sequencing technology, which can more accurately detect low-abundance lncRNAs, but this technology still has certain limitations, such as insufficient sequencing depth and sequencing errors. Additionally, circulating lncRNAs in exosomes from follicular fluid likely originate from various cells including granulosa cells, oocyte, and cumulus cells, and may shuttle between different cells and be part of the bi-directional signaling program, necessitating differentiation of lncRNAs by cell source. Consequently, differential lncRNAs identified through follicular fluid lncRNA sequencing in this study may not serve as precise biomarkers for oocyte quality, yet they do reflect ovarian follicular development and dysfunction to some extent. In future research, it will be crucial to employ the necessary technical methods to differentiate lncRNAs from exosomes of distinct cellular origins within follicles, and continue to expand the sample size and diversify the study scope, method and patients demographics to facilitate the identification of differential expression of extracellular lncRNAs in patients with varying health states complicated by PCOS, which would enable us to delve deeper into the specific mechanisms by which lncRNAs contribute to the regulation of oocyte function, as well as the onset and progression of PCOS.

Conclusions

In this study, we used high-throughput sequencing to investigate the transcriptomics of follicular fluid exosomes in patients with obesity and PCOS. The potential mechanisms of action of obesity and PCOS are reflected in the differential expression profiles of the lncRNAs. The differential lncRNA-related gene pathways mainly involved spliceosomes, Huntington's disease, Parkinson's disease, NAFLD, and ribosomal pathways. Establishment of expression profiles provides a basis for PCOS diagnosis and treatment. Related lncRNAs may serve as biomarkers for studying obesity-related PCOS and regulating oocyte quality and function, thereby providing value for future in-depth research surrounding the occurrence and development of obesity-related PCOS.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13048-024-01552-8.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Acknowledgements

We would like to thank Editage (http://www.editage.cn) for English language editing.

Author contributions

FL and HW conceived and designed this study. XX and JL contributed to the acquisition and analyses of data. XX and LD contributed to drafting the manuscript. YQ was responsible for sample collection. XX and WC contributed to manuscript revision. All authors read and approved the final submitted version of this manuscript.

Funding

This research was funded by National Natural Science Foundation of China (No. 82274573, 81703958), Natural Science Foundation of Shandong Province (No. ZR2021MH255) and the Special Fund of "Taishan Scholars" of Shandong Province (No. tsqn202103182).

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethical approval

This study had been registered with the Chinese Clinical Trial Registration Center (ChiCTR210052331||http://www.chictr.org.cn/) on October 24, 2021. The research had also been approved by The Reproductive Medicine Ethics Committee of Shandong University of Traditional Chinese Medicine Affiliated Hospital (batch number: SDSZYYSZ20211009) and to exempt the informed consents.

Human ethics and consent to participate

All procedures were carried out in accordance with relevant guide-lines and regulations and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All participants had signed informed consent forms before enrollment.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Author details

[1]First School of Clinical Medicine, Shandong University of Traditional Chinese Medicine, Jinan 250011, China
[2]Shandong Wendeng Osteopathic Hospital, Wendeng, Shandong 264400, China
[3]Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan 250014, China

Received: 11 March 2024 / Accepted: 1 November 2024
Published online: 11 November 2024

References

1. Qiao J, Wang Y, Li X, Jiang F, Zhang Y, Ma J, et al. A Lancet Commission on 70 years of women's reproductive, maternal, newborn, child, and adolescent health in China. Lancet. 2021;397(10293):2497–536.

