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ORIGINAL ARTICLE

Male Infertility

Identification and characterization of circular RNAs in the testicular tissue of patients with non-obstructive azoospermia

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Circular RNAs (circRNAs) are highly conserved and ubiquitously expressed noncoding RNAs that participate in multiple reproduction-related diseases. However, the expression pattern and potential functions of circRNAs in the testes of patients with non-obstructive azoospermia (NOA) remain elusive. In this study, according to a circRNA array, a total of 37 881 circRNAs were identified that were differentially expressed in the testes of NOA patients compared with normal controls, including 19 874 upregulated circRNAs and 18 007 downregulated circRNAs. Using quantitative real-time polymerase chain reaction (qRT-PCR) analysis, we confirmed that the change tendency of some specific circRNAs, including hsa_circ_0137890, hsa_circ_0136298, and hsa_circ_0007273, was consistent with the microarray data in another larger sample. The structures and characteristics of these circRNAs were confirmed by Sanger sequencing, and fluorescence *in situ* hybridization revealed that these circRNAs were primarily expressed in the cytoplasm. Bioinformatics analysis was used to construct the competing endogenous RNA (ceRNA) network, and numerous miRNAs that could be paired with circRNAs validated in this study were reported to be vital for spermatogenesis regulation. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses indicated that genes involved in axoneme assembly, microtubule-based processes, and cell proliferation were significantly enriched. Our data suggest that there are aberrantly expressed circRNA profiles in patients with NOA and that these circRNAs may help identify key diagnostic and therapeutic molecular biomarkers for NOA patients.

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INTRODUCTION

Infertility has become a serious public health problem that affects 10%–15% of reproductive-age couples worldwide. Approximately half of these cases are due to male factors. Male infertility is a complex pathological syndrome with a highly heterogeneous phenotype, which is often associated with spermatogenic failure.[1] Non-obstructive azoospermia (NOA) is the most severe pathological type of male infertility and is mainly attributed to spermatogenic impairment and sperm production deficiency in the testes.[2]

Previous studies have shown that most NOA may have a genetic background associated with epigenetic and environmental contributions.[3,4] Genetic disorders, including chromosomal abnormalities and gene mutations, have been shown to account for 15%–30% of male infertility cases. In addition to Klinefelter syndrome and Y chromosome microdeletions, mutations in specific genes, such as synaptonemal complex protein 3 (*SYCP3*), heat shock factor 2 (*HSF2*), spermatogenesis and oogenesis-specific basic helix-loop-helix 1 (*SOHLH1*), and testis-expressed 11 gene (*TEX11*), have been detected in men with infertility.[5–8] However, the etiology of most men with NOA remains unknown and has been defined as "idiopathic infertility".[9] It is widely believed that a considerable number of these unexplained cases may involve genetic defects.

In recent years, epigenetic regulators, especially noncoding RNAs (ncRNAs), have been found to be essential for maintaining spermatogenesis.[10] Gou *et al.*[11] reported that human *Piwi* (*Hiwi*), a small ncRNA, plays an important role in regulating histone-to-protamine transition during spermatogenesis; germline mutations of Hiwi are found in men with azoospermia, which prevent its ubiquitination and degradation. There is a dynamic change in long noncoding RNA (lncRNA) expression throughout the progression of spermatogenesis.[12] Knockout of the X-linked lncRNA *Tslrn1* in mice does not affect normal fertility but significantly decreases spermatozoa production. Deletion of *Dicer1*, an RNase III enzyme required for microRNA (miRNA) biogenesis, can lead to disrupted germ cell development and spermatogenic failure.[13] Moreover, it has been reported that many miRNAs, such as miRNA-21 (miR-21) and miR-638, are vital for germ cell differentiation and Sertoli cell maturation.[14,15]

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Circular RNAs (circRNAs) are a novel subclass of endogenous ncRNA molecules that are formed through back-splicing and joining at splice sites. This alternative splicing mechanism generates a covalently linked continuous loop.[16] Emerging studies have reported that circRNAs function as miRNA "sponges" and thus are involved in gene regulation at the posttranscriptional level.[8] In addition to binding miRNAs, circRNAs can also regulate the transcription of their parent genes, serve as mRNA traps through competition with linear splicing, or directly translate to peptides.[17] circRNAs have been shown to be ubiquitously expressed in multicellular organisms, highly conserved among species, and more stable than linear RNAs.[18] Previous studies have shown that circRNAs are crucial regulators of various biological processes, including proliferation, differentiation, migration, and apoptosis.[19] Disrupted circRNA regulation is involved in the pathogenesis of tumorigenesis, cardiovascular and metabolic diseases, and reproduction-related disorders such as polycystic ovary syndrome.[20–22] Global analysis of circRNA profiles can contribute to studies on the pathogenesis of various complex diseases and may identify promising diagnostic biomarkers and therapeutic targets.[23–25]

However, studies on the expression profiles and potential functions of circRNAs in the testes of patients with NOA remain scant. The aim of the present study was to identify and characterize circRNA profiles in the testes of NOA patients and investigate their potential role in spermatogenesis and male infertility. For this purpose, we determined the differentially expressed circRNAs in the testes of NOA patients compared with normal controls using microarray analysis and explored the potential functions of several differentially expressed circRNAs by analyzing their putative miRNA targets.

