Hindawi
Andrologia
Volume 2023, Article ID 2074931, 13 pages
https://doi.org/10.1155/2023/2074931

Research Article

Global Analysis in Nonobstructive Azoospermic Testis Identifies miRNAs Critical to Spermatogenesis

Fahimeh Piryaei ,[1] Hossein Mozdarani ,[1] Mohammad Ali Sadighi Gilani,[2] Singh Rajender ,[3,4] Renata Finelli ,[5] Mahsa Darestanifarahani,[6] Abdolazim Sarli ,[1] Poonam Mehta ,[3,4] and Ashok Agarwal [7]

[1]*Department of Medical Genetics, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran*
[2]*Department of Andrology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran*
[3]*Male Reproductive Biology Laboratory, Central Drug Research Institute, Lucknow, India*
[4]*Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, India*
[5]*CREATE Fertility, 150 Cheapside, London EC2V 6ET, UK*
[6]*Department of Medical Biotechnology, Faculty of Allied Medicine, Iran University of Medical Sciences, Tehran, Iran*
[7]*American Center for Reproductive Medicine, Global Andrology Forum, Moreland Hills, OH, USA*

Correspondence should be addressed to Hossein Mozdarani; mozdarah@modares.ac.ir

Received 19 October 2022; Revised 5 January 2023; Accepted 5 April 2023; Published 10 May 2023

Academic Editor: Vikas Kumar Roy

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Introduction. The etiology of male infertility characterized by non-obstructive azoospermia is largely unknown, especially at the molecular level. Identifying dysregulated microRNAs (miRNAs) in male infertility would be useful to achieve a more profound understanding of its pathogenesis. *Methods*. Small RNA sequencing was performed on the testicular tissues of 10 nonobstructive azoospermic patients with the Sertoli cell only syndrome (SCOS) and 8 obstructive azoospermic individuals with normal spermatogenesis. The expressions of two dysregulated miRNAs were validated by quantitative real-time polymerase chain reaction, confirming the results obtained by sequencing analysis. Bioinformatic analysis was undertaken to identify the main pathways impaired in complete spermatogenic failure. *Results*. A total of 136 miRNAs were detected to be differentially expressed in the Sertoli cell only syndrome group in comparison with the obstructive azoospermia group. Bioinformatic analysis suggested that the altered miRNAs were substantially involved in pathways related to spermatogenesis. *Conclusions*. Our study investigates the entire profile of miRNAs with emphasis on the crucial role of miRNAs in idiopathic Sertoli cell only syndrome, suggesting potential targets for employing molecular therapeutic strategies in the treatment of spermatogenic failure.

1. Introduction

Infertility affects nearly 15% of couples as a worldwide health problem, and half of the cases are attributable to male factors [1]. Around 30-40% of male infertility cases remain idiopathic, which also includes nearly 15% of azoospermic cases [2, 3]. The etiology of azoospermia is largely unknown, especially at the molecular level. Spermatogenesis is an intricately organized and complex process, which is accomplished in three successive phases: consecutive mitotic divisions of spermatogonia, reductive meiotic division of spermatocytes, and differentiation of spermatids [4]. Several protein mediators are involved in the regulation of this complicated process; however, microRNAs (miRNAs) play critical roles at the posttranscriptional stage [5–7]. miRNAs are small (18-22 nucleotides long) noncoding RNAs, which can negatively and selectively modulate gene expression. miRNAs have been described as the molecular regulators of various cellular biological events such as growth, proliferation, differentiation, and programmed cell death [8–10]. Hence, alterations in the molecular signaling in the testis may result in impaired spermatogenesis and male infertility. Identification of the

molecular players dysregulated in azoospermia/infertility would pave the way to the development of new therapeutic approaches in the future. Since miRNAs regulate a large number of genes, identification of dysregulated miRNAs in infertility would be useful in the diagnosis and treatment of infertility [11].

So far, a few studies have analyzed the miRNAs' expression levels in testicular biopsies obtained from azoospermic patients and normal individuals [12–17]. In the first study, Lian et al. performed microarray and identified altered expression of miRNAs in nonobstructive azoospermic (NOA) patients, with 19 and 154 up- and downregulated miRNAs, respectively [14]. Subsequently, another study analyzed 48 testicular biopsy samples obtained from patients with various degrees of spermatogenic impairment (germ cell arrest, mixed atrophy, and Sertoli cell only) and compared them with normozoospermic controls [12]. A number of dysregulated miRNAs were identified in the infertile group. Based on these results, Abu-Halima et al. selected a set of five miRNAs and analyzed them in a large set of infertile biopsy samples, emphasizing their value as diagnostic biomarkers of male infertility [13]. Munoz et al. screened 623 miRNAs in testicular samples of men with spermatogenic failure and identified a set of three miRNAs, including miR-122, miR-34c-5p, and miR-449a, with high predictive value for the presence of mature spermatozoa in the testis [15]. Noveski et al. analyzed testicular biopsies of infertile men with hypospermatogenesis, maturation arrest, hypospermatogenesis with azoospermia factor c (AZFc) deletion, and Sertoli cell only syndrome (SCOS), while men with normal spermatogenesis served as the control group [16]. They found some dysregulated miRNAs common among different histopathological groups. In addition, miRNA-mRNA network analysis revealed the role of several genes involved in the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) pathway. Recently, Zhang et al. used microarray to study the miRNA profile of testicular biopsies from NOA patients, finding that 129 miRNAs were aberrantly expressed in comparison to the controls [17]. In particular, this study reported that a panel composed of miR-10b-3p and miR-34b-5p may be used to predict the existence of testicular sperm in azoospermic patients.

Most of the above studies have laid the foundation for the identification of miRNA-based diagnosis of the pathogenesis that can unveil the path to therapy. Independent investigations in different populations would not only further strengthen these initial findings but also provide a way to finding consistent miRNAs that are critical to spermatogenesis and fertility. Therefore, in the current work, we performed small RNA sequencing in a selected set of patients with SCOS to uncover the global microRNA dysregulation in comparison to obstructive azoospermia (OA) individuals with normal spermatogenesis.

2. Material and Methods

2.1. Sample Collection. Azoospermic infertile men referred to the Royan Institute (Tehran, Iran) were recruited in this study after receiving their informed consent. Idiopathic NOA individuals ($n = 10$) characterized by SCOS constituted the case group while individuals with OA constituted the control group ($n = 8$). Testicular specimens of these individuals were obtained by testicular sperm extraction (TESE) procedure, performed as diagnostic biopsy for histopathological examination and sperm extraction for assisted reproductive treatment. Azoospermia was defined according to the WHO 2010 criteria [18]. No ejaculated spermatozoa were seen in two sequential semen analyses. SCOS was confirmed upon finding no spermatozoa within the testes and/or epididymis upon microscopic examination of at least 50 tiny foci in biopsy specimens. OA was specified as containing a notable number of mature spermatozoa retrieved by TESE. The diagnosis was supported by the histological reports as well as hormonal findings for the two groups. All patients enrolled in this study had primary infertility. The NOA and OA groups were age- and body mass index (BMI) matched. They were nonsmokers and did not consume alcohol regularly.

