Functional prediction and profiling of exosomal circRNAs derived from seminal plasma for the diagnosis and treatment of oligoasthenospermia

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Abstract. Oligoasthenospermia (OAZ) is the most common element contributing to male infertility. However, the etiology of OAZ remains unknown in the majority of cases. Growing evidence indicates that exosomal circular (circ)RNAs may exhibit potential as biological markers for the detection of various disorders. The available information on exosomes derived from seminal plasma is limited. The present study investigated the composition and role of circRNAs in exosomes isolated from seminal plasma of patients with OAZ. Exosomes were isolated from the seminal plasma of 12 patients with OAZ and 12 matched healthy controls. Thereafter, RNA sequencing was performed using exosomes from both groups to identify circRNAs associated with OAZ. The sequencing data revealed a total of 14,991 circRNAs. Among these, 7,635 were upregulated and 7,356 were downregulated in patients with OAZ. Gene Ontology functional enrichment analysis revealed that the differentially expressed exosomal circRNAs were primarily enriched in 'protein binding', 'intracellular organelles' and 'cellular metabolism'. Kyoto Encyclopedia of Genes and Genomes pathway analysis revealed that the differentially expressed exosomal circRNAs were enriched in 'ubiquitin-mediated proteolysis', 'endocytosis' and 'RNA transport', which are involved in spermatogenesis-related pathways. Then seven differentially expressed circRNAs were predicted and validated as putative upstream targets and their target genes also were detected by reverse transcription-quantitative PCR. CircRNA-microRNA-mRNA network was constructed to predict their potential functions. The findings provide a preliminary foundation for identifying the potential diagnostic value of critical exosomal circRNAs involved in OAZ.

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Introduction

Results obtained from the World Health Organization (WHO) demonstrate that infertility is a serious social issue (1,2). The worldwide incidence of infertility is >15%, of which, 50% is attributed to male factors (3). Oligoasthenospermia (OAZ) is considered a major contributing factor in male infertility (4). OAZ is defined as a reduction in sperm concentration (<15 million/ml) and sperm progressive motility (<32%) or total motility (<40%) based on the WHO 2010 5th criteria (5). However, the underlying mechanisms and etiology of OAZ remain to be elucidated. The molecular biomarkers of hereditary factors affecting OAZ may provide valuable information for the development of targeted treatments and may help to determine the etiology of OAZ (6). In addition, novel non-invasive biomarkers and more accurate diagnostic tools are required to identify OAZ in clinical settings (7,8). Due to the relatively accessible availability of seminal plasma and the presence of fluid from the testes, it is a prospective source of biomarkers for OAZ.

Exosomes are considered a key mediator of intercellular communication and are associated with the development and prognosis of numerous diseases, including cancer, neurodegenerative diseases, infections and autoimmune diseases (9-11). Exosomes are disk-shaped vesicles with lipid bilayer structures, ranging in size from 50-150 nm. Exosomal nucleic acid substances are delivered to target cells and act on them by regulating their gene expression and functional status (12). The presence of exosomes in various body fluids facilitates non-invasive detection. Previous studies have demonstrated that the exosomes are found in semen, amniotic fluid, blood and breast milk (13,14). Seminal plasma exosomes are involved in sperm maturation (15).

Seminal plasma exosomes also perform important sperm functions (16); for example, they induce the regulation of sperm

motility. Although exosomes are found in human seminal plasma, their specific composition and potential physiological functions remain unknown (17).

Small RNAs play an important role in spermatogenesis and early embryonic development (17,18). As novel non-coding RNAs, the specific loop-like structure of circular (circ)RNAs allows them to remain stable in living organisms and on entering exosomes (19,20). Exosomal circRNAs perform numerous physiological and pathological functions, such as regulating mRNA transcription and serving as circRNA-protein mediators and efficient micro(mi) RNA sponges (21,22). In addition, circRNAs are present in exosomes derived from the male reproductive system and expression differs between different physiological and pathological states of the testes and semen (23). Dong *et al* (24) confirm that circRNAs are stably expressed and present in semen. Ji *et al* (25) demonstrate that circRNAs derived from seminal plasma may be used to predict microscopic testicular sperm extraction outcomes in patients with idiopathic non-obstructive azoospermia. Lv *et al* (26) note that circ_0000116 expression is significantly higher in the testicular tissues of patients with non-obstructive azoospermia, compared with that in patients with obstructive azoospermia. Liu *et al* (27) report that circ_0049356 expression is decreased in seminal plasma, which leads to the hypothesis that circ_0049356 performs a highly regulated biological function in non-obstructive azoospermia. Exosomes derived from seminal plasma transport disease-specific circRNAs to spermatogenic cells (14). Further investigations into the role of exosomal circRNAs in male reproduction may provide novel insights into their potential application as non-invasive biomarkers for the early detection of diseases.

The specific role of exosomal circRNAs in OAZ remains to be elucidated. In the present study, RNA-sequencing (RNA-seq) was performed to analyze the expression profile of seminal plasma-derived exosomal circRNAs in patients with OAZ and healthy controls. In addition, bioinformatics software and verification experiments were performed to establish a circRNA-miRNA-mRNA network for predicting the interactions among exosomal circRNAs (28-30). These results may offer novel insights into the role of exosomal circRNAs in OAZ.

Materials and methods

Subjects and seminal plasma samples. Semen samples were obtained from 12 men with infertility diagnosed with OAZ (mean age, 33.5 years; range, 27-37 years) and 12 healthy controls (mean age, 31.4 years; range, 26-40 years) between March 2021 and September 2021. The clinicopathological characteristics of the patients are summarized in Table I. Patients with OAZ enrolled in the present study did not present with any clinical factors for infertility, such as anatomic malformation, genetic abnormalities, endocrine factors, varicocele, reproductive tract infections, immunological factors, testicular damage or environmental factors. All semen samples were analyzed in accordance with WHO guidelines (5).

Participants in the control group were selected based on a favorable medical assessment using a physical examination performed during the aforementioned time period. For follow-up experiments of exosomal circRNAs in seminal plasma, samples were divided for RNA-seq and additional validation experiments. Donor semen samples were acquired using manual masturbation following 3-5 days of sexual abstinence and were processed immediately. Routine semen analysis was conducted in accordance with the WHO 2010 5th criteria. To obtain seminal plasma supernatant, sperm and cellular debris were pelleted following centrifugation and subsequently collected.

Exosome isolation from seminal plasma. An ExoQuick Exosome Precipitation kit was used for isolating seminal plasma exosomes (System Bioscience) (31). The collected samples were centrifuged at 3,000 x g for 15 min at 25˚C, and the supernatant was dissolved in phosphate-buffered saline (PBS). Subsequently, 65 μl reagent was added (4:1) according to the kit instructions and immediately vortexed. The seminal plasma-reagent mixture was incubated at 4˚C for 1 h for precipitation. Subsequently, the mixture was centrifuged twice at 1,500 x g at 25˚C for 5 min using purification columns. The pellet was resuspended and stored.