PATIENTS AND METHODS

Azoospermic patients and testicular biopsies

The study was approved by the Ethics Committee of Peking University Third Hospital (Beijing, China; approval No. 2017SZ-035), and written informed consent was obtained from each participant. We obtained 61 testicular biopsy specimens from patients with NOA (37 cases with spermatogenic arrest and 24 cases with Sertoli cell-only syndrome [SCOS]) who underwent diagnostic testicular biopsy and were aged 28 years to 45 years. These patients were found to have no sperm in at least 3 separate semen analyses, including high-speed centrifugation of the entire pellet, and no obstructive causes. Diagnostic testicular biopsy was recommended to further evaluate the spermatogenesis status of the testis before microscopic testicular sperm extraction. Patients with chromosomal abnormalities were excluded, Y chromosome microdeletions, orchitis, epididymitis, varicocele, testicular torsion, and antisperm antibodies. The control group specimens were obtained from 27 patients with obstructive azoospermia who underwent vasoepididymostomy treatment and were proven to have normal spermatogenesis by pathological examination. The testicular samples were snap-frozen in liquid nitrogen and then stored at −80°C.

RNA extraction

Total RNA, including circRNAs, was isolated from testicular biopsies using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and purified with the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's specifications. RNA concentration and purity were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and RNA integrity was determined by capillary electrophoresis using the RNA 6000 Nano Lab-on-a-Chip kit and the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Only testicular samples with an RNA integrity value >6 were used for further analysis.

Microarray analysis of circRNA expression profiling

The circRNA expression profiles of 20 testicular biopsy samples (14 NOA samples and six normal controls) were obtained using the CapitalBiotech human Transcriptome Array (CapitalBio Technology Inc., Beijing, China). Briefly, extracted total RNA was reverse transcribed to double-stranded complementary DNA (cDNA) and then synthesized into cRNA, which was purified and reverse transcribed to cDNA and finally labeled with a fluorescent dye (Cy3-dCTP). The labeled cDNAs were hybridized on the CapitalBiotech human Transcriptome Array, which was designed with four identical arrays per slide (4 × 180k format) with each array targeting 170 340 human circRNA probes. The Transcriptome Array also contains 4974 Agilent control probes.

Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) biological pathway analyses

The parental gene functions of the differentially expressed circRNAs were submitted to the DAVID database (http://david.abcc.ncifcrf.gov) and analyzed using GO and KEGG annotation pathway. The numbers of targeted genes in a biological pathway and the *P* value are presented as the enrichment correlation significance.

Primer design

Divergent primers were designed for each candidate circRNA to anneal at the distal ends of its sequence. Divergent primers for the *β-actin* linear transcript were used as the negative control. As a further negative control for the divergent primers, we used genomic DNA extracted using the Qiagen DNeasy Blood and Tissue kit (Qiagen, Duesseldorf, Germany). As a positive control, we used convergent primers for the corresponding linear transcripts. The primers used in this study are listed in **Supplementary Table 1**.

Polymerase chain reaction (PCR) amplification and Sanger sequencing

Total RNA extracted from normal testicular biopsy samples was treated with RNase R and then reverse transcribed to double-stranded cDNA. The cDNA templates were PCR amplified with the corresponding divergent primers using the TransStart FastPfu DNA Polymerse kit (TransGen Biotech, Beijing, China) according to the manufacturer's protocol. PCR products were visualized after electrophoresis on a 2% ethidium bromide-stained agarose gel. To confirm the PCR results, the PCR products were purified using the Wizard SV Gel (Biodee, Beijing, China) and PCR Clean-Up System kit (Beyotime, Shanghai, China) and subsequently subjected to Sanger sequencing.

RNA isolation, cDNA synthesis, and quantitative real-time PCR (qRT-PCR)

Total RNA extraction, cDNA synthesis, and qRT-PCR were performed as previously described in 21 normal controls and 47 NOA samples.[26] cDNA synthesis was conducted by the following protocol with an abm kit (G490, abm, Zhenjiang, China). (1) Thaw RNA templates and the 5X All-In-One RT MasterMix on ice, and mix solutions gently. (2) Prepare a 10 μl mixture (total RNA, mRNA, 5X All-In-One RT MasterMix and nuclease-free H_2O) in a PCR tube on ice. (3) Mix the components well and collect by brief centrifugation, and incubate the tube at 25°C for 10 min. (4) Perform cDNA synthesis by incubating the tube for 15 min at 42°C. (5) Stop the reaction by heating it at 85°C for 5 min followed by chilling on ice. For RNase R (Lucigen, Middleton, WI, USA) treatment, 1 μg of total RNA was incubated for 30 min at 37°C with 4 U μg^{-1} RNase

R, and enzyme activity was killed at 65°C for 20 min. The expression level of the circRNAs was calculated using the $2^{-\Delta\Delta CT}$ method. *β-actin* was used as the internal control for normalization of the data.