2. Zhou Z, Zheng D, Wu H, Li R, Xu S, Kang Y, et al. Epidemiology of infertility in China: a population-based study. BJOG. 2018;125(4):432–41.
3. The Endocrine Metabolism Branch of the Chinese Medical Association. Consensus of Endocrine experts on the diagnosis and treatment of polycystic ovarian syndrome. Chin J Endocrinol Metabolism. 2018;34(1):1–7.
4. Luo Lilan. Infertility and infertility. Beijing: People's Health Publishing House, 2009:300–9.
5. Choudhury AA, Rajeswari VD. Polycystic ovary syndrome (PCOS) increases the risk of subsequent gestational diabetes mellitus (GDM): a novel therapeutic perspective. Life Sci. 2022;310:121069.
6. Azziz R, Introduction. Determinants of polycystic ovary syndrome. Fertil Steril. 2016;106(1):4–5.
7. Zhan W, Tang W, Shen X, Xu H, Zhang J. Exposure to bisphenol A and its analogs and polycystic ovarian syndrome in women of childbearing age: a multicenter case-control study. Chemosphere. 2023;313:137463.
8. Escobar-Morreale HF. Polycystic ovary syndrome: definition, aetiology, diagnosis and treatment. Nat Rev Endocrinol. 2018;14(5):270–84.
9. Li L, Chang HY. Physiological roles of long noncoding RNAs: insight from knockout mice. Trends Cell Biol 2014,24(10):594–602.
10. 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.
11. Huang MJ, Zhao JY, Xu JJ, Li J, Zhuang YF, Zhang XL. lncRNA ADAMTS9-AS2 controls human mesenchymal stem cell chondrogenic differentiation and functions as a ceRNA. Mol Ther Nucleic Acids. 2019;18:533–45.
12. Gao M, Liu X, Gu H, Xu H, Zhong W, Wei X, et al. Association between single nucleotide polymorphisms, TGF-β1 promoter methylation, and polycystic ovary syndrome. BMC Pregnancy Childbirth. 2024;24(1):5.
13. Yu L, Wang C, Liu M, Xia L, Liu T, Che Q, et al. Follicular fluid-derived exosomal LncRNA LIPE-AS1 modulates steroid metabolism and survival of granulosa cells leading to oocyte maturation arrest in polycystic ovary syndrome. J Assist Reprod Genet. 2024;41(5):1387–401.
14. Geng X, Zhao J, Huang J, Li S, Chu W, Wang WS, et al. lnc-MAP3K13-7:1 inhibits ovarian GC proliferation in PCOS via DNMT1 downregulation-mediated CDKN1A promoter hypomethylation. Mol Ther. 2021;29(3):1279–93.
15. Che Y, Yu J, Li YS, Zhu YC, Tao T. Polycystic ovary syndrome: challenges and possible solutions. J Clin Med. 2023;12(4):1500.
16. Huang X, Pan J, Wu B, Teng X. Construction and analysis of a lncRNA (PWRN2)-mediated ceRNA network reveal its potential roles in oocyte nuclear maturation of patients with PCOS. Reprod Biol Endocrinol. 2018;16(1):73.
17. Guo Y, Peng X, Cheng R, Chen H, Luo X. Long non-coding RNA-X-inactive specific transcript inhibits cell viability, and induces apoptosis through the microRNA-30c-5p/Bcl2-like protein 11 signaling axis in human granulosa-like tumor cells. Bioengineered. 2022;13(6):14107–17.
18. Yu L, Wang C, Zhang D, Liu M, Liu T, Pan B, et al. Exosomal circ_0008285 in follicle fluid regulates the lipid metabolism through the miR-4644/ LDLR axis in polycystic ovary syndrome. J Ovarian Res. 2023;16(1):113.
19. Jeppesen DK, Fenix AM, Franklin JL, Higginbotham JN, Zhang Q, Zimmerman LJ, et al. Reassessment Exosome Composition Cell. 2019;177(2):428–e44518.
20. Farooqi AA, Desai NN, Qureshi MZ, Librelotto DRN, Gasparri ML, Bishayee A, et al. Exosome biogenesis, bioactivities and functions as new delivery systems of natural compounds. Biotechnol Adv. 2018 Jan-Feb;36(1):328–34.
21. Salomon C, Nuzhat Z, Dixon CL, Menon R. Placental exosomes during Gestation: Liquid biopsies carrying signals for the regulation of human parturition. Curr Pharm Des. 2018;24(9):974–82.
22. Tamaddon M, Azimzadeh M, Tavangar SM. microRNAs and long non-coding RNAs as biomarkers for polycystic ovary syndrome. J Cell Mol Med. 2022;26(3):654–70.
23. Chudzicka-Strugała I, Gołębiewska I, Banaszewska B, Brudecki G, Zwoździak B. The role of individually selected diets in obese women with PCOS-A review. Nutrients. 2022;14(21):4555.