Transmission electron microscopy (TEM) of exosomes. The isolated exosomes were washed twice using PBS. Subsequently, 2% osmium solution was added and exosomes were fixed for 2 h at 4˚C. The exosomes were dehydrated with 1 ml each of 50, 70, 80 and 90% ethanol. Samples were left for 15 min after each dehydration step. Subsequently, the samples were dehydrated twice (20 min each time) with 1 ml of 100% ethanol, which was replaced twice with 1 ml of acetone. Samples were incubated with the solution for 2 h and polymerized. Uranyl acetate and lead acetate were used for staining the plates for 10 min 4˚C. After washing, the morphological features of the isolated exosomes were captured using a TEM (HT7800, Hitachi, Ltd.).

Nanoparticle tracking analysis (NTA). The NP100 nanopores (NanoSight NS500; Zetaview) of the measurement system were calibrated using particles of known size (CPC100 standard solution) and washed twice with PBS. The exosome sample was diluted 1,000 times with PBS and subsequently added to the nanopores for the recording and tracking of each visible particle.

Western blotting analysis. A lysis solution (Santa Cruz Biotechnology, Inc.) was added to the isolated exosomes in a volume ratio of 1:1. A BCA protein concentration kit was used (Santa Cruz Biotechnology, Inc.) for determining the supernatant protein concentration after centrifuging at 12,000 x g for 5 min at 4˚C. The proteins in each sample were normalized to 30 μg per lane, and were loaded onto 12% sodium dodecyl sulfate polyacrylamide gel. After electrophoresis, the proteins were transferred to the polyvinylidene fluoride (PVDF) membrane (MilliporeSigma). The membrane was blocked by 5% skimmed milk for 2 h at 4˚C and subsequently incubated with TBST-diluted antibodies (containing 0.1% Tween-20) against tumor susceptibility gene TSG101 (1:1,000; ab30871; Abcam), CD63 (1:1,000; ab68418; Abcam) and GAPDH (1:5,000; ab9485; Abcam) at 4˚C overnight. Following primary incubation, the membrane was incubated with an HRP-labelled secondary antibody (1:2,000; ab205718; Abcam) for 1 h at 4˚C and ECL reagents A and B were added in a 1:1 ratio for

Table I. The basic clinical characteristics of HC and OAZ.

Clinical characteristic	HC	OAZ	P-value
Age (year)	31.42±3.53	33.51±4.11	0.195
Testes volume (ml)	16.82±2.39	16.37±3.13	0.696
Follicle Stimulating Hormone (IU L^{-1})	4.48±3.01	5.61±2.28	0.311
Luteinizing Hormone (IU L^{-1})	3.83±2.07	4.63±1.36	0.275
Testosterone (ng ml^{-1})	5.63±2.74	4.46±1.52	0.209
Semen volume (ml)	4.30±1.83	5.06±2.56	0.412
pH	7.40±0.16	7.37±0.14	0.603
Sperm concentration (x10^6 ml^{-1})	75.62±24.18	7.58±2.44	<0.001
Progressive motility (%)	42.55±6.55	11.56±4.79	<0.001
Normal morphology(%)	7.58±2.75	6.13±1.92	0.148

Data were the mean ± SD. HC, Healthy control; OAZ, oligoasthenospermia.

2 min (MilliporeSigma). Western blot bands were quantified by ImageJ software (https://imagej.nih.gov/ij/download.html; v 1.8, National Institutes of Health).

RNA library construction and RNA-seq. The Ribo-Zero rRNA Removal kit (Illumina, Inc.) was used to remove rRNA from the total RNA according to the manufacturer's instructions. The removed rRNA was pretreated using a TruSeq Stranded Total RNA Library Prep kit (Illumina, Inc.) and sequencing libraries were constructed. The qualitative and quantitative analysis of RNA libraries were performed using an Agilent BioAnalyzer 2100 system (Agilent Technologies, Inc.). Following successful sequencing and library construction, double-end sequencing (2x150 bp) was performed using the Illumina Hiseq 4000 sequencing platform (Illumina, Inc.).

CircRNA sequencing analysis. Following RNA-seq, double-end reads were harvested. Cutadapt (version 1.9.3; https://cutadapt.readthedocs.io/en/stable/installation.html) software was used to de-join and remove low-quality reads. The high-quality reads were produced and guaranteed using STAR software (http://star.mit.edu/, v2.5.1b). CircRNAs were identified using DCC software (https://dccwiki.com/DCC_Software, v0.4.4). CircBase (www.circbase.org) and Circ2Traits databases (https://gyanxet-beta.com/cricdb/) were used to annotate the identified circRNAs. The differentially expressed circRNAs were identified using EdgeR software (https://bioconductor.org/packages/release/bioc/html/edgeR.html, version3.16.5, Bioconductor) and the data were standardized (Table SI). The expression of any circRNA with a fold change >2.0 and P<0.05 was considered significantly differential. Gene Ontology (GO) (http://www.geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses (https://www.genome.jp/kegg/) were performed on differentially expressed circRNA-associated genes to predict the functions of the circRNAs. Information regarding the biological processes, cellular components and molecular functions of circRNA-targeting genes was obtained via GO analysis and KEGG analysis was conducted to investigate pathways associated with these genes.

Reverse transcription-quantitative (RT-q) PCR. The expression of candidate circRNAs in seminal plasma exosomes and target-genes were analyzed using the $2^{-\Delta\Delta Cq}$ method (32). Briefly, total exosomal RNA was extracted from 20 μl of exosome suspension using TRIzol® (Thermo Fisher Scientific, Inc.). QuantiTect Reverse Transcription Kit (Qiagen, Germany) was used for the reverse transcription according to the manufacturer's instructions. The expression levels of the candidate circRNAs and target genes were validated using qPCR SYBR Green Master Mix (Takara Bio, Inc.) using a Roche LightCycler 480 qPCR System (v 1.5.0.39; Roche Applied Science). The PCR was completed in following steps: 95˚C for 3 min; 95˚C for 10 sec and 60˚C for 1 min (95˚C for 15 sec for 40 cycles). All reactions were performed in triplicate. The results are expressed as the mean ± standard deviation. Statistical analysis was conducted using SPSS 24.0 software (IBM Corp.). The primers used to target circRNAs are listed in Table II and the primers for target genes are listed in Table III. *GAPDH* was used as the internal reference.