Fluorescence in situ hybridization (FISH)

The expression and location of candidate circRNAs in testicular tissue were examined by FISH. Cy3-labeled probes for hsa_circ_0137890, hsa_circ_0136298, and hsa_circ_0007273, which were designed and synthesized by GenePharma (Shanghai, China), were applied for RNA FISH. The oligonucleotide sequences are provided in **Supplementary Table 2**. The FISH assay was conducted with an RNA FISH kit (Paraffin Section; GenePharma) according to the manufacturer's instructions. To determine the expression and location of candidate circRNAs in normal testicular tissue, 4-μm-thick sections were cut from paraffin-embedded blocks, processed, hybridized, and analyzed. Briefly, paraffin sections of testicular tissue samples were deparaffinized with 100% xylene and rehydrated with a graded ethanol series. The sections were treated with proteinase K and then denatured. The sections were then incubated with a prehybridization solution at 37°C for 30 min, and the probes were added to the slides and hybridized overnight. Next, the slides were washed with phosphate-buffered saline, incubated with 4′,6-diamidino-2-phenylindole (DAPI; Harveybio, Beijing, China) to stain the cell nuclei, and then analyzed using a Nikon inverted fluorescence microscope (Nikon, Tokyo, Japan).

Construction of the circRNA-miRNA network

circRNAs can serve as miRNA "sponges" by binding miRNA response elements (MREs) and protecting target mRNAs from miRNA-mediated cleavage. The circRNA-miRNA interactions were predicted based on miRanda-3.3 software (http://www.bioinformatics.com.cn/), which is an algorithm used to identify potential genomic targets for miRNA. The miRanda scanning algorithm was performed as previously described, with a defined threshold of a match score >160.[27] These circRNA-miRNA pairs were then used to construct the network using the open-source bioinformatics software Cytoscape (https://cytoscape.org/).

Statistical analyses

All statistical analyses were performed with SPSS 19.0 (SPSS Inc., Cambridge, MA, USA). All data are presented as the mean ± standard deviation (s.d.). The differences between the NOA group and normal control group were analyzed using Student's *t*-test, and $P < 0.05$ was considered statistically significant. The raw microarray data were analyzed for data summarization, normalization, and quality control using GeneSpring software V13.0 (Agilent Technologies, Santa Clara, CA, USA). The significantly up- and downregulated circRNAs were determined with the threshold set at $P < 0.05$ (Student's *t*-test) and a fold change ≥2. Cluster analysis was performed with hierarchical and average linkage to display the differential circRNA expression profiles among the samples. A volcano plot was drawn according to the *P* value and log2 (fold change).

RESULTS

Characteristics of study subjects

We analyzed a total of 88 testicular biopsies from NOA patients and normal controls. The demographics and characteristics of the subjects are listed in **Table 1**. There were no significant differences in age or serum testosterone between the azoospermic group and the normal control group. However, increased serum FSH and LH levels and decreased testicular volume were observed in azoospermic men compared with the controls.

Differentially expressed circRNA profiles in the testes of NOA patients

We first analyzed the global profile of circRNAs in testis samples from 14 patients with NOA and six normal testicular samples by circRNA microarray. Volcano plots showed that circRNA expression visibly differed between the NOA and control groups (**Figure 1a**). Based on microarray analysis, 19 874 circRNAs were statistically significantly upregulated with a set filter of fold change >2.0 ($P < 0.05$), and 18 007 circRNAs were downregulated. The differentially expressed circRNA profiles are shown in a hierarchical heatmap (**Figure 1b**). The top 20 up- or downregulated circRNAs in testicular samples from NOA patients compared with those from normal controls are shown in **Supplementary Table 3**. Based on parental gene location, although the differentially expressed circRNAs were widely distributed on all chromosomes, chromosomes 1, 2, and 15 produced most of the differentially expressed circRNAs (**Supplementary Figure 1**).

Validation of candidate circRNAs

To validate the microarray analysis results and explore the characteristics of circRNAs in spermatogenesis, we choose three dysregulated conserved circRNAs at random. The expression of hsa_circ_0137890, hsa_circ_0136298, and hsa_circ_0007273 was analyzed using a larger group of testicular samples collected from 61 NOA patients and 27 normal controls. The structures and characteristics of

Table 1: Clinical characteristics of non-obstructive azoospermia and normal control groups

Characteristic	*NOA (n=61)*	*NC (n=27)*	*P*
Age (year)	29.8±3.3	31.9±6.6	NS
Testicular volume (ml)	9.2±2.3	15.4±4.1	<0.01
FSH (mIU ml^{-1})	15.9±6.9	5.8±2.9	<0.01
LH (mIU ml^{-1})	6.7±3.9	3.9±1.3	<0.01
T (nmol l^{-1})	9.7±4.4	11.6±4.0	NS

All data are presented as the mean±s.d. A $P<0.05$ was considered statistically significant. NS: not significant; FSH: follicle-stimulating hormone; LH: luteinizing hormone; T: testosterone; NOA: non-obstructive azoospermia; NC: normal control; s.d.: standard deviation