Individuals with infertility related to defined medical conditions, such as pathologies involving vas deferens or epididymis, undescended testes (UDT), cryptorchidism, varicocele, a positive history of hormone therapy, retrograde ejaculation, current or previous infection with mumps, chromosome abnormalities, and AZF microdeletions were excluded from the study. After collecting, testicular samples were stored in RNALater® Solution (Behnogen, Iran) overnight at 4°C and placed at -80°C until further processing.

2.2. RNA Extraction. The total RNA was isolated from each sample using RiboEx reagent (GeneAll, Korea), following the manufacturer's recommendations. The elution was made in 30 μL of nuclease-free water. The quality and quantity of all RNA samples were evaluated by denaturing agarose gel electrophoresis and NanoDrop™ 1000 spectrophotometer, respectively.

2.3. Small RNA Library Preparation and Sequencing. To undertaken sequencing, equal amounts of RNA samples were pooled to obtain 2 OA (4 samples each) and 3 NOA (4 : 3 : 3 samples each) groups. RNA integrity (RIN) of samples was tested using Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit, USA), and samples with RIN values > 7 were considered acceptable for sequencing. BGISEQ-500 small RNA (sRNA) sample preparation kit was used to prepare the sRNA libraries. RNA (18-30 nt) molecules were ligated with adaptors; reverse transcribed and unique indices were incorporated during PCR amplification. After polyacrylamide gel electrophoresis (PAGE), the PCR amplicons were purified and dissolved in EB solution. The samples were loaded on the BGISEQ-500 platform at Yingbioteck (Shanghai, China), and sequencing by synthesis was conducted to generate single-end 50 base-pair reads [19].

2.4. Mapping Reads. After trimming the adaptors and removing the contamination, the clean reads ≥ 18 nucleotides were mapped to the*miRBase* version 21 and Rfam databases. We used Bowtie2 [20] and cmsearch [21] to map filtered reads to the reference genome as well as other sRNA

Table 1: Primer sequences for qRT-PCR of selected miRNAs and U6.

miRNA ID	Primer	Sequence
miR-449a-5p (MIMAT0001541)	RT	5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACCAGC-3′
	F	5′-AGGCGTGGCAGTGTATTGTT-3′
miR-888-5p (MIMAT0004916)	RT	5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGACTG-3′
	F	5′-GGCGCGTACTCAAAAAGCTG-3′
U6 (gene ID: 26827)	RT	5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAATATG-3′
	F	5′-ATGACGCAAATTCGTGAAGC-3′
	R	5′-CAGTGCAGGGTCCGAGGTA-3′

Abbreviation: RT: reverse transcription; F: forward; R: universal reverse.

Table 2: Demographic characteristics of subjects diagnosed with azoospermia.

Study groups	Number of subjects	Average age (years)	Average weight (MBI)	FSH (mIU/mL)	LH (mIU/mL)	Testosterone (ng/mL)
NOA	10	34.8 ± 5.20	24.69 ± 2.8	24.43 ± 13.30	13.40 ± 8.93	2.47 ± 0.31
OA	8	36.1 ± 5.64	24.18 ± 2.40	7.57 ± 3.62	7.07 ± 2.54	4.59 ± 2.47

Abbreviation: FSH: follicle stimulating hormone; LH: luteinizing hormone; OA: obstructive azoospermia; NOA: nonobstructive azoospermia.

Table 3: Summary of sequencing data for each sample.

Sample name	Known miRNA count	Sequence type	Raw tag count	Clean tag count	Percentage (%)	Mapped tag	Mapping rate (%)
NOA1	1330	SE50	23968286	21664941	90.39	20430103	94.30
NOA2	1323	SE50	23886191	22100843	92.53	21279937	96.29
NOA3	1301	SE50	23245544	203391 63	87.50	19370664	95.24
OA1	1403	SE50	24604847	22857389	92.90	21942492	96.00
OA2	1238	SE50	23072834	21066578	91.30	20318453	96.45

databases. Differentially expressed miRNA analysis was performed by DESeq2 algorithm, with a cutoff of $\log_2 FC > 1$.

2.5. Bioinformatic Analyses for Differentially Expressed miRNAs. In order to annotate and categorize the target genes attributed to cellular component (CC), biological process (BP), and molecular function (MF), Gene Ontology (GO) analysis (http://geneontology.org) was performed. Moreover, the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis was used to categorize the genes targeted by the *differentially expressed small RNAs* (DESs) on the basis of the metabolic or signal transduction pathways they were involved in. Softwares including Miranda [22] and TargetScan (http://www.targetscan.org/mmu_61/) [23] were used for predicting the targets. The default parameters were as follows: cutoff score ≥ 140 and energy ≤ −20 kcal/mol for Miranda and energy < −25 for TargetScan. The common targets identified by the two databases were used as the final miRNA target prediction results. We excluded the genes targeted by both low (<10 RPM) and highly (>100 RPM) abundant miRNAs to overcome the dominant contribution of these miRNA families leading to targeted gene downregulation as compared to those expressed at a moderate levels.

2.6. cDNA Synthesis and Quantitative Reverse Transcription PCR. To validate the expression level of selected miRNAs in the testis samples, cDNA synthesis for the same individual samples ($n = 18$) was performed with 1 μg of total RNA, utilizing TaKaRa PrimeScript II 1st strand cDNA synthesis kit (Takara Bio, Japan). The primers were designed according to the stem-loop RT method introduced by Chen et al. [24], and the details are described in Table 1. Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted using SYBR green (Exiqon, Denmark) on StepOnePlus™ Real-Time PCR thermal cycler (Applied Biosystems™, USA). PCR were run in triplicate, and reverse transcriptase reactions, including RT minus controls and nontemplate controls, were run in duplicate. The values of cycle threshold (Ct) were computed, and the data were analyzed using the$\Delta\Delta$Ct method. As there are no reliable miRNAs as reference genes for the testis, U6 snRNA was used as an endogenous reference based on previous reports. The detailed methods are provided in our previous paper [25].

2.7. Statistical Analyses. Statistical analyses were performed utilizing SPSS v. 18.0.1 (SPSS Inc., Chicago, IL). The Student's t-test was used to compare the average expression level of chosen miRNAs between the NOA and OA groups. P values < 0.05 were considered statistically significant.