CircRNA-miRNA-mRNA interaction analysis. Based on miRanda (http://www.microrna.org) and TargetScan (www.targetscan.org), circRNA-miRNA interactions were predicted to probe and identify the potential functions of the selected seven circRNAs. Detailed annotation of differentially expressed circRNAs was performed. In addition, using StarBase (https://starbase.sysu.edu.cn/) and miRDB (http://mirdb.org/), the regulatory network was further refined based on circRNA-targeting miRNAs. Cytoscape software (https://cytoscape.org/, v 3.0) was used to construct the network based on five miRNAs most likely associated with circRNAs and five genes most likely associated with miRNAs.

Statistical analysis. Data are presented as the mean ± standard deviation. SPSS 24.0 software (IBM Corp.) was used for statistical analysis. Significant differences between two groups were analyzed using a unpaired student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of exosomes from seminal plasma. TEM was performed to characterize the morphology and structure of the seminal plasma exosomes obtained from patients with OAZ. As shown in Fig. 1A and B, the extracted vesicles were

Table II. The primers of the seven candidate circRNAs and GAPDH.

CircRNA	Primer type	Primer sequence (5'-3')
chr3:132050491-132051188+	Forward	GTTTCCCCCAGAAGGTGTC
(novel)	Reverse	TTCATAATGCTGCTCCATGC
chr18:51686135-51731527-	Forward	AATGGACAGTGGAGATGAAGC
(hsa_circ_0005584)	Reverse	AGGTACCTTGCCAACTGAGG
chr3:138289160-138291826-	Forward	CTAAGTAGGGCTTGCCACCA
(hsa_circ_0003823)	Reverse	AGGGGCTACCGGAAACATAG
chr4:170428188-170459062-	Forward	AATGCACGTGCTGCTGTACT
(hsa_circ_0125759)	Reverse	CTGCATAGCTTCCCGTTTTC
chr1:40529899-40530231+	Forward	CCTGGCCCTTATGTGAAAGA
(hsa_circ_0009142)	Reverse	AACTTGCTGCCTCGGTTCT
chr12:130827535-130846146+	Forward	GGTCAATCGCAGGATTTGTT
(novel)	Reverse	ATAATTCCCCCTCTGCTGGT
chr1:31452909-31468067-	Forward	TGTCCCCAATCCATACATCA
(hsa_circ_0002452)	Reverse	ATCACTGTCTGCATCCCTTG
GAPDH	Forward	GGCCTCCAAGGAGTAAGACC
	Reverse	AGGGGAGATTCAGTGTGGTG

circRNA, circular RNA.

Table III. Primers of seven target-genes.

CircRNA	Target-genes	Primer type	Target-genes primer sequence (5'-3')
chr3:132050491-132051188+	*ACPP*	Forward	GCCGTATCCCCTCATGCTAC
(novel)		Reverse	TACACTCCGTGGACCAGTCT
chr18:51686135-51731527-	*SAE1*	Forward	AGATCCCGGAGCTCAGTTCT
(hsa_circ_0005584)		Reverse	CTGGAGCAGCAAGTCAGACA
chr3:138289160-138291826-	*CEP70*	Forward	TCAGCTAGAGCAAAGCCGAG
(hsa_circ_0003823)		Reverse	AATGCTGGCACTTCACCTGT
chr4:170428188-170459062-	*MYO9B*	Forward	CCCTAGAGCACTCCTCACCT
(hsa_circ_0125759)		Reverse	TCTGGAACTTGACGTGCTCC
chr1:40529899-40530231+	*CAP1*	Forward	GAAGTTGGAGCGAGCTCTGT
(hsa_circ_0009142)		Reverse	GCTGACAGCTGACAGGTGAT
chr12:130827535-130846146+	*SEPT1*	Forward	GCCTCTTCCTCACCAACCTC
(novel)		Reverse	AAAGCCAGGTGTGTCCACAA
chr1:31452909-31468067-	*NEK1*	Forward	AGTGACATTTGGGCTCTGGG
(hsa_circ_0002452)		Reverse	GAGACACCAAACTGCGGAGA

circRNA, circular RNA; *ACPP*, acid phosphatase, prostate; *SAE1*, SUMO1 activating enzyme subunit 1; *CEP70*, centrosomal protein 70; *MYO9B*, myosin IXB; *CAP1*, cyclase associated actin cytoskeleton regulatory protein 1; *SEPTIN1*, septin 1; *NEK1*, NIMA related kinase 1.

100-150 nm in diameter and spherical, with a double-layered plasma membrane structure. This was consistent with the expected characteristics of exosomes.

NTA was used to analyze the size and concentration of exosomes isolated from seminal plasma samples. As shown in Fig. 1C and D, the main peak of exosome size in the obtained nanoparticles was located at 175 nm, when the concentration of exosomes was the highest. Therefore, it could be inferred that the particles obtained via ultracentrifugation were exosomes.

Results of the western blotting analysis demonstrated that two groups of exosomes were obtained from nanoparticles. The two exosomal landmark proteins (TSG101 and CD63) and the internal reference for normalization (GAPDH) were expressed in both groups (Fig. 1E).

Characteristics of exosomal circRNAs. A total of 14,991 differentially expressed circRNAs were identified in seminal plasma-derived exosomes via high-throughput sequencing, with 7,635 upregulated and 7,356 downregulated circRNAs. These circRNAs were mainly concentrated on chromosomes 1,