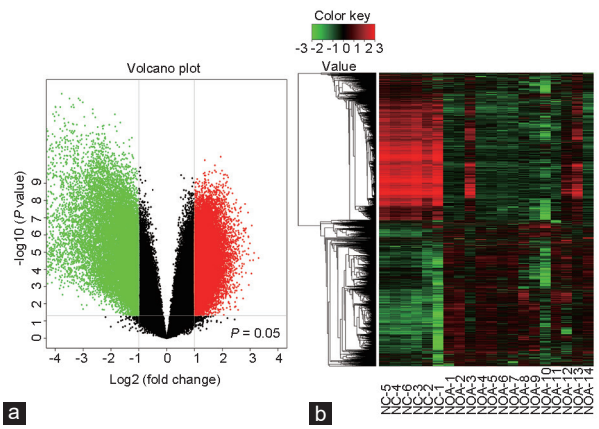

Figure 1: Identification of differentially expressed circRNAs in testicular tissues of patients with NOA compared with NC. (**a**) Volcano plot visualizing the differentially expressed circRNAs. The vertical lines indicate two fold upregulation and downregulation. The horizontal line represents $P = 0.05$. (**b**) The heatmap analysis showed that a total of 37 881 circRNAs were dysregulated between testis samples from patients with NOA and the control samples (fold change ≥2, $P < 0.05$). Red represents upregulation, and green represents downregulation. NOA: non-obstructive azoospermia; NC: normal control; circRNAs: circular RNAs.

these circRNAs are shown in **Figure 2**. The head-to-SH-tail splicing junctions were confirmed by Sanger sequencing of the reverse transcription PCR (RT-PCR) product (**Figure 2a–2c**). FISH against hsa_circ_0137890, hsa_circ_0136298, and hsa_circ_0007273 revealed that these circRNAs were primarily expressed in the cytoplasm (**Figure 2d**). As shown in **Figure 3**, the expression levels of these three circRNAs were significantly decreased in the NOA patients compared with the normal controls, which is in accordance with the microarray data.

The circRNA-miRNA interaction network

Given that circRNAs have been widely reported to function as sponges for miRNAs and that hsa_circ_0137890, hsa_circ_0136298, and hsa_circ_0007273 are stable and located in the cytoplasm, we next identified the potential circRNA-sponged miRNAs of the three validated circRNAs theoretically predicted based on conserved MREs using miRanda analysis. Furthermore, the top 30 miRNAs for each circRNA are presented as a network constructed using Cytoscape software (**Figure 4**). Numerous miRNAs, including miR-221 and miR-762, that could be paired with circRNAs validated in this study were reported to be vital for spermatogenesis regulation, indicating that these circRNAs in NOA may be related to miRNA-mediated gene regulation.

GO and KEGG analyses

To explore changes in the patterns and associated functions of the differentially expressed circRNAs in NOA patients, GO and KEGG enrichment analyses were performed for the NOA and normal groups. GO analysis predicted the functional roles of the parental genes, which were divided into three groups: biological processes, cellular components, and molecular functions. For the biological processes, the significantly enriched GO terms of the differentially expressed genes were axoneme assembly, microtubule-based process, and plasmacytoid dendritic cell cytokine production; for cellular components, the most common terms were dynein complex, cilium, and inner dynein arm; and microtubule motor activity, ferric iron binding, and insulin-like growth factor II binding were the most enriched molecular function terms (**Supplementary Figure 2a–2c**). Based on KEGG pathway analysis, the parental genes of the dysregulated circRNAs were involved in the G2/M DNA replication checkpoint, cyclin B2-mediated events, and endosomal/vacuolar pathway (**Supplementary Figure 2d**). Importantly, most of these biological pathways are closely associated with the pathophysiological process of NOA.