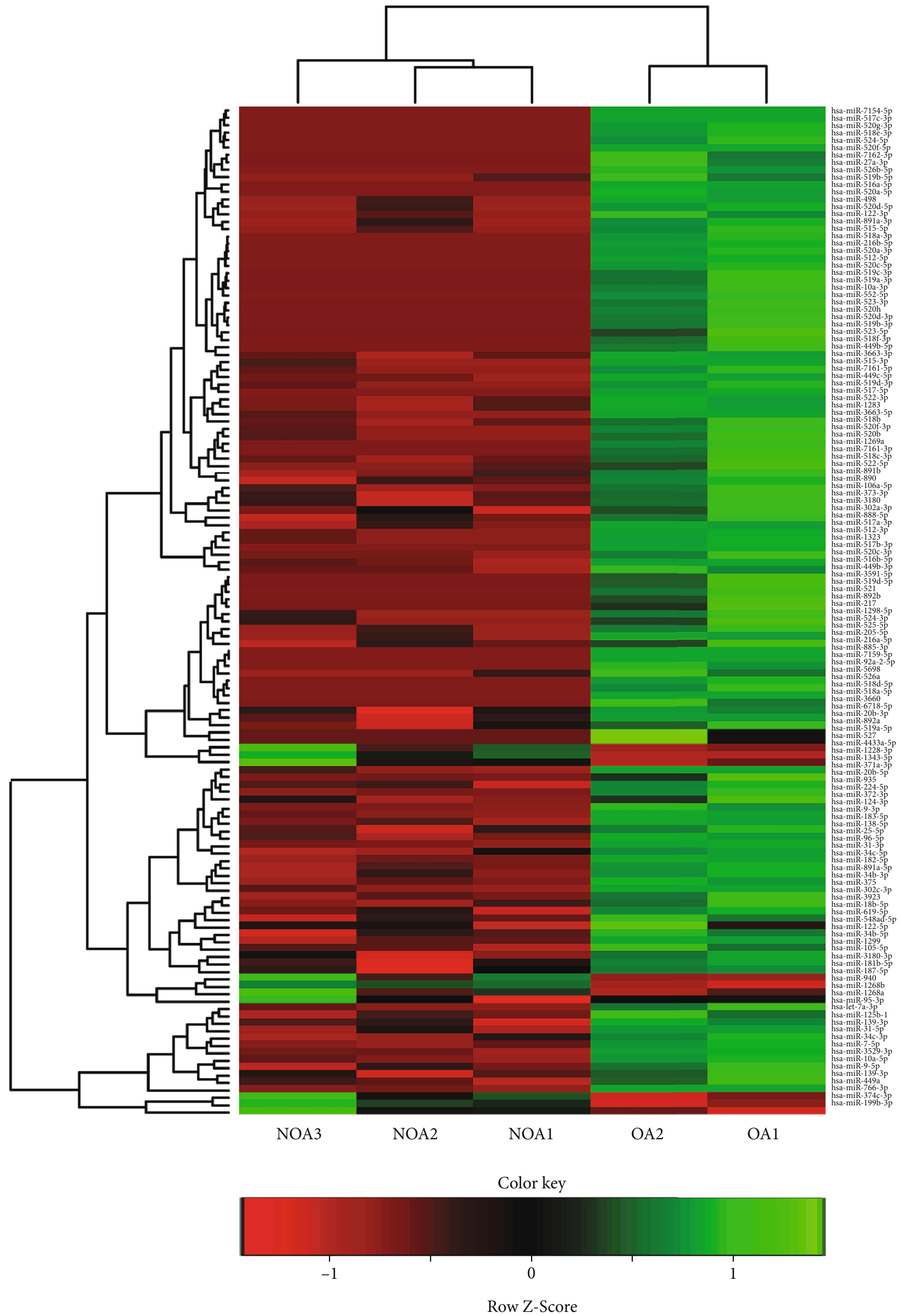

Figure 1: A heatmap of miRNA expression values depicting miRNA clustering between NOA and OA samples based on differentially expressed miRNAs in all pairwise of cluster plan (Padj < 0.05). Positive and negative numbers indicate upregulation and downregulation, respectively.

Table 4: Expression of differentially expressed miRNAs in testicular samples of SCOS patients compared to that of OA individuals.

	miRNA ID	log_2FoldChange (case/control)	*P* value	Padj	Up-/downregulation	Genome location
1	hsa-miR-449a	-8.95712	8.55*E*-66	1.56*E*-62	Down	Chr5
2	hsa-miR-34c-3p	-5.42921	8.53*E*-30	5.19*E*-27	Down	Chr11
3	hsa-miR-375	-5.76047	4.47*E*-28	1.63*E*-25	Down	Chr2
4	hsa-miR-517b-3p	-9.10218	1.50*E*-26	4.57*E*-24	Down	Chr19
5	hsa-miR-512-3p	-8.37851	1.51*E*-25	3.44*E*-23	Down	Chr19
6	hsa-miR-34c-5p	-5.15346	9.05*E*-24	1.84*E*-21	Down	Chr11
7	hsa-miR-520c-3p	-8.11517	1.25*E*-23	2.29*E*-21	Down	Chr19
8	hsa-miR-516b-5p	-6.93696	2.19*E*-23	3.63*E*-21	Down	Chr19
9	hsa-miR-1323	-7.97416	9.51*E*-23	1.45*E*-20	Down	Chr19
10	hsa-miR-34b-3p	-5.32971	9.42*E*-22	1.23*E*-19	Down	Chr11
11	hsa-miR-7-5p	-4.68621	6.21*E*-21	7.09*E*-19	Down	Chr9
12	hsa-miR-449b-3p	-7.00788	1.09*E*-19	1.16*E*-17	Down	Chr5
13	hsa-miR-519d-3p	-7.51046	1.47*E*-19	1.49*E*-17	Down	Chr19
14	hsa-miR-517-5p	-7.92481	6.45*E*-19	6.20*E*-17	Down	Chr19
15	hsa-miR-182-5p	-4.76507	1.21*E*-17	1.00*E*-15	Down	Chr7
16	hsa-miR-891a-5p	-5.35565	5.57*E*-16	4.42*E*-14	Down	ChrX
17	hsa-miR-34b-5p	-4.84987	8.12*E*-16	6.18*E*-14	Down	Chr11
18	hsa-miR-518b	-6.39722	1.15*E*-15	8.04*E*-14	Down	Chr19
19	hsa-miR-515-3p	-6.46087	1.25*E*-15	8.46*E*-14	Down	Chr19
20	hsa-miR-522-3p	-6.18912	1.41*E*-15	9.19*E*-14	Down	Chr19
21	hsa-miR-3663-5p	-5.72328	1.53*E*-15	9.64*E*-14	Down	Chr10
22	hsa-miR-517a-3p	-5.86253	2.84*E*-15	1.73*E*-13	Down	Chr19
23	hsa-miR-7161-5p	-6.70005	5.67*E*-15	3.34*E*-13	Down	Chr6
24	hsa-miR-449c-5p	-6.24951	1.12*E*-14	6.36*E*-13	Down	Chr5
25	hsa-miR-96-5p	-3.51773	1.48*E*-14	8.20*E*-13	Down	Chr7
26	hsa-miR-516a-5p	-7.06944	2.63*E*-14	1.41*E*-12	Down	Chr19
27	hsa-miR-498	-5.88303	1.48*E*-12	7.70*E*-11	Down	Chr19
28	hsa-miR-522-5p	-6.59192	2.33*E*-12	1.18*E*-10	Down	Chr19
29	hsa-miR-1283	-5.5631	2.46*E*-12	1.21*E*-10	Down	Chr19
30	hsa-miR-1299	-4.89852	3.20*E*-12	1.53*E*-10	Down	Chr9
31	hsa-miR-520a-5p	-6.62105	3.50*E*-12	1.64*E*-10	Down	Chr19
32	hsa-miR-520g-3p	-6.41133	2.16*E*-11	9.88*E*-10	Down	Chr19
33	hsa-miR-1269a	-6.41876	2.85*E*-11	1.27*E*-09	Down	Chr4
34	hsa-miR-520f-3p	-5.94296	3.26*E*-11	1.42*E*-09	Down	Chr19
35	hsa-miR-7154-5p	-6.35628	3.71*E*-11	1.57*E*-09	Down	Chr11
36	hsa-miR-520d-5p	-5.54841	4.33*E*-11	1.79*E*-09	Down	Chr19
37	hsa-miR-3529-3p	-2.86549	5.13*E*-11	2.08*E*-09	Down	Chr15
38	hsa-miR-520b	-5.98042	6.80*E*-11	2.70*E*-09	Down	Chr19
39	hsa-miR-122-3p	-5.83444	1.26*E*-10	4.89*E*-09	Down	Chr18
40	hsa-miR-517c-3p	-6.17555	1.98*E*-10	7.51*E*-09	Down	Chr19
41	hsa-miR-888-5p	-4.62783	2.69*E*-10	1.00*E*-08	Down	ChrX
42	hsa-miR-7161-3p	-6.21697	2.76*E*-10	1.01*E*-08	Down	Chr6
43	hsa-miR-518e-3p	-6.09464	3.98*E*-10	1.43*E*-08	Down	Chr19
44	hsa-miR-183-5p	-3.26591	4.81*E*-10	1.69*E*-08	Down	Chr7
45	hsa-miR-31-5p	-3.61452	8.69*E*-10	2.99*E*-08	Down	Chr9
46	hsa-miR-124-3p	-3.03982	9.42*E*-10	3.13*E*-08	Down	Chr8
47	hsa-miR-519b-5p	-5.69616	9.35*E*-10	3.13*E*-08	Down	Chr19
48	hsa-miR-25-5p	-3.09462	2.03*E*-09	6.62*E*-08	Down	Chr7