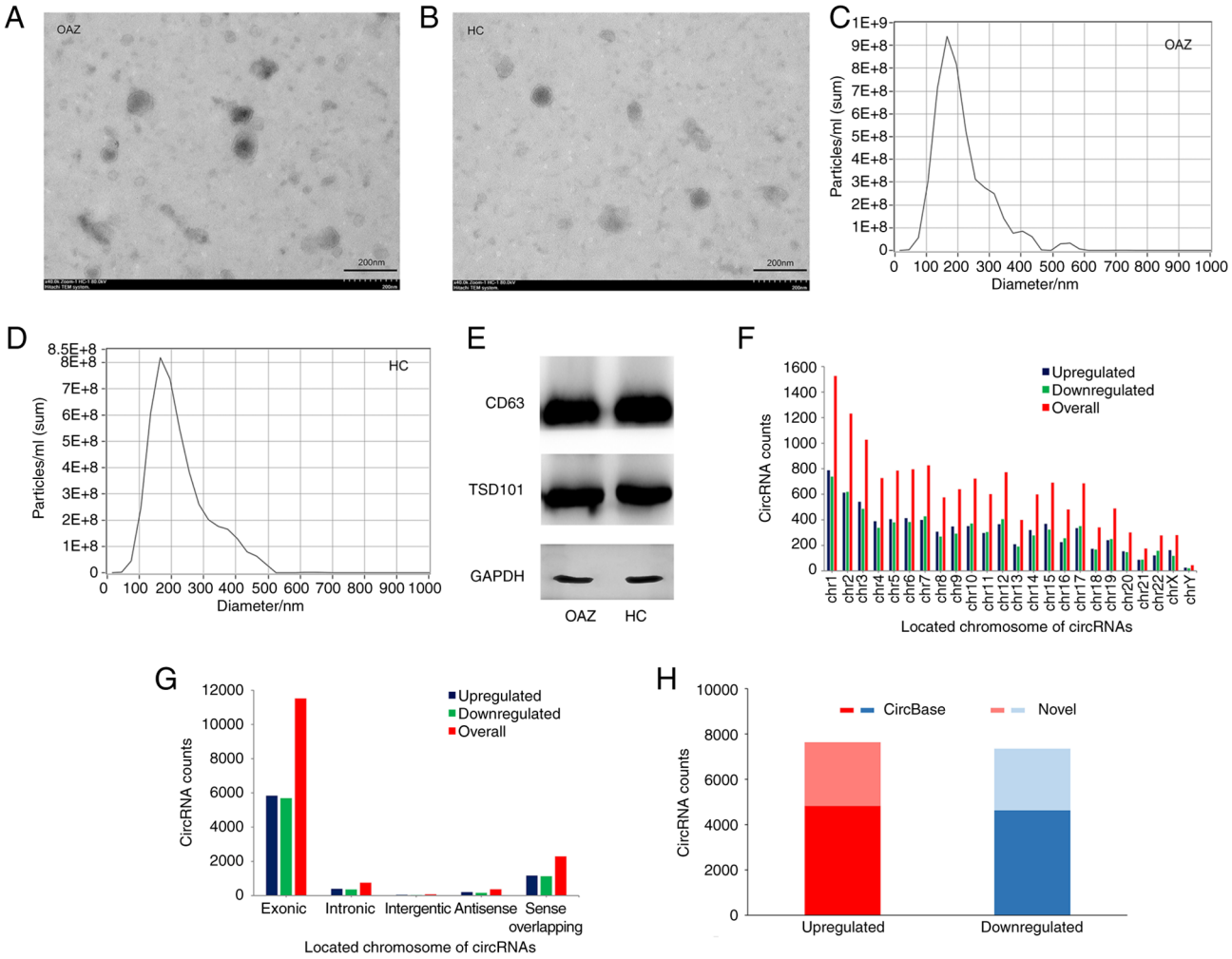

Figure 1. Overview of exosomal circRNAs derived from seminal plasma. Exosomes were characterized as cup-shaped structures with a diameter of 50-150 nm using electron microscopy. Exosomes in the (A) OAZ and (B) HC group. Size distribution of isolated exosomes in the (C) OAZ and (D) HC group was detected using NTA analysis. (E) Markers CD63, TSG101 and GAPDH were detected in exosomes using western blot analysis. (F) Chromosomal distribution of exosomal circRNAs. (G) Distribution of different types of exosomal circRNAs. (H) The proportion of circBase and novel circRNAs (14,991 circRNAs identified). circRNA, circular RNA; OAZ, oligoasthenospermia; HC, healthy control; NTA, nanoparticle tracking analysis; TSG, tumor susceptibility gene.

2 and 3 (Fig. 1F) and 80% of them were exons (Fig. 1G). These results suggested that exon-derived circRNAs and chromosomes 1, 2 and 3 are closely associated with the pathogenesis of OAZ. As novel circRNAs, 2,811 upregulated and 2,731 downregulated were verified. Meanwhile, in the circRNA database, 4,824 upregulated and 4,625 downregulated were identified (Fig. 1H).

Distribution of circRNAs differential expression. Differential expression of exosomal circRNAs was analyzed using edgeR software. A model was based on negative binomial distribution, with a corrected P<0.05 as the screening threshold for significant differences. Volcano plots, scatter plots and dendrograms were drawn to visualize the distribution of differential expression.

The expression of all differentially expressed circRNAs was analyzed via hierarchical clustering. A heat map was generated to obtain an overview of differential expression and to distinguish the differential expression of exosomal circRNAs derived from seminal plasma between the two groups. The distribution of circRNAs in healthy controls and patients with OAZ was almost identical after normalization. The different shades indicate the level of circRNA expression, with red indicating upregulated gene expression and green indicating downregulated gene expression (Fig. 2A).

A volcano plot was drawn to visualize the overall distribution of differential expression of exosomal circRNAs. The differential ploidy and corrected P-values on the same graph facilitated the screening of differentially expressed genes. The differentially expressed circRNAs were distributed on both sides of the dotted line, with red dots on the left side representing downregulated circRNAs and red dots on the right side representing upregulated circRNAs. According to the volcano plot, the number and fold difference of upregulated circRNAs were comparable to those of the downregulated circRNAs; however, P-value distribution was not significantly different between the healthy controls and patients with OAZ (Fig. 2B).

In the scatter plot, the values represented the average normalized signal values (in logarithmic terms) for each group of samples. CircRNAs above and below the ploidy change line indicated a fold change >2.0. CircRNAs were considered significantly differentially expressed if they were upregulated or downregulated at least 2-fold (Fig. 2C).