24. Xu Y, Zhu H, Li W, Chen D, Xu Y, Xu A, et al. Targeting adipokines in polycystic ovary syndrome and related metabolic disorders: from experimental insights to clinical studies. Pharmacol Ther. 2022;240:108284.
25. Xiang S, Xia MF, Song JY, Liu DQ, Lian F. Effect of Electro-acupuncture on expression of IRS-1/PI3K/GLUT4 pathway in ovarian granulosa cells of infertile patients with polycystic ovary syndrome-insulin resistance of phlegm-dampness syndrome. Chin J Integr Med. 2021;27(5):330–5.
26. Tang ZX, Yu LJ, Ping YZ, Wang YR. Serum levels of miR-194-3p、miR-296-3p and AMH and diagnostic value of patients with polycystic ovary syndrome [J]. China Maternal Child Health Res. 2022;33(12):57–62.
27. Butler AE, Hayat S, Dargham SR, Malek JA, Abdullah SA, Mahmoud YA, et al. Long non-coding RNA expression in non-obese women with polycystic ovary syndrome and weight-matched controls. Reprod Biomed Online. 2020;41(4):579–83.
28. Salama EA, Adbeltawab RE, El Tayebi HM. XIST and TSIX: Novel Cancer Immune biomarkers in PD-L1-Overexpressing breast Cancer patients. Front Oncol. 2020;9:1459.
29. Kim CG, Lee JK, Cho GJ, Shin OS, Gim JA. Small RNA sequencing of small extracellular vesicles secreted by umbilical cord mesenchymal stem cells following replicative senescence. Genes Genomics. 2023;45(3):347–58.
30. Wu X, Pang Q, Zhi F, Mao X, Hu Y. Overexpression of long non-coding RNA ACTA2-AS1 inhibits the viability, proliferation, migration and invasion of colorectal cancer cells. Tissue Cell. 2022;76:101769.
31. Wu S, Zhu J, Jiang T, Cui T, Zuo Q, Zheng G, et al. Long non-coding RNA ACTA2-AS1 suppresses metastasis of papillary thyroid cancer via regulation of miR-4428/KLF9 axis. Clin Epigenetics. 2024;16(1):10.
32. Wang N, Li J, Xin Q, Xu N. USP30-AS1 contributes to mitochondrial quality control in glioblastoma cells. Biochem Biophys Res Commun. 2021;581:31–7.
33. Zong RQ, Zhang HY, Li XY, Li YR, Chen Y. Overexpressed histocompatibility minor 13 was Associated with Liver Hepatocellular Carcinoma Progression and Prognosis. Genet Res (Camb). 2022;2022:7067743.
34. Koedoot E, Smid M, Foekens JA, Martens JWM, Le Dévédec SE, van de Water B. Co-regulated gene expression of splicing factors as drivers of cancer progression. Sci Rep. 2019;9(1):5484.
35. Ek I, Arner P, Rydén M, Holm C, Thörne A, Hoffstedt J, et al. A unique defect in the regulation of visceral fat cell lipolysis in the polycystic ovary syndrome as an early link to insulin resistance. Diabetes. 2002;51(2):484–92.
36. Xu J, Bao X, Peng Z, Wang L, Du L, Niu W, et al. Comprehensive analysis of genome-wide DNA methylation across human polycystic ovary syndrome ovary granulosa cell. Oncotarget. 2016;7(19):27899–909.
37. Saedi S, Panahi R, Orak N, Jafarzadeh Shirazi MR. Comparative Meta-analysis of Adipose Tissue Transcriptomics Data in PCOS patients and Healthy Control Women. Reprod Sci. 2023;30(6):1823–33.
38. Villalón-García I, Povea-Cabello S, Álvarez-Córdoba M, Talaverón-Rey M, Suárez-Rivero JM, Suárez-Carrillo A, et al. Vicious cycle of lipid peroxidation and iron accumulation in neurodegeneration. Neural Regen Res. 2023;18(6):1196–202.
39. Doycheva I, Ehrmann DA. Nonalcoholic fatty liver disease and obstructive sleep apnea in women with polycystic ovary syndrome. Fertil Steril. 2022;117(5):897–911.
40. Targher G, Rossini M, Lonardo A. Evidence that non-alcoholic fatty liver disease and polycystic ovary syndrome are associated by necessity rather than chance: a novel hepato-ovarian axis? Endocrine. 2016;51(2):211–21.
41. Nakanishi N, Osuka S, Kono T, Kobayashi H, Ikeda S, Bayasula B, et al. Upregulated ribosomal pathway impairs follicle development in a polycystic ovary syndrome mouse model: Differential Gene expression analysis of oocytes. Reprod Sci. 2023;30(4):1306–15.
42. Bai X, Ma J, Wu X, Qiu L, Huang R, Zhang H, et al. Impact of visceral obesity on structural and functional alterations of gut microbiota in polycystic ovary syndrome (PCOS): a pilot study using metagenomic analysis. Diabetes Metab Syndr Obes. 2023;16:1–14.

Publisher's note