Table 4: Continued.

	miRNA ID	log_2FoldChange (case/control)	*P* value	Padj	Up-/downregulation	Genome location
49	hsa-miR-122-5p	-4.46093	2.12*E*-09	6.80*E*-08	Down	Chr18
50	hsa-miR-520f-5p	-5.88763	2.37*E*-09	7.45*E*-08	Down	Chr19
51	hsa-miR-524-5p	-5.87427	2.74*E*-09	8.48*E*-08	Down	Chr19
52	hsa-miR-515-5p	-5.41886	3.00*E*-09	9.11*E*-08	Down	Chr19
53	hsa-miR-518c-3p	-5.24506	3.34*E*-09	1.00*E*-07	Down	Chr19
54	hsa-miR-523-3p	-5.85544	4.30*E*-09	1.27*E*-07	Down	Chr19
55	hsa-miR-520h	-5.74672	8.56*E*-09	2.48*E*-07	Down	Chr19
56	hsa-miR-7162-3p	-5.73587	1.38*E*-08	3.94*E*-07	Down	Chr10
57	hsa-miR-891b	-4.21921	1.44*E*-08	4.04*E*-07	Down	ChrX
58	hsa-miR-374c-3p	3.487852	1.94*E*-08	5.38*E*-07	Up	ChrX
59	hsa-miR-27a-3p	-5.62798	2.81*E*-08	7.65*E*-07	Down	Chr19
60	hsa-miR-520d-3p	-5.57633	3.40*E*-08	9.12*E*-07	Down	Chr19
61	hsa-miR-519b-3p	-5.57408	3.60*E*-08	9.53*E*-07	Down	Chr19
62	hsa-miR-526b-5p	-5.51974	4.80*E*-08	1.25*E*-06	Down	Chr19
63	hsa-miR-520c-5p	-5.48718	5.46*E*-08	1.40*E*-06	Down	Chr19
64	hsa-miR-523-5p	-5.54306	7.26*E*-08	1.84*E*-06	Down	Chr19
65	hsa-miR-891a-3p	-4.74652	8.79*E*-08	2.20*E*-06	Down	ChrX
66	hsa-miR-890	-4.23732	1.05*E*-07	2.58*E*-06	Down	ChrX
67	hsa-miR-518a-3p	-5.3572	1.39*E*-07	3.32*E*-06	Down	Chr19
68	hsa-miR-9-3p	-2.49706	1.40*E*-07	3.32*E*-06	Down	Chr1
69	hsa-miR-216b-5p	-5.34256	1.54*E*-07	3.60*E*-06	Down	Chr2
70	hsa-miR-512-5p	-5.32522	1.74*E*-07	4.01*E*-06	Down	Chr19
71	hsa-miR-520a-3p	-5.28657	2.28*E*-07	5.21*E*-06	Down	Chr19
72	hsa-miR-224-5p	-2.37211	2.66*E*-07	5.98*E*-06	Down	ChrX
73	hsa-miR-518f-3p	-5.27129	3.19*E*-07	7.10*E*-06	Down	Chr19
74	hsa-miR-449b-5p	-4.28293	5.69*E*-07	1.25*E*-05	Down	Chr5
75	hsa-miR-519a-5p	-5.251	1.03*E*-06	2.23*E*-05	Down	Chr19
76	hsa-miR-552-5p	-5.05373	1.09*E*-06	2.34*E*-05	Down	Chr1
77	hsa-miR-519c-3p	-5.04066	1.34*E*-06	2.83*E*-05	Down	Chr19
78	hsa-miR-10a-3p	-4.91927	2.64*E*-06	5.46*E*-05	Down	Chr17
79	hsa-miR-138-5p	-2.53821	2.65*E*-06	5.46*E*-05	Down	Chr16
80	hsa-miR-31-3p	-3.10722	2.66*E*-06	5.46*E*-05	Down	Chr9
81	hsa-miR-519a-3p	-4.90331	3.06*E*-06	6.21*E*-05	Down	Chr19
82	hsa-miR-3663-3p	-4.18958	4.91*E*-06	9.84*E*-05	Down	Chr10
83	hsa-miR-139-3p	-2.69096	5.79*E*-06	0.000115	Down	Chr11
84	hsa-miR-935	-2.36926	8.48*E*-06	0.000166	Down	Chr19
85	hsa-miR-20b-3p	-3.63398	1.39*E*-05	0.000271	Down	ChrX
86	hsa-miR-302c-3p	-2.57249	1.45*E*-05	0.000278	Down	Chr4
87	hsa-miR-18b-5p	-2.66115	1.83*E*-05	0.000347	Down	ChrX
88	hsa-miR-106a-5p	-3.00341	2.22*E*-05	0.000413	Down	ChrX
89	hsa-miR-92a-2-5p	-4.50185	2.74*E*-05	0.000505	Down	ChrX
90	hsa-miR-3923	-2.46461	3.83*E*-05	0.000699	Down	Chr3
91	hsa-miR-3180-3p	-2.72251	4.28*E*-05	0.000774	Down	Chr16
92	hsa-miR-619-5p	-2.49174	4.40*E*-05	0.000788	Down	Chr12
93	hsa-miR-181b-5p	-3.40787	5.23*E*-05	0.000926	Down	Chr9
94	hsa-miR-371a-3p	-1.87004	6.39*E*-05	0.001121	Down	Chr19
95	hsa-miR-525-5p	-3.98509	7.77*E*-05	0.00135	Down	Chr19
96	hsa-miR-7159-5p	-4.2429	9.71*E*-05	0.001656	Down	Chr6

Table 4: Continued.