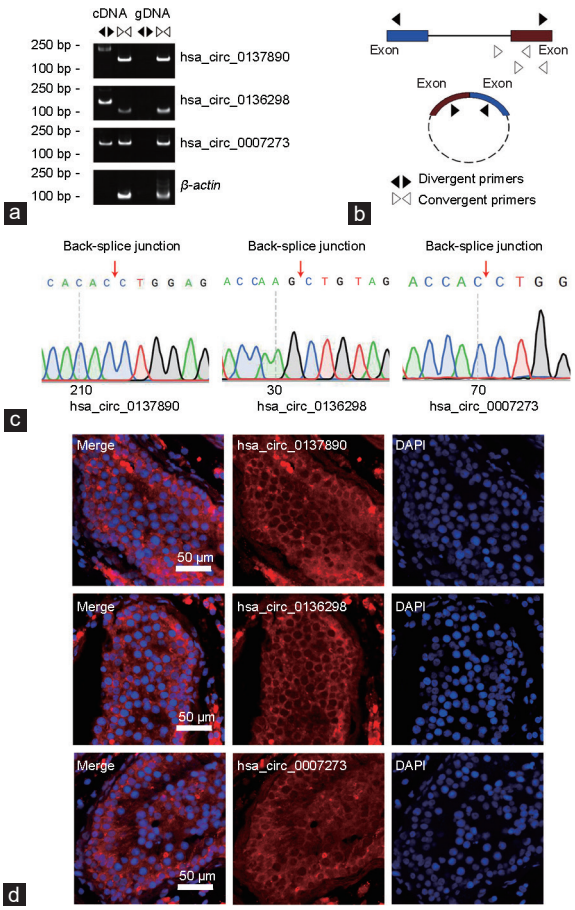

Figure 2: Characterization of selected circRNAs in human testicular tissue. (**a**) Divergent and convergent primers were designed to amplify selected circRNAs. (**b**) The expression of these circRNAs and the corresponding linear mRNAs in cDNA and gDNA were shown by gel electrophoresis. (**c**) The back-splice junctions of selected circRNAs were identified by Sanger sequencing. (**d**) Evaluation of the subcellular localization of selected circRNAs. Scale bars = 50 μm. The FISH assay showed that hsa_circ_0137890, hsa_circ_0136298, and hsa_circ_0007273 were predominantly distributed in the cytoplasm of testicular tissue cells. bp: base pair; circRNAs: circular RNAs; cDNA: complementary DNA; gDNA: gemonic DNA; mRNAs: messenger RNAs; DAPI: 4',6-diamidino-2-phenylindole; FISH: fluorescence *in situ* hybridization.

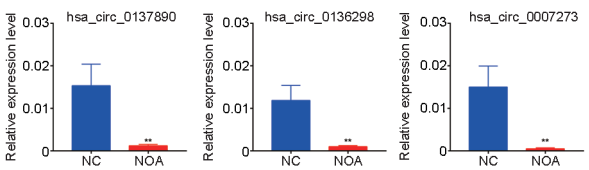

Figure 3: qRT-PCR validation of selected circRNAs. The expression of 3 circRNAs between the NOA group and the normal group was measured by qRT-PCR. $^{**}P < 0.01$ versus NC group. qRT-PCR: quantitative real-time polymerase chain reaction; NOA: non-obstructive azoospermia; NC: normal control; circRNAs: circular RNAs.

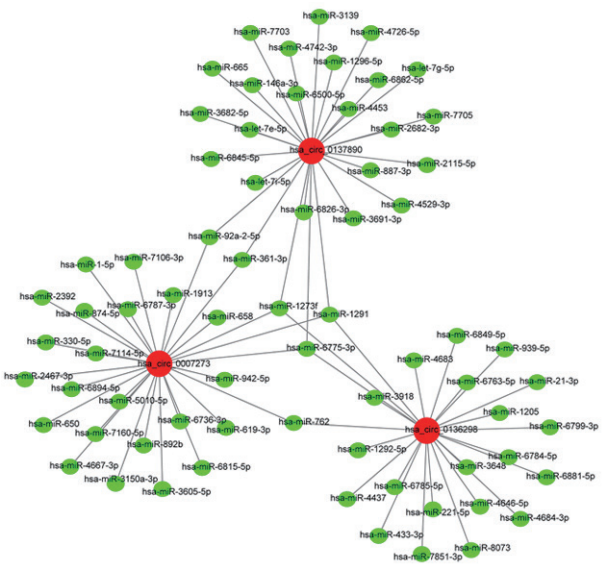

Figure 4: Construction of the circRNA-miRNA network map according to bioinformatics analysis. The red round nodes represent significantly downregulated circRNAs, and the green round nodes represent miRNAs. circRNAs: circular RNAs; miRNAs: microRNAs.

DISCUSSION

Spermatogenesis is a complex developmental process in which diploid spermatogonia differentiate into haploid spermatozoa through a round of tightly orchestrated events within the seminiferous tubules. This process requires dynamic regulation of stage-specific and spermatogenic cell-specific gene expression, which is mainly controlled by transcription factors, miRNAs, and lncRNAs.[10,12] Recent studies have demonstrated that circRNAs are emerging as important regulatory factors.[19] However, there are few studies on circRNA expression patterns in NOA patients by microarray. In the current study, a total of 37 882 circRNAs were significantly different compared with the normal controls, including 19 874 statistically upregulated and 18 008 downregulated circRNAs. Bioinformatics analysis indicated that these differentially expressed circRNAs may play a role in the processes of spermatogenic failure and NOA pathogenesis.

Based on circRNA-seq studies, it is estimated that thousands of circRNAs are expressed in various tissues and cells among mammalian organisms.[28] Compared to other organs, the abundance and diversity of circRNA expression in human and mouse testes is only second to that in the brain.[29] CircRNA levels are dynamically regulated in neurons and are essential for maintaining normal brain function.[30,31] The expression of circRNAs is spatiotemporally modulated during neural development or neuronal activity. In addition, dysregulated circRNAs may contribute to the initiation and progression of various neurodegenerative diseases. For example, deficiency in the circRNA cirRS-7-mediated "sponge effect" in the hippocampal region of Alzheimer's disease patients results in redundant miR-7 and subsequent downregulated expression of ubiquitin conjugating enzyme E2A, which serves as a neuroprotector by eliminating amyloid peptides.[32] A global trend of circRNA accumulation was observed in aging *Caenorhabditis elegans* and mouse brains, and this upregulation was largely independent of linear RNA changes in the host genes.[30,33] Lin *et al.*[34] found that the production of circRNAs during different spermatogenic stages is dynamically regulated instead of random.[34] Thus, it has been hypothesized that these abundant circRNAs also take an active part in the regulation of spermatogenesis.