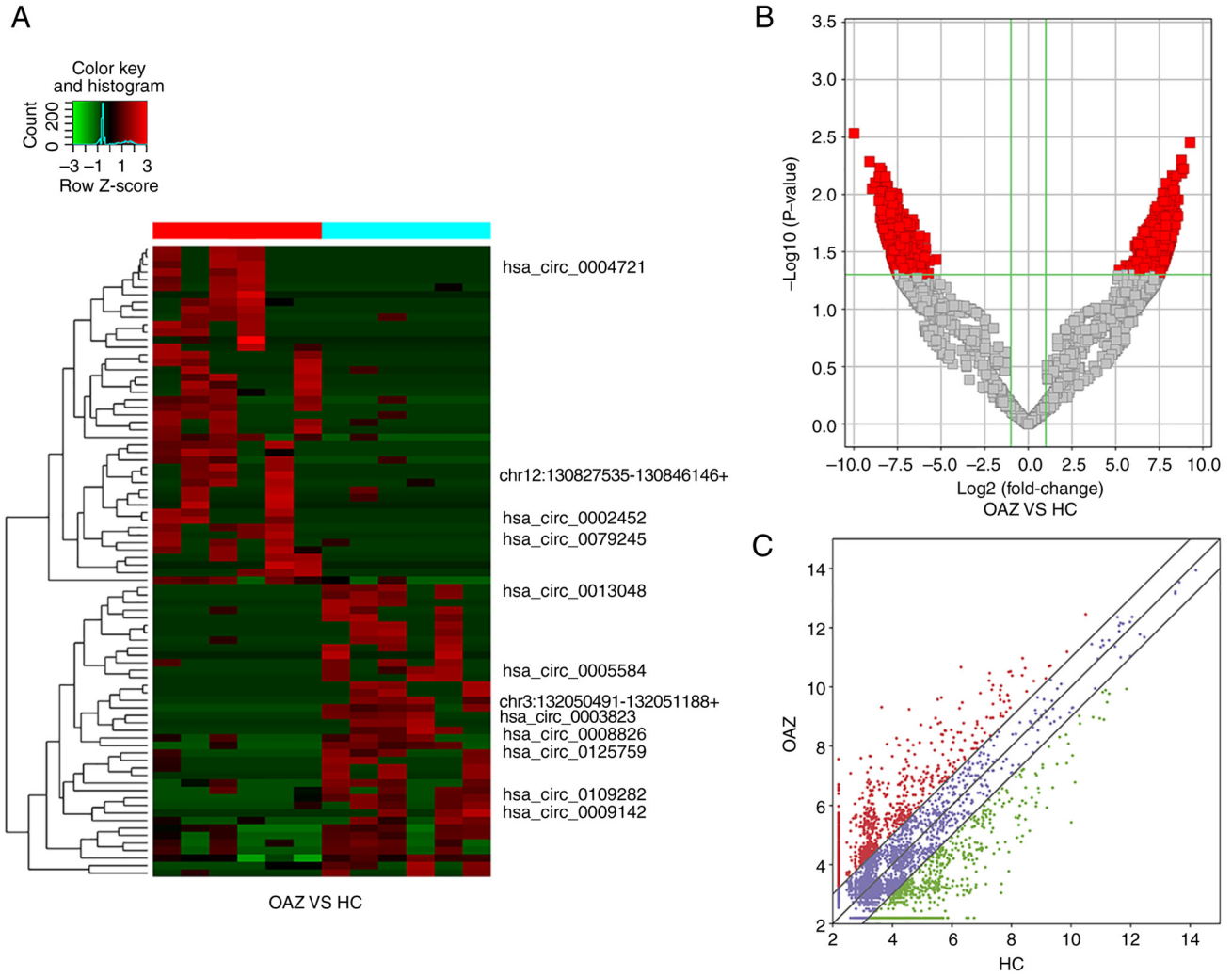

Figure 2. Hierarchical clustering analysis. (A) Expression of total exosomal circRNAs was detected in the healthy control and OAZ groups, the expression levels are indicated using different colors. Hierarchical clustering analysis was performed using twelve samples across two groups. (B) Volcano plots demonstrated the differential expression of exosomal circRNAs in the seminal plasma of OAZ and healthy controls using fold-change and P-values. X-axis, log2 (fold change); Y-axis, -log10 (P-values); red points indicate differentially expressed circRNAs with statistical significance. (C) Scatter plots exhibited differential expression of exosomal circRNAs in the OAZ and healthy control groups. The distribution of clots demonstrated a fold-change >2.0. circRNA, circular RNA; OAZ, oligoasthenospermia; HC, healthy control.

GO and KEGG pathway analysis. Bioinformatics analysis, including GO functional enrichment and KEGG analyses were performed. The associated functions of differentially expressed exosomal circRNAs in seminal plasma obtained from patients with OAZ were analyzed. Results of the GO analysis were presented as scatter plots. These circRNAs were mainly enriched in organelle organization and metabolic processes in the biological process enrichment analysis, including 'cellular metabolism', 'cellular component organization', 'cellular macromolecule metabolism', 'single-organism organelle organization', 'organic substance metabolism' and 'nucleobase-containing compound metabolism' (Fig. 3A and D). The cellular component enrichment analysis revealed that ~90% of the exosomal circRNAs were enriched in intracellular and membrane-bound organelles in healthy controls and patients with OAZ (Fig. 3B and E).

These results were consistent with the properties of exosomes, as they are mainly released by cells through the fusion of intracellular vesicles with the cell membrane, or secreted by cells through parietal secretion (9,13). Consequently, the main components of exosomal circRNAs originate from the cell membrane and cytoplasm, with few components originating from the nucleus. Molecular functional enrichment analysis demonstrated that the majority of circRNAs served as binding functional proteins, such as ATP binding, adenyl nucleotide binding, adenyl ribonucleotide binding and poly(A) RNA binding (Fig. 3C and F). These functions play an essential role in sperm motility and capacitation and also exhibit numerous enzyme-related activities, indicating that the identified differentially expressed exosomal circRNAs are physiologically rich in functions and are synergistically involved in the regulation of spermatogenesis.

RT-qPCR validation. RT-qPCR results obtained during the present study were consistent with results of the RNA-seq analysis. RT-qPCR validation results of exosomal circRNAs obtained from 20 pairs of plasma samples with significant differences between the OAZ and healthy control were presented in Fig. 4A. The expression of seven target-genes