	miRNA ID	log_2FoldChange (case/control)	*P* value	Padj	Up-/downregulation	Genome location
97	hsa-miR-885-3p	-4.2429	9.71*E*-05	0.001656	Down	Chr3
98	hsa-miR-3180	-2.54454	0.000124	0.002094	Down	Chr16
99	hsa-miR-10a-5p	-1.79915	0.00022	0.003683	Down	Chr17
100	hsa-miR-766-3p	2.208073	0.000223	0.003698	Up	ChrX
101	hsa-miR-105-5p	-2.88496	0.000231	0.003802	Down	ChrX
102	hsa-miR-5698	-3.71593	0.000345	0.005509	Down	Chr1
103	hsa-miR-205-5p	-3.65805	0.000391	0.006046	Down	Chr1
104	hsa-miR-373-3p	-2.62875	0.000389	0.006046	Down	Chr19
105	hsa-miR-95-3p	-1.51027	0.000391	0.006046	Down	Chr4
106	hsa-miR-1228-3p	3.219571	0.000463	0.007105	Up	Chr12
107	hsa-miR-let-7a-3p	-1.77104	0.000495	0.007533	Down	Chr22
108	hsa-miR-216a-5p	-3.30972	0.00052	0.007847	Down	Chr2
109	hsa-miR-526a	-3.83598	0.00057	0.008526	Down	Chr19
110	hsa-miR-892b	-3.83139	0.000605	0.008977	Down	ChrX
111	hsa-miR-524-3p	-3.60166	0.000611	0.008987	Down	Chr19
112	hsa-miR-518d-5p	-3.81687	0.000616	0.008999	Down	Chr19
113	hsa-miR-1298-5p	-3.55837	0.000649	0.009403	Down	ChrX
114	hsa-miR-940	1.860381	0.000766	0.01101	Up	Chr16
115	hsa-miR-6718-5p	-2.77769	0.000941	0.013423	Down	Chr18
116	hsa-miR-217	-3.71504	0.000957	0.013542	Down	Chr2
117	hsa-miR-139-3p	-1.49141	0.001097	0.015399	Down	Chr11
118	hsa-miR-3591-5p	-3.64572	0.001207	0.016811	Down	Chr18
119	hsa-miR-521	-3.61742	0.00132	0.018256	Down	Chr19
120	hsa-miR-199b-3p	2.152197	0.001346	0.018465	Up	Chr9
121	hsa-miR-527	-3.62189	0.001436	0.019552	Down	Chr19
122	hsa-miR-519d-5p	-3.57545	0.001548	0.02092	Down	Chr19
123	hsa-miR-20b-5p	-1.48311	0.001842	0.024713	Down	ChrX
124	hsa-miR-518a-5p	-3.50258	0.001966	0.026189	Down	Chr19
125	hsa-miR-548ad-5p	-2.61279	0.002084	0.027561	Down	Chr2
126	hsa-miR-125b-1	-1.54534	0.002191	0.028763	Down	Chr11
127	hsa-miR-372-3p	-1.55022	0.002313	0.030151	Down	Chr19
128	hsa-miR-1343-5p	3.178463	0.002607	0.033748	Up	Chr11
129	hsa-miR-1268a	2.390785	0.0027	0.034697	Up	Chr15
130	hsa-miR-302a-3p	-2.19397	0.003068	0.039153	Down	Chr4
131	hsa-miR-892a	-2.80124	0.003278	0.041539	Down	ChrX
132	hsa-miR-1268b	2.381553	0.003613	0.045472	Up	Chr17
133	hsa-miR-4433a-5p	2.533323	0.00378	0.047246	Up	Chr2
134	hsa-miR-3660	-3.29753	0.003885	0.048236	Down	Chr5
135	hsa-miR-187-5p	1.909446	0.003939	0.048566	Up	Chr18
136	hsa-miR-9-5p	-1.29072	0.003965	0.04857	Down	Chr1

3. Results

3.1. Patients' Characteristics. The demographic characteristics of NOA and OA patients are presented in Table 2. The serum levels of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in NOA patients were higher than those in OA patients, whereas the testosterone level in the NOA group was lower than that in the OA group.

3.2. Small RNA Sequencing and Mapping of Reads to Human Reference Genome. Before carrying out data analysis, low-quality tags were removed, and after trimming, over 20 million reads were detected in each sample. The sequencing data for each sample, including the mapping ratio, are summarized in Table 3. The legnth distribution analysis showed that sRNAs had length between 18 and 30 nucleotides. The coverage percentage after cleaning was more than 90% in 4 samples.

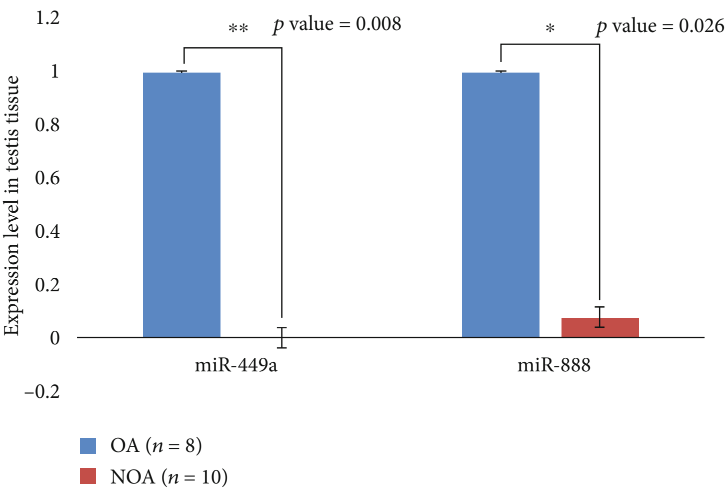

Figure 2: The comparison of expression levels of selected differentially expressed miRNAs between testicular tissue samples of the NOA and OA groups. $^{*}P < 0.05$ and $^{**}P < 0.01$.

3.3. Global miRNA Profiles between Azoospermia and Normozoospermia. Using the pheatmap function, hierarchical clustering analysis of the miRNA's expression variation illustrated a set of aberrantly expressed miRNAs in the NOA patients (Figure 1). In total, 136 known miRNAs showed significant differential expression in the testis tissue between the NOA and OA individuals. Specifically, we observed that 120 miRNAs were downregulated in NOA patients in comparison to OA men, whereas 10 miRNAs were upregulated (adjusted $P < 0.05$, using fold change threshold values of ≥1 and ≤−1). The altered miRNAs were distributed across all chromosomes, except chromosomes 13, 14, 20, 21, 22, and Y. The maximum numbers of dysregulated miRNAs were mapped to chromosomes 11, 19, and X (Table 4).

3.4. Validation of Small RNA Sequencing Results by Real-Time PCR. To validate the small RNA sequencing findings, qRT-PCR was conducted for two miRNAs; namely, miR-449a and miR-888, in individual tissue samples from NOA ($n = 10$) and OA ($n = 8$) patients. These miRNAs were chosen according to the fold change (FC). Both of these miRNAs were significantly downregulated in NOA samples ($P < 0.05$) (Figure 2). These findings were fully concordant with the differential expression observed by sequencing.