The potential role of circRNAs in the pathophysiology of NOA has yet to be elucidated. Ge *et al.*[35] suggested that the testicular circRNA expression profile is altered in NOA patients and inferred that circRNAs might play important roles in regulating spermatogenesis.[35] In a recent study, Zhu *et al.*[36] explored the circRNA expression pattern in the testicular tissues of patients with SCOS and found that host genes of the differentially expressed circRNAs (DEcircRNAs) were enriched in biological processes related to the cell cycle and intercellular communication. In the present study, we found a unique differential circRNA expression profile in the testes of patients with NOA compared with the control group. The biological functions of the parental genes of the differentially expressed circRNAs were predicted based on the GO and KEGG databases.[22] Most of the affected biological processes and pathways are involved in the spermatogenesis process, indicating that these dysregulated circRNAs may contribute to the development of NOA. qRT-PCR validation in a larger independent set of samples showed that the differentially expressed circRNAs had the same change tendency.

To further investigate the potential biological functions of candidate circRNAs in NOA, the circRNA-miRNA gene networks were identified by miRanda analysis. We found numerous miRNAs that could be paired with circRNAs reported to be vital for spermatogenesis regulation. For example, miR-221 is an hsa_circ_0136298-targeted miRNA that plays a critical role in maintaining the undifferentiated state in mammalian male germ cells through regulation of KIT expression.[37] Overexpression of miR-211 led to reduced expression of the c-Kit protein in spermatogonia cell lines. hsa_circ_0000116 was found to be overexpressed in NOA patients compared with normal controls, and it may affect fertility function through an hsa_circ_0000116-miR-449-autophagy-related competing endogenous RNA (ceRNA) network.[38] miR-762 potentially binds to hsa_circ_0136298 and hsa_circ_0007273 and has been shown to be involved in the regulation of proliferation and apoptosis of immature porcine Sertoli cells by directly binding the mRNA of the ring finger protein 4 gene and accelerating DNA damage repair.[39] Let-7e-5p, let-7 g-5p, and let-7i-5p were matched with hsa_circ_0137890; the expression of these let-7 family miRNAs is remarkably high in type B spermatogonia cells and primary spermatocytes, and they have been reported to target vital genes that participate in cell differentiation and cell cycle regulation.[40] Thus, we hypothesize that one possible mechanism of circRNAs in NOA may be related to miRNA-mediated gene regulation. However, the underlying mechanism of circRNA-miRNA interactions and their target gene interactions need to be further studied.

Emerging studies have demonstrated that circRNA expression is abundant in the mammalian transcriptome, and circRNAs have been identified as novel diagnostic and therapeutic biomarkers in various diseases, such as preeclampsia, Hashimoto's thyroiditis, cardiovascular diseases, and carcinomas.[20,21,24,25] Our results indicate that hsa_circ_0137890, hsa_circ_0136298, and hsa_circ_0007273 might serve as promising biomarkers in NOA; however, further studies with larger sample numbers are essential to evaluate the sensitivity and specificity of these circRNAs as novel biomarkers of NOA. It is hypothesized that circRNAs may carry disease characteristics into biological fluids such as blood and seminal plasma.[23] Because of their circularized structure and resistance to RNase digestion, numerous testis-derived circRNAs could be stably examined in seminal plasma, as they are barely degraded even after 24 h at room temperature.[41] Malcher *et al.*[42] reported that a set of specific genes can be used in molecular diagnosis to assess the degree of spermatogenic failure in patients with idiopathic NOA. Exosomal miRNAs in seminal plasma originating from azoospermia can predict the existence of spermatozoa in testicular tissue.[43] Lv *et al.*[38] demonstrated a potential connection between negative sperm retrieval outcome and a high level of hsa_circ_0000116 expression. We hypothesize that circRNAs may have greater potential and serve as better genetic biomarkers than other RNAs. The differential expression of circRNAs tested in testicular tissue harvested from biopsy revealed that it may act as a potential diagnostic and therapeutic tool of NOA upstream. Meanwhile, given the invasive nature of testicular biopsy, the next step would be evaluation of circRNA within the seminal fluid, which would avoid invasive testicular biopsy in these patients. Thus, it will be interesting to explore the expression patterns of circRNAs in the seminal plasma of infertile men with different pathologic types in the future.

CONCLUSIONS

In the present study, we found abundant differentially expressed circRNAs in NOA patients, which may participate in biological pathways related to spermatogenesis and contribute to the pathogenesis of NOA. The identification of circRNAs with altered expression is critical for understanding the pathological characteristics of azoospermia, which in turn will aid in the development of novel potential diagnostic and therapeutic targets. Further studies are required to determine the function of the candidate circRNAs in male infertility.