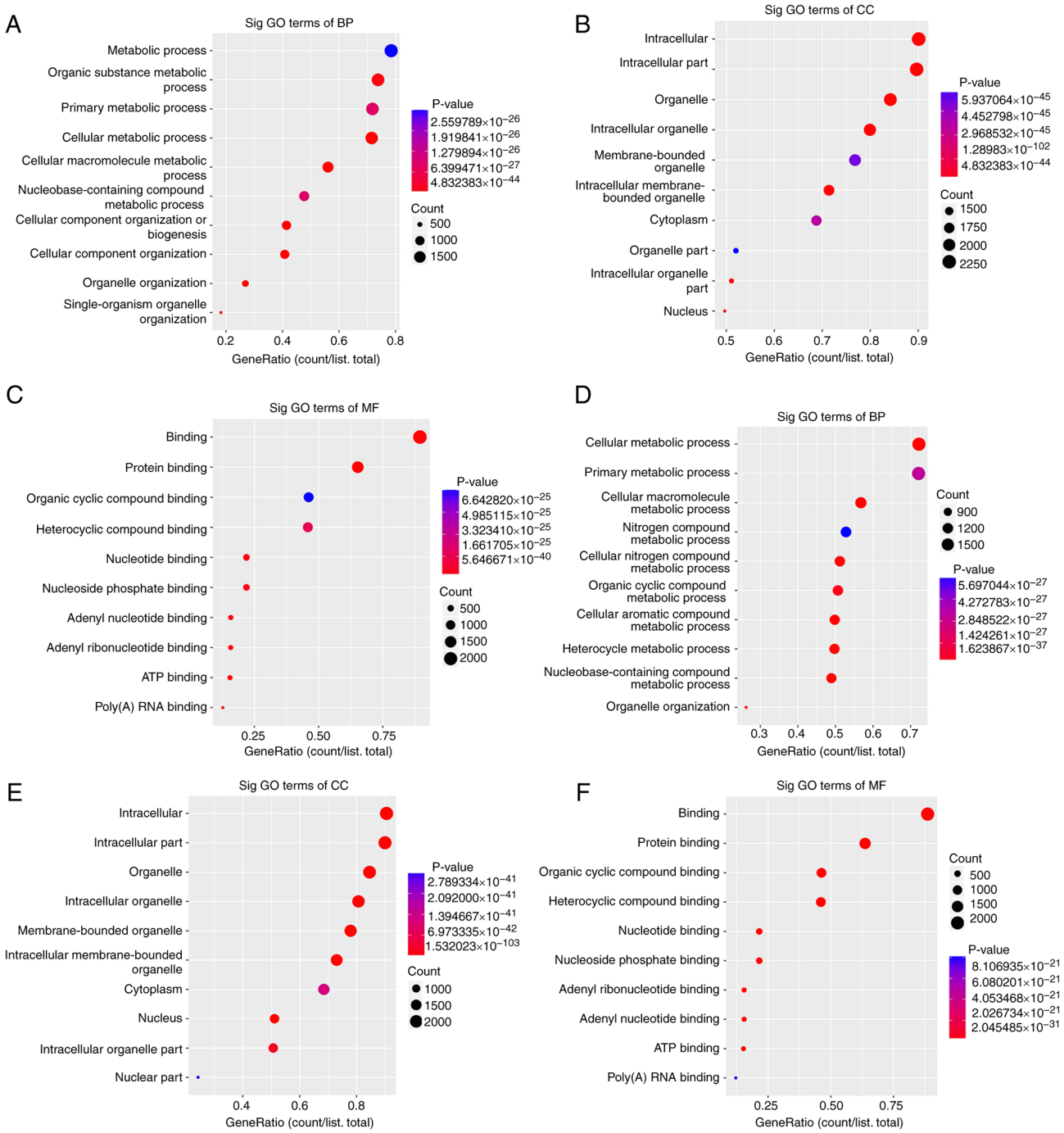

Figure 3. GO analysis results of differentially expressed circRNAs. Downregulated circRNAs in (A and D) biological process, (B and E) cellular component and (C and F) molecular function. GO, gene ontology; circRNA, circular RNA; BP, biological process; CC, cellular component; MF, molecular function.

were presented in Fig. 4B. All of them had the significantly differential expression between OAZ and healthy control groups.

KEGG pathway analysis contributed to further understanding the exosomal circRNA biofunctions and revealed the major signal transduction and metabolic pathways associated with OAZ. The top 10 pathways associated with exosomal circRNAs were 'ubiquitin-mediated proteolysis', 'endocytosis', 'RNA transport', 'protein processing in the endoplasmic reticulum system', 'cell cycle', 'circadian rhythm', 'lysine degradation', 'inositol phosphate metabolism' and 'thyroid hormone signaling pathway' (Fig. 5A). The majority of these pathways are closely associated with the pathophysiological process of OAZ. Phosphatidylinositol signaling in the testis is important for the formation of normal sperm plasma membrane structures, affecting healthy fertilization (33). Results of a previous study demonstrate the differential expression of lysine acetyltransferase and lysine deacetylase in healthy spermatozoa and sperm with poor motility (34). Therefore, exosomal circRNAs may also participate in the aforementioned functional pathways.

CircRNA-miRNA-mRNA interaction analysis. CircRNAs possess multiple miRNA binding sites and primarily regulate gene expression by serving as miRNA

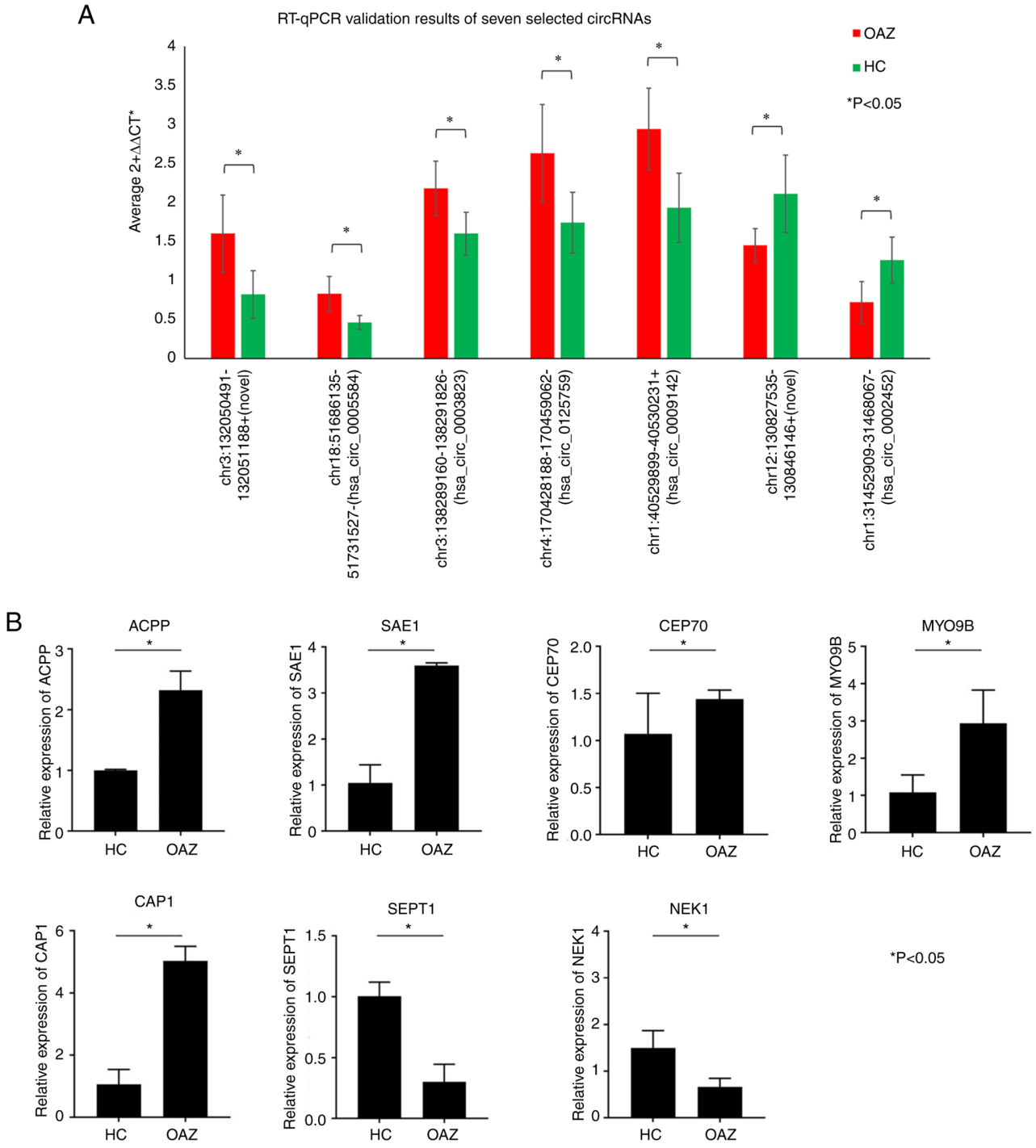

Figure 4. RT-qPCR results. (A) RT-qPCR validation results of exosomal circRNAs between the OAZ and HC (*P<0.05). (B) Expression of seven target-genes. Seven target-genes of these circRNAs were selected, and the differential expression was significant between OAZ and HC (*P<0.05). OAZ, oligoasthenospermia; HC, healthy control.