3.5. GO and Pathway Analyses for Targets of Differentially Expressed miRNAs. To explore the functions of the genes regulated by the differentially expressed miRNAs, the target genes were subjected to Gene Ontology (GO) and pathway analyses. The GO analysis demonstrated the target genes to be enriched in GO terms of 25 biological processes, 18 molecular functions, and 16 cellular components (Figure 3). Cellular process, cell and cell part, and binding were the most enriched terms in the main categories of cellular components, biological processes, and molecular functions, respectively.

In addition, pathway enrichment analysis of the DES target genes was performed based on the KEGG database, and the results were reported as a bar plot for the statistics of KEGG term types (Figure 4). The most enriched pathway was signal transduction.

4. Discussion

In this study, we analyzed the miRNA expression profile in the testicular tissues of idiopathic azoospermic patients with SCOS and compared it with OA individuals with normal spermatogenesis. Using small RNA sequencing, 136 miRNAs were recognized to be differentially expressed between the two groups. Most of these miRNAs belonged to the ascertained families, such as miR-515, miR-449, miR-34, miR-891, miR-743, miR-25, and miR-302, which are widely known and expressed as determined clusters. Specific or preferential expression of a number of miRNAs in the testis suggests their roles in spermatogenesis. In fact, some of the GO terms and KEGG pathways obtained in this study relate to sperm production, cell growth and death, signal transduction, replication and repair, energy metabolism, endocrine system, hormone secretion, reproductive process, cell motility, cell junction, and antioxidant activity (Figures 3 and 4).

We found that a remarkable number of miR-515 family members located on chromosome 13 were downregulated in SCOS patients. These primate-specific miRNA family members have been illustrated to significantly modulate several biological processes and pathways, such as cell motility (GO:0048870), cell proliferation (GO:0008283), cell death (GO:0008219), apoptotic signaling pathways (GO:0097190), mitotic cell cycle (GO:0000278), cell cycle arrest (GO:0007050), DNA metabolic process (GO:0006259), G2/M transition of the mitotic cell cycle (GO:0000086), response to stress (GO:0006950), and the MAPK signaling pathway (has04010) [26]. Among targeted genes of dysregulated miRNAs, some are implicated in male infertility. For example, miR-520a-3p regulates a plenty of genes, such as *CDKN1A*, *CCND1*, *MAPK9*, *MAPK13*, *PIR3R1*, *AKT3*, *MDM2*, *FBXO25*, *SOS2*, and *SMAD2* [27], some of which have been demonstrated to be associated with impaired sperm production.

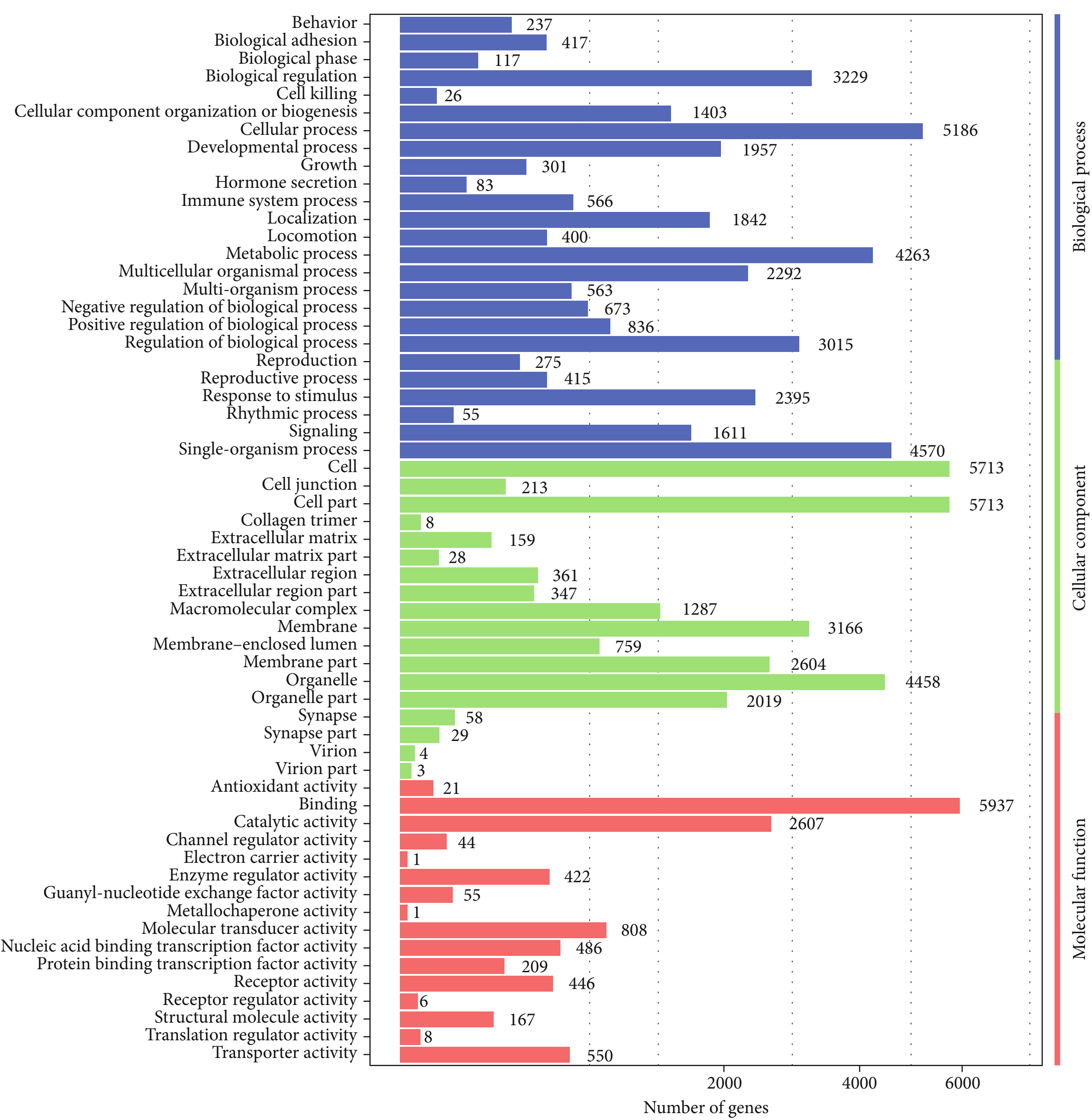

Figure 3: The GO enrichment analysis for the targets of differentially expressed miRNAs.

CDKN1A, targeted by both miR-520h and miR-519c-3p, is found to be upregulated in undifferentiated spermatogonia due to DNA damage [28]. Similarly, the overexpression of *CDKN1A* has been reported in SCOS patients [29]. Down-regulations of its targetting miRNAs, miR-520h and miR-519c-3p, in our study is an interesting corroborating observation *CCND1* is another gene, which is downregulated in SCOS patients, and is targeted by miR-520a-3p, miR-518a-5p, and miR-520c-3p. Strikingly, certain miR-515 family members also control the MAPK pathway genes (Figure 5). Several studies have demonstrated that the MAPK pathway is involved in the differentiation and maturation of male germ cells. Specifically, cascade activation of MAPKs is suggested to be involved in regulating transcription and ectoplasmic specialization, flagellar motility of mature spermatozoa, acrosome reaction, and hyperactivation. For instance, p38δ (MAPK13) isoform of p38 mitogen-activated protein kinase is predominantly expressed and activated in XY germ cells [30]. Normal spermatogenesis is critically dependent on the integrity and maintenance of the blood-testis barrier (BTB) as well as the adhesion between germ and Sertoli cells in the seminiferous tubules [31].