AUTHOR CONTRIBUTIONS

ZZ, HL, HW, and HJ contributed to the design and conception of the study. LZ, ZZ, and HW performed the experiment. LZ and HW contributed significantly to the analysis and manuscript preparation. LZ, ZZ, and HW performed the data analyses and wrote the manuscript. JMM and DFL polished the language and structurally revised the manuscript. HTZ, YZY, and LMZ helped perform the analysis with constructive discussions. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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Supplementary Information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

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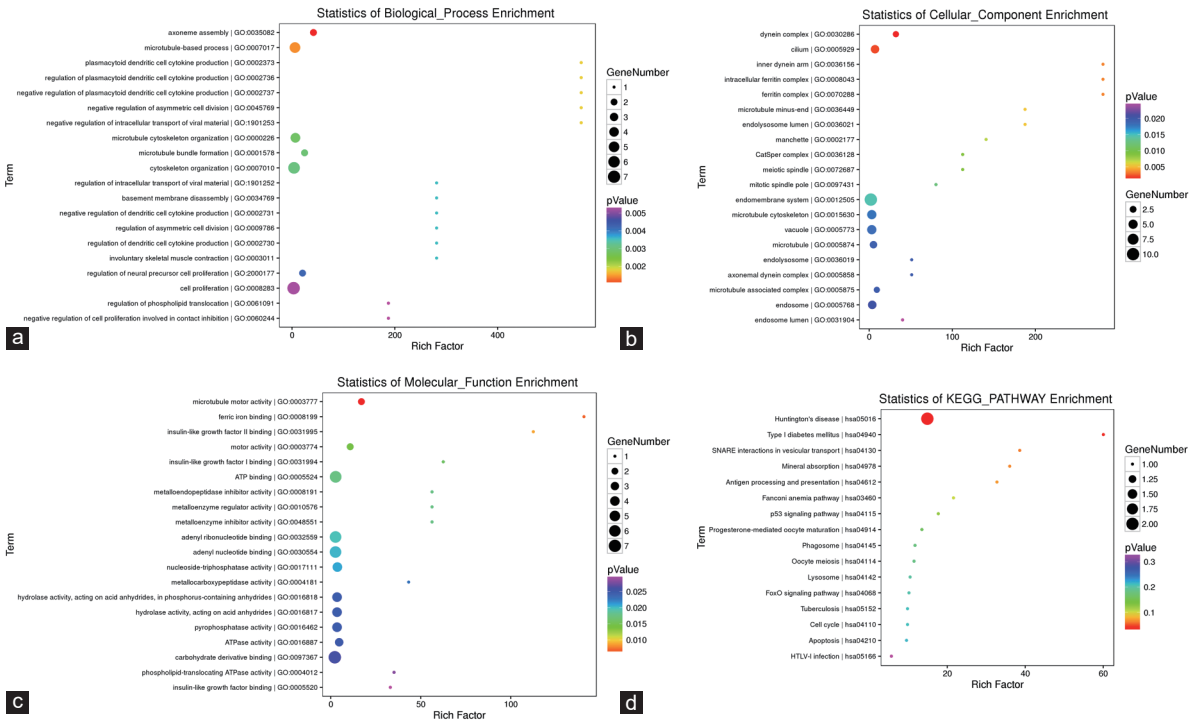
Supplementary Figure 1: The Circos plot showing the distribution of differentially expressed circular RNAs on the chromosomes. Red indicates upregulation, and green indicates downregulation.



Supplementary Figure 2: GO and KEGG pathway analysis of the parental genes of the differentially expressed circRNAs. Top 20 GO terms of the gene enrichment analysis in patients with NOA versus a control group for (**a**) biological processes, (**b**) cellular components, and (**c**) molecular functions. (**d**) The top 20 enriched KEGG pathways of the parental genes of the differentially expressed circRNAs. GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; circRNA: circular RNA; NOA: non-obstructive azoospermia.

Supplementary Table 1: Primers used for polymerase chain reaction amplification

Oligo name	*Sequence (5'–3')*	*Amplicon*
hsa_circ_0137890 Div-F	GAGCTTTTGTTGGGGCGACA	241 bp
hsa_circ_0137890 Div-R	ACAGTCTTGCCTCTTTTGTTCTTG	
hsa_circ_0136298 Div-F	GCATGAAACCCTCATCCACT	137 bp
hsa_circ_0136298 Div-R	ACCGATCTCCGATTTTGATG	
hsa_circ_0007273 Div-F	AGCTGGTTTGGCTTGAGAGA	146 bp
hsa_circ_0007273 Div-R	ATACTGGCGCTCAGGTTGTT	
hsa_circ_0137890 Con-F	TGCTAATGATGATCGCCATGT	192 bp
hsa_circ_0137890 Con-R	TCTCCGGCCTCATCTTTCTT	
hsa_circ_0136298 Con-F	GACAATTTAGCGGCACGGAT	95 bp
hsa_circ_0136298 Con-R	TTGCCGGTTTTGCGAATG	
hsa_circ_0007273 Con-F	TAAGTCGGAGCGAGTTGGG	152 bp
hsa_circ_0007273 Con-R	GGGTTTCTGAGTCGGGTCCTT	
β-actin Div-F	AAAGGCGAGGCTCTGTGCT	N/A
β-actin Div-R	GGGCTTACCTGTACACTGACTTGA	
β-actin Con-F	TTGTTACAGGAAGTCCCTTGCC	101 bp
β-actin Con-R	ATGCTATCACCTCCCCTGTGTG	