sponges. These competitively bind to miRNAs and suppress miRNA regulation of target genes, thereby indirectly regulating gene expression (35). Based on results obtained using TargetScan and miRanda, the association between circRNAs and miRNAs were predicted to investigate the underlying features of the selected seven circRNAs. The top five miRNAs with the highest correlation with the validated circRNAs were identified using the miRanda software and are listed in Table IV. Based on the association between miRNAs and circRNAs and between miRNAs and mRNAs, a circRNA-miRNA-mRNA network was constructed using Cytoscape (Fig. 5B).

Discussion

The present study provided a preliminary foundation for identifying the potential diagnostic value of critical exosomal circRNAs derived from seminal exosomes involved in OAZ. Thus, the corresponding expression profiles and differentially expressed circRNAs must be determined in the seminal plasma

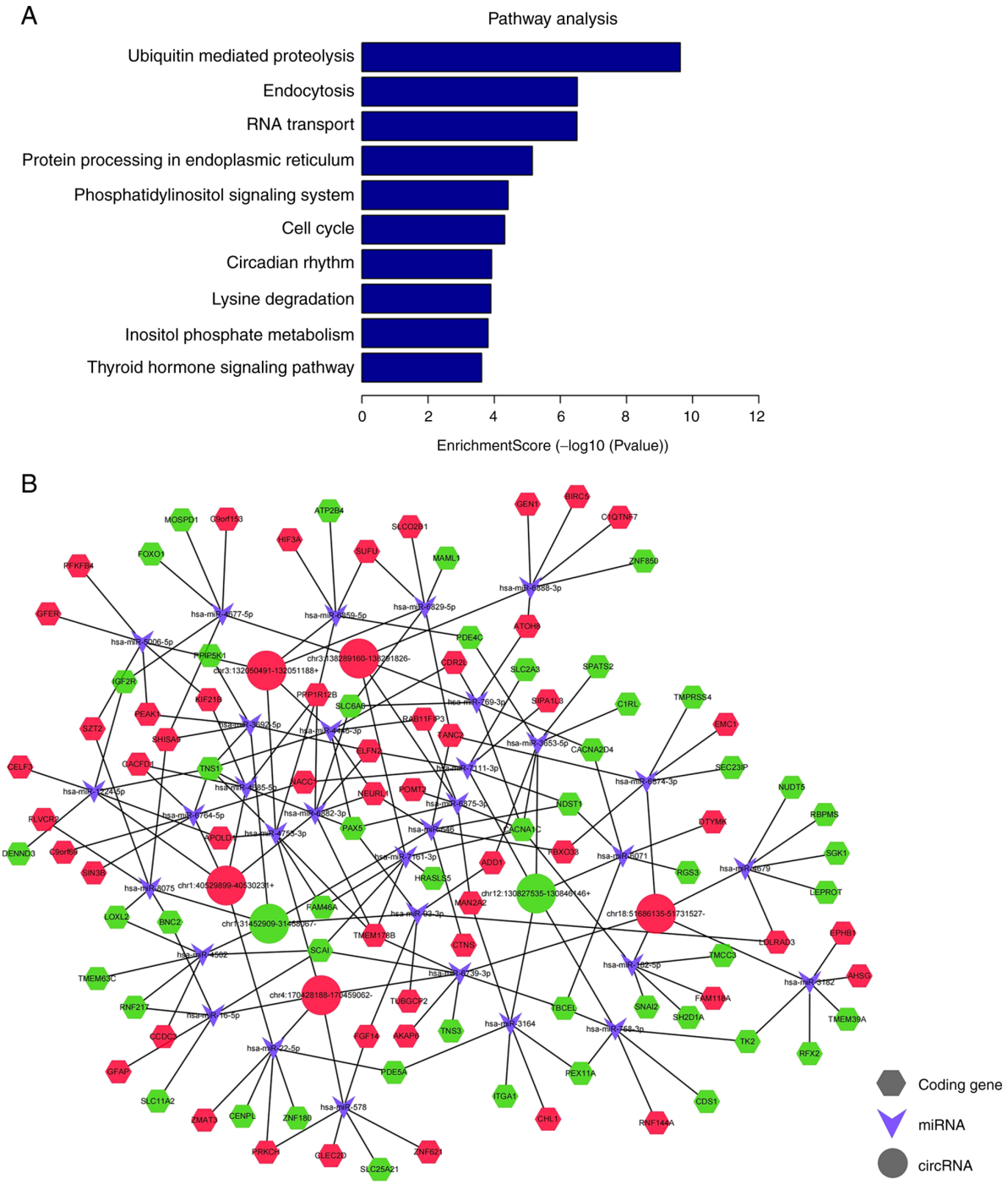

Figure 5. KEGG pathway analysis of exosomal circRNAs and a network diagram of circRNA-miRNA-mRNA interactions based on the differential expression of seven predicted circRNAs. (A) The top 10 KEGG signaling pathway of exosomal circRNAs obtained from the seminal plasma of patients with OAZ. (B) Red represents upregulated circRNAs, green represents downregulated circRNAs, lines represent interactions; arrows represent miRNA; red hexagons represented upregulated mRNA, green hexagons represented downregulated mRNA. KEGG, Kyoto Encyclopedia of Genes and Genomes; circRNA, circular RNA; miRNA, microRNA; OAZ, oligoasthenospermia.

of patients with OAZ. The expression profiles of circRNAs in human seminal plasma-derived exosomes were analyzed and circRNAs that were associated with OAZ were screened. These circRNAs may serve as potential markers for the prognostic evaluation of patients with OAZ. In addition, results obtained from GO and KEGG analyses indicated that circRNAs may participate in sperm motility and spermatogenesis. However, these circRNAs may not be available for clinical application and their effects are indeterminate. They may also be associated with tumors (36-38) and no clinical data is available at present. In addition, the role of circRNAs as therapeutic targets for the treatment of diseases remains to be fully elucidated (35). Previous studies have demonstrated that circRNAs may be used as potential biomarkers for different diseases (39,40), including cell-free saliva containing >400 circRNAs, that may be used for non-invasive diagnosis (41).