Accompanied by the ERK MAPK pathway activation, p38 may lead to the loss of germ cells from the epithelium by inducing the BTB disruption [32]. Moreover, p38 has been evidenced to play an essential role in sperm motility

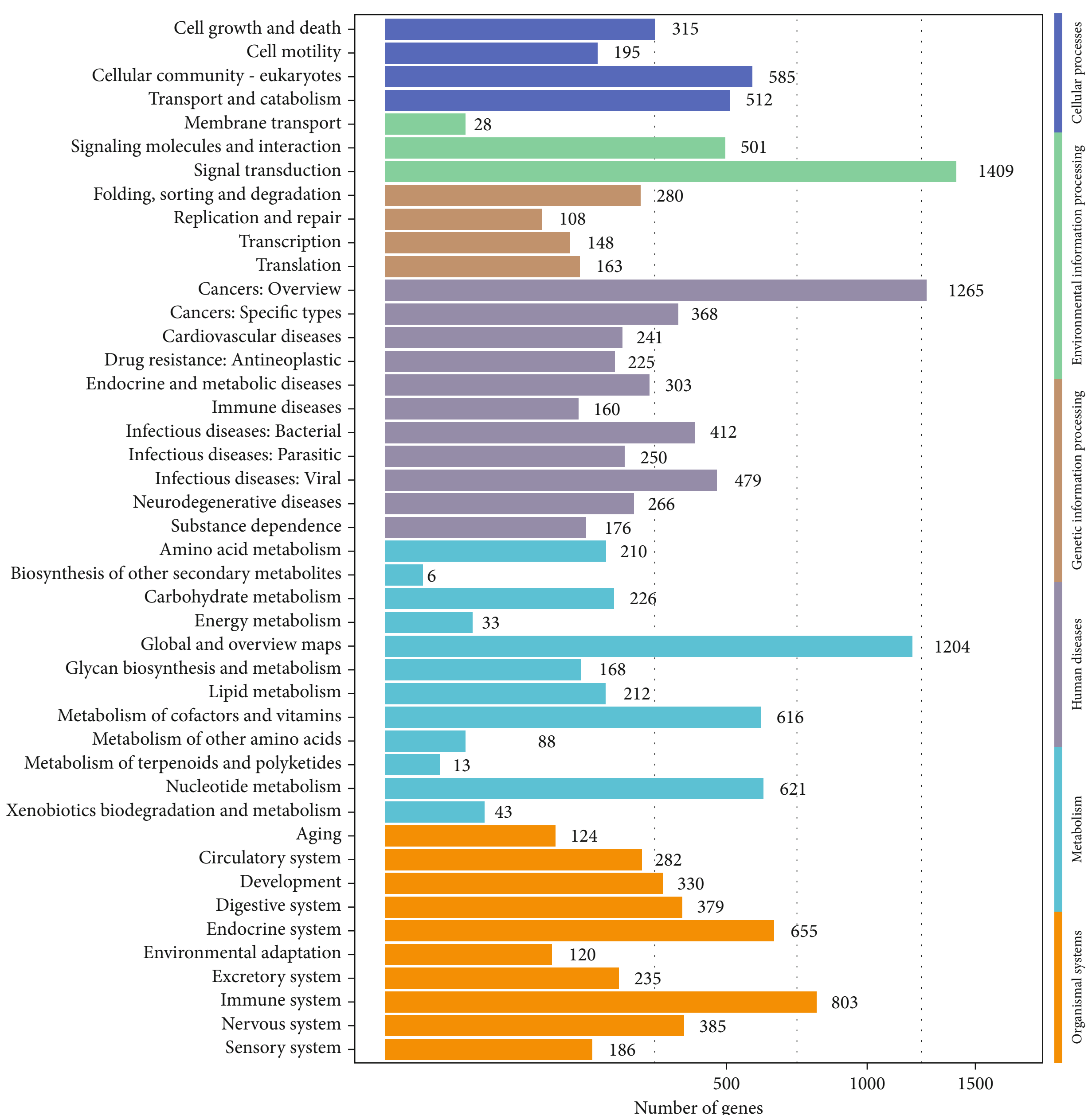

Figure 4: The KEGG classification. The *X* represents the number of differentially expressed genes. The *Y* axis indicates the second KEGG pathway terms. These terms are grouped in the top pathway terms illustrated by different colors on the right side of the graph.

[33]. Homozygous deletion of*PRKACA* (*Prkaca*$^{-}$), another gene in this pathway, has been shown to result in substantial malformations in head morphology and forward motility of mature sperm in mice [34]. Two heat shock proteins (HSPs), *HSPA8* and *HSPA1B*, targeted by the miR-515 family members, are involved in the MAPK pathway. *HSPA8* is recognized to contribute to the maintenance of TSSK6 structure expressed in the late stages of spermiogenesis. *Tssk6* disruption is linked with diminished sperm number, impaired condensation of DNA, and abnormal sperm shape and motility in male mice [35]. Rac1 in the Sertoli cells acts in the progression of spermatogenesis. Conditional knockout of Rac1 in adult mice results in spermatogenic arrest at the round spermatids stage and severe polarity disruption and increased apoptosis in the Sertoli cell [36]. The above information suggests that the miR-515 family regulates spermatogenesis through the MAPK pathway; however, the precise role of each miRNA is yet to be unveiled as the functions of many genes targeted by this family remain unknown. Dysregulation of miR-515, miR-518a-3p, miR-518d-5p, miR-518e-3p and -5p, miR-518b, miR-517-5p, miR-519d-3p, miR-520a-5p, miR-520f-5p, and miR-520h is consistent with their dysregulation in Klinefelter syndrome patients [37].

We observed that the miR-34 (miR-34b-3p, miR-34b-5p, miR-34c-3p, and miR-34c-5p) and miR-449 (miR-449a, miR-449b-3p, miR-449b-5p, and miR-449c-5p) family