Div: divergent; Con: convergent; F: forward primer; R: reverse primer; N/A: not available

Supplementary Table 2: Primers used for RNA fluorescence *in situ* hybridization probe preparation

Gene name	*Sequence (5'–3')*
hsa_circ_0137890	GCTTGTTCTCTGGAACACACCTGGAGCTATGTAGCCAGGA
hsa_circ_0136298	GCATGCAGCAAGACCTACAGCTTGGTAAGCAAGTTTAAAT
hsa_circ_0007273	TGGAGTGCAGCTTGTACCACCTGGGTTTCTGAGTCGGGTC

Supplementary Table 3: Differentially expressed circular RNAs in non-obstructive azoospermia

Target ID	*Up or down*	*Fold change*	*P*	*circRNA chromosome*	*circRNA genomic length (bp)*	*circRNA spliced sequence length (bp)*
hsa_circ_0123861	Up	9.876119	0.000163	chr3	238	238
hsa_circ_0064062	Up	8.834428	9.66E-05	chr3	101 241	3724
hsa_circ_0123657	Up	8.636109	0.000203	chr3	75 190	3188
hsa_circ_0123813	Up	8.273904	0.000829	chr3	3918	508
hsa_circ_0123663	Up	7.965585	0.00021	chr3	2396	294
hsa_circ_0001261	Up	7.685515	0.000114	chr3	4619	179
hsa_circ_0124661	Up	7.671506	6.07E-05	chr3	28 215	425
hsa_circ_0120106	Up	7.668067	1.78E-05	chr2	19 861	19 861
hsa_circ_0120096	Up	7.609506	1.62E-06	chr2	6350	564
hsa_circ_0077060	Up	7.337683	0.000107	chr6	19 649	1006
hsa_circ_0069860	Up	7.2116	0.000775	chr4	265 512	2797
hsa_circ_0075466	Up	7.130919	1.22E-05	chr6	3301	3301
hsa_circ_0084847	Up	7.067317	9.31E-06	chr8	23 830	1104
hsa_circ_0064075	Up	7.043871	8.56E-06	chr3	6978	756
hsa_circ_0124659	Up	7.017778	7.93E-05	chr3	15 969	418
hsa_circ_0120098	Up	6.922312	1.02E-05	chr2	28 902	28 902
hsa_circ_0123460	Up	6.869542	0.000306	chr3	83 742	559
hsa_circ_0054324	Up	6.823968	9.81E-06	chr2	14 227	420
hsa_circ_0120101	Up	6.794642	1.76E-05	chr2	53 502	53 502
hsa_circ_0034350	Up	6.706725	8.38E-05	chr15	393	393
hsa_circ_0097144	Down	332.746	1.24E-05	chr12	22 547	557
hsa_circ_0131661	Down	219.1307	2.8E-07	chr6	20 714	754
hsa_circ_0123328	Down	180.0503	5.68E-09	chr3	219	219
hsa_circ_0066031	Down	141.3431	6.47E-07	chr3	882	673
hsa_circ_0110558	Down	118.999	0.000248	chr1	40 530	777
hsa_circ_0026777	Down	114.0156	3.17E-05	chr12	8636	357
hsa_circ_0118010	Down	103.5574	6.89E-06	chr2	25 859	1023
hsa_circ_0127671	Down	96.66126	1.68E-06	chr5	33 161	1769
hsa_circ_0105755	Down	91.91886	6.69E-06	chr16	3868	618
hsa_circ_0106653	Down	88.93248	0.000192	chr17	5976	571
hsa_circ_0106817	Down	80.23748	0.000213	chr17	138	138
hsa_circ_0104727	Down	75.98965	9.07E-08	chr15	29 519	793
hsa_circ_0031979	Down	73.08445	6.48E-08	chr14	104	104
hsa_circ_0104722	Down	71.59193	1.55E-07	chr15	98 020	944
hsa_circ_0073631	Down	71.53524	2.87E-06	chr5	14 153	1208
hsa_circ_0105173	Down	70.78257	3.75E-05	chr16	79 145	3656
hsa_circ_0091620	Down	70.5545	0.001407	chrX	2082	705
hsa_circ_0104732	Down	69.95805	1.47E-07	chr15	20 243	651
hsa_circ_0043542	Down	65.85173	0.000361	chr17	1676	335
hsa_circ_0097146	Down	65.09275	3.03E-07	chr2	63 910	1558

circRNA: circular RNAs