The mRNA-miRNA-circRNA axis affects disease development by inhibiting or enhancing signaling pathways (42). In

Table IV. Bioinformatics analysis showed the top five miRNA most closely related to each circRNA.

circRNA	miRNA(1)	miRNA(2)	miRNA(3)	miRNA(4)	miRNA(5)
chr3:132050491-132051188+	hsa-miR-6829-5p	hsa-miR-5006-5p	hsa-miR-4446-3p	hsa-miR-4753-3p	hsa-miR-6859-5p
chr18:51686135-51731527-	hsa-miR-6739-3p	hsa-miR-6874-3p	hsa-miR-4679	hsa-miR-5182	hsa-miR-182-5p
chr3:138289160-138291826-	hsa-miR-4677-5p	hsa-miR-6888-3p	hsa-miR-769-3p	hsa-miR-6875-3p	hsa-miR-7161-3p
chr4:170428188-170459062-	hsa-miR-578	hsa-miR-22-5p	hsa-miR-16-5p	hsa-miR-4753-3p	hsa-miR-6739-3p
chr1:40529899-40530231+	hsa-miR-22-5p	hsa-miR-1224-5p	hsa-miR-6764-5p	hsa-miR-4685-5p	hsa-miR-6882-3p
chr12:130827535-130846146+	hsa-miR-7111-3p	hsa-miR-3653-5p	hsa-miR-6071	hsa-miR-758-3p	hsa-miR-3164
chr1:31452909-31468067-	hsa-miR-8075	hsa-miR-3692-5p	hsa-miR-646	hsa-miR-93-3p	hsa-miR-4502

circRNA, circular RNA; miRNA, microRNA.

the present study, an mRNA-miRNA-circRNA network was predicted based on the seven identified circRNAs and the presence of this network exerted a negative feedback effect on the regulation of gene expression. In turn, this may have constituted a post-transcriptional level of regulation, furthering the understanding of the mode of gene expression regulation. The present study also revealed a distinct expression profile of exosomal circRNAs in seminal plasma, suggesting their potentially significant feature in the diagnosis and prognosis of OAZ, particularly as the cyclic structure remains stable in bodily fluids. This stability is attributed to the availability of exosomes as carriers that protect circRNAs from degradation by RNA enzymes. In addition, due to the amount of exosomal circRNAs that are constantly altered during spermatogenesis, results of the present study provide an accurate reflection of the pathological changes in the reproductive system (43,44). Therefore, circRNAs are considered highly potential disease biomarkers.

Exosomes are a novel delivery medium that carry numerous specific messages long distances for the transmission of information *in vivo* (10). They promote a communication method that mediates paracrine and endocrine secretions, including the packaging and transfer of various functional elements, such as circRNAs (45). Only exosomes can be continuously released by cells, as all other forms of extracellular vesicles are solely released by activated or apoptotic cells. Exosomes derived from seminal plasma, particularly in the acquisition of sperm motility and maturation of spermatozoa, may play a critical role (46). Although the regulatory functions of circRNAs and exosomes are not independent of each other, the secretory execution of circRNAs is more dependent on exosomes as they are adequate biological carriers and prolong the half-life of circRNA functions (47). In addition, the function of non-coding RNA biomarkers is often dependent on exosomes for detection in seminal plasma. To date, the identified species and functions of circRNAs represent only a minority of total circRNAs and numerous circRNAs are yet to be identified. Based on the combination of molecular biology, bioinformatics and different detection algorithms, highly accurate circRNAs may be identified by searching circRNA databases, such as RNABase (http://www.rnabase.org), deepBase (http://biocenter.sysu.edu.cn/deepBase/index), circBase and Circ2Traits. As a class of RNAs with regulatory functions, circRNAs may serve an important role in the study of male reproduction (17).

Notably, the sperm count was significantly different between healthy control and OAZ patients. However, there was no significant difference in the amount of seminal plasma exosomes between two groups. Exosomes derived from OAZ and healthy control displayed similar shape, size and electron density according to the electron microscopy. NTA confirmed that the exosomes displayed very similar size profiles among the two populations. Human semen contains a large number of extracellular microvesicles (EVs) containing exosomes, which are secreted from male reproductive system glands such as epididymis epithelium cell, prostate epithelium and other accessory glands. The EVs produced by the prostate gland are generally called prostasomes and produced by the epididymis are commonly referred to as epididymosomes (48). They are considered as the main source of exosomes and participated in the transport of nucleic acids, proteins and

lipids and act as a signal transduction carrier for abnormal cellular pathology (15,49). In the present study, as the criteria, the patients with genitourinary inflammation and prostate problems were excluded. OAZ had no significant effect on the number of seminal plasma exosomes. Similar reports could also be found in other studies (8,14).

Regarding future perspectives, the specific mechanisms underlying exosomal secretion and the partitioning of circRNAs requires further and continuous investigation. The delivery of exosomal circRNAs may facilitate the correspondence and exchange of intercellular hereditary material and markedly affect the biological behavior of spermatogenesis. Therefore, an in-depth investigation into the delivery of exosomal circRNAs is required to understand their role in OAZ. Regarding clinical applications, exosomes exhibit an advantage in acting as nanoscale vesicles that protect the endogenous 'cargo'-circRNA from degradation. Therefore, further investigations into the use of exosomal circRNAs as biomarkers and effective drug carriers are required for the development of targeted treatment options for male reproduction.

The present study had some limitations. Due to limited semen from patients, the data need to be enlarged. Although the prognostic value of the seven circRNAs was determined, the concrete molecular mechanism was not clarified by biological experiments. Further validation of the biological function and mechanism of these circRNAs miRNAs and genes need to be performed. The mimics of downregulated selected circRNAs and inhibitors of upregulated selected circRNAs need to be treated in OAZ cell lines or OAZ animal models to validate their functions as a potential direction in our future studies to assess whether they can serve as novel biomarkers or therapeutic targets.

In conclusion, the present study demonstrated that abundant critical exosomal circRNAs are involved in testicular development or spermatogenesis. The present study provides a theoretical basis for the development of highly sensitive and specific diagnostic markers and gene-targeted therapies for infertility.

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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.

Authors' contributions

RuifY designed the experiments and finished the data analysis. DY and RuipY performed the experiments. DY, RuifY and CX acquired and managed clinic information. DY, RuipY and CX contributed to collecting tissue specimens and writing the manuscript. CX and RuifY reviewed and revised the manuscript. All authors read and approved the final manuscript. All the authors confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the bioethics committees of the Second Hospital of Shandong University (approval number: KYLL-2021KJA-0245). Written informed consent was provided to the participating patients and healthy individuals. The experimental protocol was established according to the World Medical Association Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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