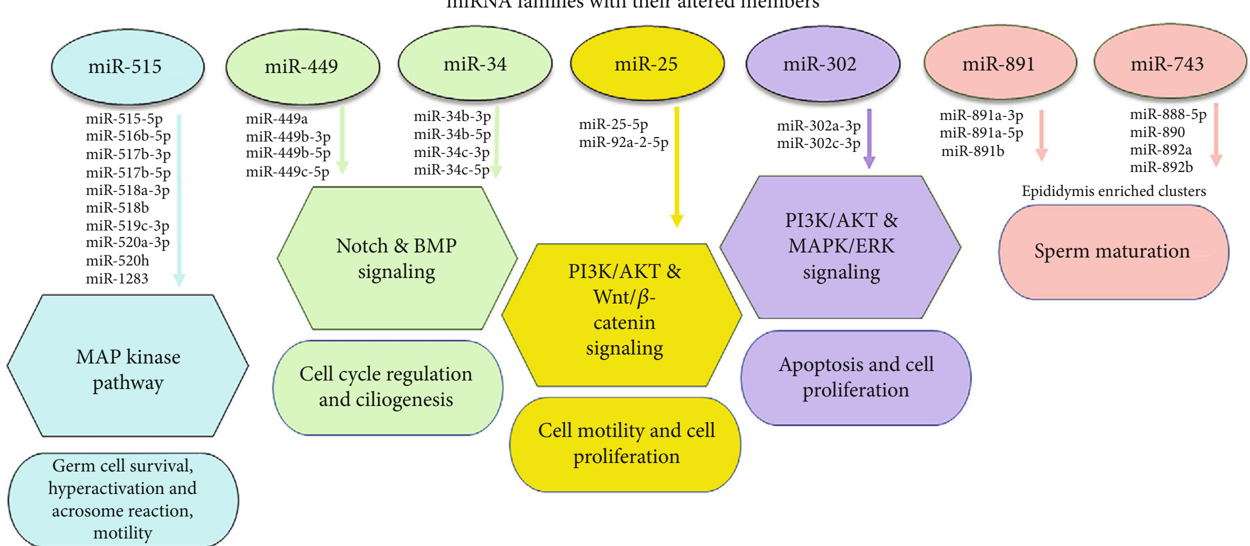

Figure 5: The roles of major miR families downregulated in SCOS.

members located on chromosomes 5 and 11, respectively, were significantly dysregulated in SCOS patients. In a previous study, drastic reductions in the expressions of both the miR-34 and miR-449 families were observed in three groups of patients with impaired spermatogenesis, including SCOS, meiotic arrest, and mixed atrophy [12]. In another study, altered expressions of some members of the miR-449b and miR-34b/c families were observed even in patients who carried AZF microdeletions [16]. Both the above studies on testicular miRNAs were microarray based. In contrast to Abu-Halima et al. [13], our study was on a different population and used miRNA sequencing, which is an advanced and more sensitive method of gene expression analysis. In comparison to Noveski et al. [16], our samples were ruled out for Y-deletions.

The miR449 family members (miR-449b and miR-449c) were not detectable in azoospermic patients with the Klinefelter syndrome [37]. Consistent dysregulation of these miRNAs across three studies signifies their role in spermatogenesis, making them excellent candidates for further investigations. Yuan et al. demonstrated that male miR-34b/c- or miR-449-knockout mice displayed normal spermatogenesis, resulting in normal fertilization and consequently normal preimplantation development, while miR-449 and miR-34b/c double knockout (miR-dKO) mice developed infertility in the form of severe spermatogenic impairment and oligoasthenoteratozoospermia (OAT) syndrome [38]. All these miRNAs, except miR-449b-3p, are involved in the regulation of hundreds of genes, which are parts of common biological processes and pathways, including mitotic cell cycle (GO:0000278), cell death (GO:0008219), response to stress (GO:0006950), cell cycle (GO:0007049), apoptosis (hsa04210), p53 signaling pathway (hsa04115), fatty acid metabolism (hsa01212), and fatty acid biosynthesis (hsa00061) [26]. Nonetheless, more investigations on these two families are needed since aberrations of single family did not lead to infertility. The miR-34 and miR-449 families are involved in ciliogenesis and cell cycle regulation through BMP and notch signaling, suggesting their importance in spermatogenesis [39] (Figure 5).

Two other families, including miR-891 (hsa-miR-891a-3p, hsa-miR-891a-5p, and hsa-miR-891b) and miR-743 (hsa-miR-888-5p, hsa-miR-890, hsa-miR-892a, and hsa-miR-892b), are located on the X chromosome as their members appear to express in the miR-888 cluster. No related GO terms or KEGG pathways have yet been identified for these miRNAs, although some roles in spermatogenesis have been suggested. miR-891b, miR-890, miR-892a, and miR-892b are speculated to be involved in sperm maturation insomuch that their expressions are predominately observed in the epididymis of human and other primates [40, 41], indicating their contributionto sperm maturation (Figure 5). miR-92a-2-5p and miR-25-5p, belonging to the miR-25 family, act at the mitotic G1/S transition checkpoint (GO:0044819). miR-92a-2-5p also plays a role in cell motility (GO:0048870) and cell proliferation (GO:0008283) [26]. miRNA-106a, located on chromosome X, is indispensable for self-renewal of the SSCs by targeting Ccnd1 and Stat3 in mouse [42]. The miR-25 and miR-302 families have been investigated for their roles in cell division and apoptosis in cancer, which suggests their significant contributions to the regulation of cell cycle and apoptosis in the testis (Figure 5); however, further investigations are required to understand their exact roles in spermatogenesis.

5. Conclusions

In summary, this is the first study analyzing the expression profile of miRNAs through small RNA sequencing in the testicular tissue of idiopathic SCOS patients. We detected 136 differentially expressed miRNAs. While the differences in the germ cell composition in obstructive versus nonobstructive azoospermia cases could be behind the alterations identified in this study, irrespective of the reasons behind these differences, the identification of the missing/downregulated miRNAs would put forth the miRNAs that are critical

to infertility treatment in the future. These miRNAs may be used as prognostic markers for the treatment of azoospermia using stem cell therapy in the future. Further studies on these miRNAs may be required to investigate their biological functions in the testis. We admit the limitation of a limited sample size in this study, which was due to the difficulty in obtaining testicular biopsy samples.

Abbreviations

AZFc: Azoospermia factor c
BP: Biological process
CC: Cellular component
DESs: Differentially expressed small RNAs
FC: Fold change
GO: Gene Ontology
MF: Molecular function
miRNA: MicroRNA
NOA: Nonobstructive azoospermia
OA: Obstructive azoospermia
RPM: Reads per million
SCOS: Sertoli cell only syndrome
TESE: Testicular sperm extraction
TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand.

Data Availability

The data from which the findings of this study have been extracted are available from the corresponding author upon reasonable request.

Ethical Approval

The current research was first approved by the Ethical Committee of Tarbiat Modares University (IR.TMU.REC.1395.382).

Consent

All individuals referring to the Royan Institute (Tehran, Iran) were recruited in this study after giving informed consent.

Disclosure

A preprint version of this manuscript has previously been published [43].

Conflicts of Interest

The authors declare that there is no conflict of interest.

Authors' Contributions

F.P. designed the scientific work, accomplished the laboratory experiments, analyzed the data, and wrote the manuscript. A.S. and M.D.F. carried out the laboratory experiments. M.A.S.G. confirmed the clinical diagnosis and selected the patients. R.S. and R.F. edited the manuscript, tables, and figures. A.A. edited the manuscript. HM designed the research plan, supervised the scientific work, and edited the manuscript. P.M. investigated and added the mechanistic details of miRNAs and added a figure depicting the same. All authors have read and authorized the final version of manuscript. Singh Rajender, Renata Finelli, Mahsa Darestanifarahani, and Abdolazim Sarli contributed equally to this work.

Acknowledgments

This study was partially supported by the research department of the Tarbiat Modares University and was partially self-funded. The authors thank Dr. Faramarz Farrahi, Dr. Marjan Sabbaghian, and all the staff of Male Infertility Clinic at the Royan Institute for sample collection. In addition, we would like to appreciate all the participants for their voluntary contributions to make this work possible.

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